Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

Presented by
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Born in: Leverkusen, Germany
Oral-examination: 05.03.2018

Inhibition of glucocorticoid signaling and synthesis detected by an *in vivo* zebrafish larva screening system:

A novel tool for endocrine disruptor risk evaluation

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Summary

Pharmaceutical residues in aquatic systems constitute a major environmental risk as pharmaceuticals are designed to interact with specific biological processes. These processes are mostly conserved among all vertebrate groups (e.g. signal transduction, metabolism), thus, drugs may have similar effects in fish as they have in mammals. In particular, endocrine disruptive chemicals (EDCs) are of high ecotoxicological concern as they may interfere with hormonal signaling via multiple pathways and can affect health and reproduction of organisms. EDCs which directly interfere with the reproductive system via sexual steroid hormones such as estrogens and androgens have been in the focus of ecotoxicological risk assessment for decades, but potential interactions with other hormonal groups are so far poorly investigated. One of these neglected hormonal groups are Glucocorticoids (GCs), a subclass of steroid hormones, which regulate metabolism and immune function. Drug interference with the GC pathway may thus hamper an organism's survival, but research efforts examining the complex interactions of these compounds with this pathway are so far limited.

In this thesis, I developed an *in vivo* testing approach, which enables to detect EDCs of the GC system and to investigate their mechanisms of action. I have applied a bioluminescence-based test system with transgenic zebrafish larvae, the GRIZLY assay, to screen an FDA-approved drug library for compounds that may affect the GC pathway. By means of conducting three assay modes I aimed to identify inhibitors of GC signaling*in vitro* and *in vivo* as well as disruptors of GC biosynthesis *in vivo*.

I detected 29 compounds that showed significant inhibitory activity in at least one of the assay modes. Interestingly, I also found five superactivators of GC signaling, substances which increased and/ or extended the bioluminescence signal activity. Concentration-dependent retests validated high reliability of the screen performance. The combined evaluation of compound effects in three assay modes enabled me to pre-categorize the potential substance mechanisms of action according to either direct interference with GC signaling activity or disruption of GC biosynthesis. In order to follow up on the compound mechanisms of action, I conducted chemical and gene expression analysis experiments with selected *in vivo* inhibitors and superactivators. The obtained results for substance effects on larva-internal steroid levels and target gene expression, combined with the GRIZLY outcomes, allowed me to group the compounds according to their potential mechanisms of action. For example, anti-inflammatory drugs interfered with GC signaling without affecting the GC biosynthesis pathway. Estrogens and retinoids inhibited GC signaling and synthesis, while for progestins both inhibitory and stimulatory effects were observed, suggesting a complex interaction of this compound class with the GC pathway.

Overall, I validated that the GRIZLY assay is a highly suitable *in vivo* test system to detect pharmaceutical interference with the GC system via various effect pathways. The combined evaluation of three different GRIZLY modes furthermore allows for pre-categorization of compound effect mechanisms, which facilitates the selection of specific follow-up experiments for an in-depth risk assessment for effects of EDCs on the GC system. Moreover, my results show that several of the most-widely prescribed drugs which enter aquatic systems may interfere with GC signaling and steroid hormone biosynthesis in fish. Given the GC regulation of metabolism and the immune system, this may possibly lead, especially under chronic exposure, to a weakened ability of organisms to cope with stressors. In the long-term view, pharmaceutical residues in the environment might thus lead to a reduced biological fitness, which may constitute an additional, so far largely neglected burden for aquatic Organisms.

Zusammenfassung

Arzneimittelrückstände in aquatischen Systemen stellen ein hohes Umweltrisiko dar, weil Pharmazeutika speziell für die Interaktion mit spezifischen biologischen Prozessen konzipiert sind. Da diese Prozesse größtenteils in allen Wirbeltiergruppen konserviert sind (z.B. Signaltransduktion, Metabolismus) können Arzneimittel in aquatischen Organismen ähnliche Effekte wie in Säugetieren zeigen. Insbesondere endokrin disruptive Chemikalien (EDCs) sind hierbei von hoher ökotoxikologischer Relevanz, da sie mit Hormonsignalwegen über zahlreiche Wirkungspfade interagieren und somit die Gesundheit und Reproduktionsfähigkeit von Organismen beeinträchtigen können. EDCs, die direkt über Sexualhormone wie zum Beispiel Östrogene und Androgene mit Reproduktionsmechanismen interagieren, sind seit Jahrzehnten im Fokus der ökotoxikologischen Risikobewertung, jedoch sind mögliche Interaktionen mit anderen Hormongruppen bislang nur wenig erforscht. Eine dieser wenig beachteten Hormongruppen ist die der Glukokortikoide (GCs), eine Unterklasse der Steroidhormone, welche den Metabolismus und Immunfunktionen regulieren. Medikamente, die mit dem GC Signalweg interagieren, könnten daher die Überlebensfähigkeit eines Organismus beeinträchtigen, jedoch sind wissenschaftliche Bemühungen, die komplexen Substanzinteraktionen mit diesem Signalweg zu untersuchen, bislang begrenzt.

In dieser Doktorarbeit habe ich einen *in vivo* Testansatz entwickelt, der es erlaubt, EDCs des GC-Systems zu identifizieren und ihre Wirkmechanismen zu untersuchen. Ich habe ein ein biolumineszenzbasiertes Testsystem mit transgenen Zebrabärblingslarven angewendet, den GRIZLY Assay, um eine Bibliothek aus von der FDA zugelassenen Medikamenten auf Substanzen zu durchmustern, die Effekte auf den GC-Signalweg haben können. Mithilfe der Durchführung dreier Assay-Modi war es mein Ziel, *in vitro* und *in vivo* Inhibitoren des GC-Signals und Disruptoren der GC-Biosynthese zu identifizieren.

Ich habe dabei 29 Substanzen ermittelt, die signifikant inhibierende Aktivität in mindestens einem der Assay-Modi zeigten. Interessanterweise habe ich auch fünf Superaktivatoren gefunden, also Substanzen, die die Biolumineszenzsignalaktivität intensiviert und/ oder zeitlich verlängert haben. Konzentrationsabhängige, Nachtests validierten eine hohe Verlässlichkeit des Verfahrens. Die kombinierte Auswertung von Substanzeffekten in drei Assay-Modi ermöglichte es mir, potentielle Wirkmechanismen entweder als direkte Interaktionen mit der GC-Signalaktivität oder als indirekte Interaktionen mit der GC-Biosynthese zu prä-kategorisieren. Um diese Substanzwirkmechanismen weiter zu verfolgen habe ich chemisch-analytische und Genexpressionsanalyse-Experimente mit ausgewählten *in vivo* Inhibitoren und Superaktivatoren durchgeführt. Die Resultate der Substanzeffekte auf larveninterne Steroidlevel und Zielgenexpression, in Kombination mit den GRIZLY-Ergebnissen, erlaubten es mir, die Substanzen

entsprechend ihrer potentiellen Wirkmechanismen zu gruppieren. Beispielsweise interagierten entzündungshemmende Pharmazeutika mit dem GC-Signal ohne die GC-Biosynthese zu beeinträchtigen. Östrogene und Retinoide inhibierten das GC-Signal und die Synthese, während für Progestine sowohl inhibitorische als auch stimulierende Effekte zu beobachten waren, welche komplexe Interaktionen dieser Substanzklasse mit dem GC-Wirkungspfad andeuten.
Insgesamt validierte ich den GRIZLY Assay als ein geeignetes *in vivo* Testsystem, um Arzneimittelinterferenz mit dem GC-System über verschiedene Wirkmechanismen zu detektieren. Die kombinierte Evaluation von drei verschiedenen GRIZLY-Modi erlaubt es darüber hinaus, Substanzeffektmechanismen zu prä-kategorisieren, was es vereinfacht, spezifische Folgeexperimente für eine tiefergreifende Risikobewertung der Effekte von EDCs auf das GC-System auszuwählen. Darüberhinaus zeigen meine Ergebnisse, dass einige der meistverschriebenen Arzneimittel, die in aquatische Systeme gelangen, möglicherweise mit dem GC-Signal und der Steroidhormonbiosynthese in Fischen interferieren können. Sie könnten so potenziell Metabolismus und Immunfunktion beeinträchtigen und insbesondere unter chronischen Expositionsbedingungen zu einer verminderten Fähigkeit des Organismus, mit anderen Stressoren zurecht zu kommen, führen. Auf lange Sicht könnten Arzneimittelrückstände als eine zusätzliche, bislang größtenteils vernachlässigte Umweltbelastung daher zu einer reduzierten biologischen Fitness von aquatischen Organismen führen.

Acknowledgements

This work would have not been possible without the support of the following people: First of all I would like to thank my supervisor Dr. Thomas Dickmeis for giving me the opportunity to do my Ph.D. in his group and to work on this interesting project. I am thankful for his advice and support in the laboratory, for helpful discussions and for his critical proofreading of my thesis.

I wish to express my sincere gratitude to my first supervisor and thesis advisory commitee member Prof. Dr. Uwe Strähle for his valuable scientifc advice during my Ph.D. Furthermore, I would like to thank the other members of my thesis advisory committee, Prof. Dr. Andrew Cato and Dr. Gerald Brenner-Weiss for their advice and feedback on my progress.

I am very grateful to Christin Lederer for running the lab during the first two years of my Ph.D. and for her technical assistance in the fishhouse and on the bench. My deepest thanks also to all other current and former members of the Dickmeis group, Oksana Breus, Mojtaba Ebrahimi, Andrea Schink, Theresa Liepert, Philipp Haitz, Christina Thiemann, Aloa Machado de Souza, Christin Lederer, Marisa Einloft, Meltem and Benjamin Weger, as well as to my collegues on the 4th and 5th floor, for their support and helpful discussions. An especially big thanks to Iris, Alina and Regina for their friendship, scientific exchange and helpful advice at any time.

Many thanks to our close collaborators, Dr. Gerald Brenner-Weiss and Michael Nusser, for the steroid measurements in zebrafish larvae. Also a special thanks to Markus Reischl, for his patience in answering my numerous questions concerning normalization and statistical analysis of screen data.

My sincere thanks to Nadine Borel and her team for taking excellent care of the fish and to the members of EZRC and the administration team of the ITG for keeping everythin running. I would also like to acknowledge the “Bundesinstitut für Risikobewertung” for financial support and the interest in my project and I am very grateful for the training provided by the BioInterfaces International Graduate School and the Karlsruhe House of Young Scientists (KHYS), which was very benificial for my Ph.D.

I am deeply grateful to my family and friends for all their support and patience at all times. My loving gratitude goes to Vince, who gave me most of all patience and support during the last years.

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Chapter 1

1. Introduction

Pharmaceutical residues in aquatic systems are a major issue for environmental risk assessment (Corcoran, Winter, and Tyler 2010; Fent, Weston, and Caminada 2006). As many drugs act specifically on target mechanisms that are conserved within all vertebrate groups (e.g. enzymes and receptors), a pharmaceutical may have similar effects in fish as it has in mammals (Christen et al. 2010; Corcoran et al. 2010; Fent et al. 2006; Gunnarsson et al. 2008). Especially compound effects on the endocrine system are of high ecotoxicological concern, as they may interfere with hormonal signaling via a multitude of pathways and can affect health and reproduction of an organism (WHO 2002). Endocrine disrupting chemicals (EDCs), which directly interfere with the reproductive system and with sexual steroid hormonessuch as estrogens and androgens, have been in the focus of ecotoxicological risk assessment for decades (Sanderson 2006), but drug interactions with other hormonal groups are poorly investigated.

One of the hormone groups that was rather disregarded within environmental risk evaluation up to now is that of glucocorticoids (GCs), a subclass of steroid hormones, which plays important roles in various physiological processes, including major drug targets such as metabolism, inflammation, cardiovascular function and the nervous system (Bamberger et al. 1996). Although GCs are involved in these vital processes and pharmaceutical interference with them may hamper an organism's survival, research efforts targeting the complex compound interactions are so far limited. Therefore, in this study, an *in vivo* testing approach was developed, which enables to detect EDCs of the GC system and to investigate their mechanisms of action.

1.1 Steroid hormones

Steroid hormones are small organic molecules which are structurally derived from cholesterol. They are endogenously synthesized by specific organs and secreted into the bloodstream, where they circulate either freely or bound to plasma carrier proteins. Upon uptake from the systemic circulation into target tissues, they act as signaling molecules. Within cells, they can exert their physiological effects via different mechanisms, whereof their binding to steroid hormone receptors and regulation of target gene transcription are the best-investigated steroid hormone signaling pathways.

In all vertebrates, steroid hormones mediate major physiological processes such as development, metabolism, inflammation, homeostasis and tissue function, and regulate important behavioral conditions within the central nervous system, reproduction processes and

the adrenal axis (Evans 2005; Godowski et al. 1987; Laudet 1997; Mangelsdorf et al. 1995; Neave 2008; O'Malley 1990, 2005; Thornton 2001).

1.1.1 Types of steroid hormones

Steroid hormones can be divided into two groups, sex steroids and corticosteroids. Sex steroids mainly regulate sexual differentiation, reproduction and patterns of behavior. They consist of three different types, androgens, estrogens and progestogens. To the group of corticosteroids belong mineralocorticoids and GCs. While mineralocorticoids maintain salt and water balances in the body, GCs regulate primarily developmental and metabolic processes, immune functions and stress (Adcock, Ito, and Barnes 2004; De Bosscher et al. 2005; Capper, Rae, and Auchus 2016; Weikum et al. 2017). For all types of steroid hormones, the chemical structure of the respective major active ligand in humans and of their common precursor cholesterol is shown in Figure 1. Each hormone type has its own cognatereceptors, designated as estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR), mineralocorticoid receptor (MR) and GC receptor (GR). One major signaling pathway, common for all types of steroid hormones, is binding to their specific receptor, which functions as a ligand-regulated transcription factor and induces or represses target gene transcription (Africander, Verhoog, and Hapgood 2011; De Bosscher et al. 2005; Heitzer et al. 2007; Karin et al. 1998; Louw-du Toit et al. 2017; Ronacher et al. 2009; Weikum et al. 2017).

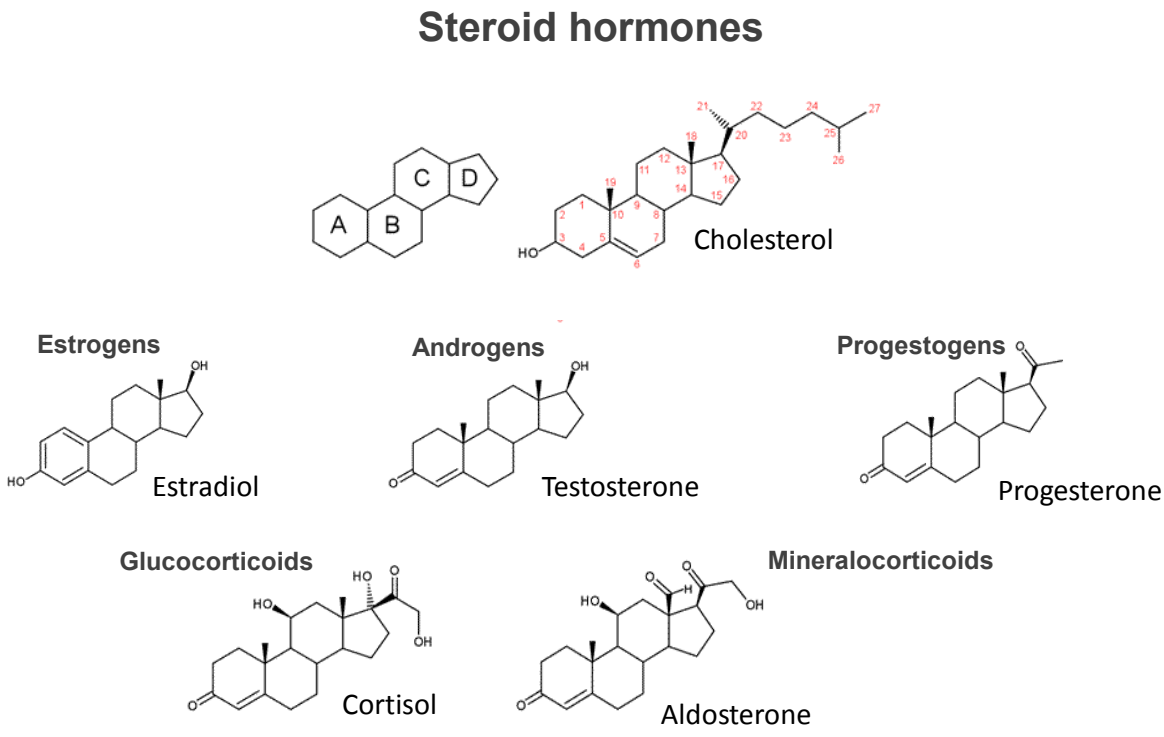


Figure 1: Groups of steroid hormones
The five types of steroid hormones and their major active form in humans (name and chemical structure): estrogens (Estradiol), androgens (Testosterone), progestogens (Progesterone), glucocorticoids (Cortisol) and mineralocorticoids (Aldosterone). All steroid hormones are structurally derived from their common precursor, cholesterol (top).

1.1.2 Steroid hormone production

Endogenously, steroid hormones and their precursors are synthesized in steroidogenic cells of several organs including adrenal gland, gonads (ovary and testis), placenta, brain, liver, intestine, skin, kidneys and adipose tissue. Adrenal, testis, ovary and the central nervous system are specialized for *de novo* steroid production. While the adrenal gland primarily synthetizes GCs (*zona fasciculata*), mineralocorticoids (*zona glomerulosa*) and precursors of androgens (*zona reticularis*), the gonads are responsible for the biosynthesis of the sex steroids. The other – peripheral – tissues, predominantly take steroid hormones up from the blood circulation and convert them into active or inactive metabolites (Capper et al. 2016; Louw-du Toit et al. 2017; Miller and Auchus 2011; Sanderson 2006).

1.2 Glucocorticoids

GCs have a broad physiologic role as they regulate important biological processes including cell cycle, development, metabolism, immune and stress response. In humans, the major active endogenous GC mediating these effects is Cortisol (Cort). GCs were discovered in the 1930s in extracts from adrenal glands. In the 1950s, their physiological effects were studied by Edward Kendall, Tadeus Reichstein and Philip Hench, who were jointly awarded the Nobel Prize in Physiology or Medicine for their work. These effects were found in the 1970s to be mediated via GC binding to specific GRs, and in the 1980s it was discovered that the GR can bind to specific DNA sequences and regulate the expression of target genes via transcriptional activation and repression (Beato, Herrlich, and Schütz 1995; Heitzer et al. 2007; Karin et al. 1998; Ward et al. 1951; Yamamoto 1985).

1.2.1 Regulation of glucocorticoid synthesis

The production of endogenous GCs is regulated in all vertebrates via a major endocrine system that consists of three parts. In mammals, hypothalamus, pituitary and adrenal gland compose the hypothalamic-pituitary-adrenal (HPA) axis, which controls the release of Cort via complex interactive mechanisms between the single parts (Capper et al. 2016). The HPA axis maintains the homeostasis of the metabolic, immune, cardiovascular, reproductive and central nervous system and controls numerous processes including digestion, energy storage, mood, emotions and expenditure. It regulates dynamic changes of Cort levels in the blood, which underlie a circadian rhythmicity. In humans, the Cort concentration peaks between 6 and 8 o´clock in the morning, in order to increase physiological and metabolic activity, and declines throughout the day with the lowest level around midnight (Chan and Debono 2010; Debono et al. 2009; Dickmeis 2009; Weitzman et al. 1971).

Not only basal GC levels are controlled by the HPA axis, it is also susceptible to external stressors that can induce the production and release of Cort. Such stressors may imply physiological and psychological challenges, such as illness and inflammation, physical activity, stress or anxiety. When the HPA axis is stimulated to increase the endogenous Cort production, neuropeptides

including the corticotropin-releasing hormone (CRH), are synthesized from neuroendocrine cells localized within the paraventricular nucleus (PVN) of the hypothalamus. CRH is released into the hypophyseal portal system, a vascular networkin the brain that connects the hypothalamus with the anterior pituitary. Via this system, CRH is transported to the anterior pituitary, where it induces the production of adrenocorticotropic hormone (ACTH) within corticotropic cells. ACTH is secreted into the blood stream and enters the adrenal gland, where it stimulates the biosynthesis and the release of GCs within the adrenal cortex. From the adrenal cortex, Cort enters the blood stream and is transported into the cells of target organs, where it mediates physiological changes that enable the organism to adapt to the stressor (Oakley and Cidlowski 2013; Papadimitriou and Priftis 2009).

If the endogenous Cort biosynthesis needs to be reduced, the HPA axis can decrease the production rate via negative feedback-loops. For instance, high levels of Cort have a negative feedback effect on the hypothalamus and the pituitary gland where the synthesis and secretion of CRH and the production of ACTH are reduced (Jacobson 2005). Long-term activation of the HPA axis can thus be avoided by this classical negative feedback mechanism. The function of the HPA axis and the negative feedback loops is shown schematically in **Figure 2**.

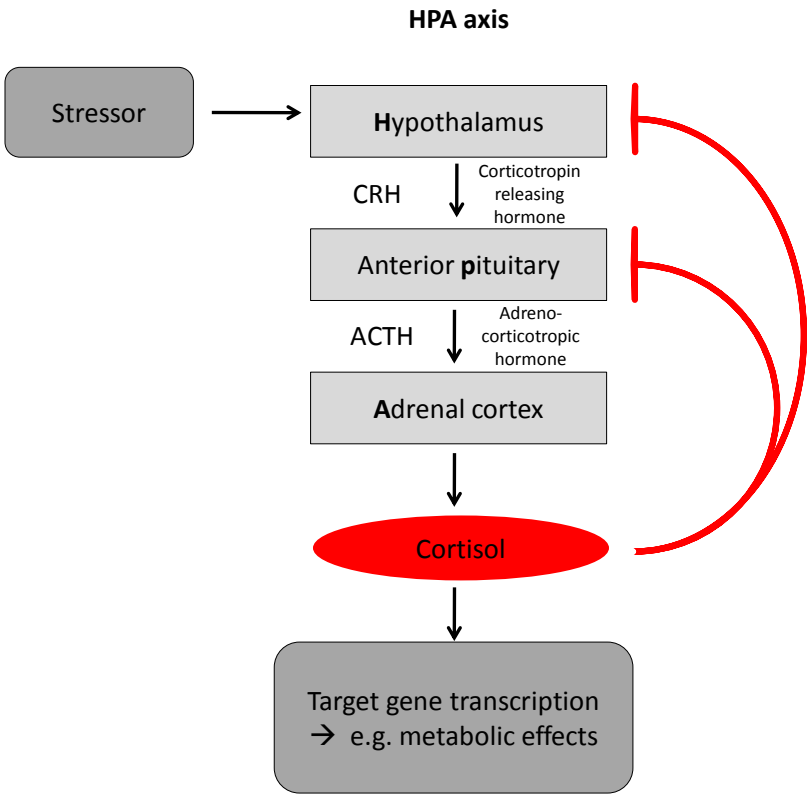


Figure 2: Negative feedback mechanism via the hypothalamic-pituitary-adrenal (HPA) axis
The HPA axis is stimulatedby an external stressor (e.g. low blood glucose, inflammation) to increase the production of Cort, corticotropin releasing hormone (CRH) is synthesized within the hypothalamus. CRH is transported to the anterior pituitary, where it induces the production of adrenocorticotropic hormone (ACTH). Via the blood ACTH enters the adrenal cortex, where it stimulates Cort biosynthesis. Cort is released into the blood and transported into target tissues where it can mediate gene transcription regulation in order to counteract the effects of the stressor, e.g. by induction of glucose production or by repression of the inflammatory response. Cort production can be inhibited via negative feedback loops by which Cort mediates the decrease of CRH and ACTH synthesis and secretion.

1.2.2 Glucocorticoid biosynthesis

Upon stimulation via the HPA axis, GCs are produced in the *zona fasciculata* of the adrenal cortex. Within the steroid hormone synthesis pathway, described as steroidogenesis, the common precursor of all steroid hormones, cholesterol, is converted via multiple serial steps into downstream steroids. With each metabolization step, a new steroid hormone is produced, until the required metabolite is obtained. In this way, progestogens are synthetized first and serve as precursors for all other groups of steroid hormones, GCs, mineralocorticoids, androgens, estrogens and their intermediate products.

This reaction cascade is catalyzed by a number of steroidogenic enzymes. Two classes of enzymes are involved in the steroid hormone biosynthesis pathway; P450 monooxygenases (CYPs), containing heme as a cofactor and catalyzing irreversible reaction steps, and hydroxysteroid dehydrogenases (HSDs), which mediate reversible reactions (Louw-du Toit et al. 2017). In Figure 3, important metabolization steps involved in the production of GCs are shown in the context of the steroid biosynthesis pathway.

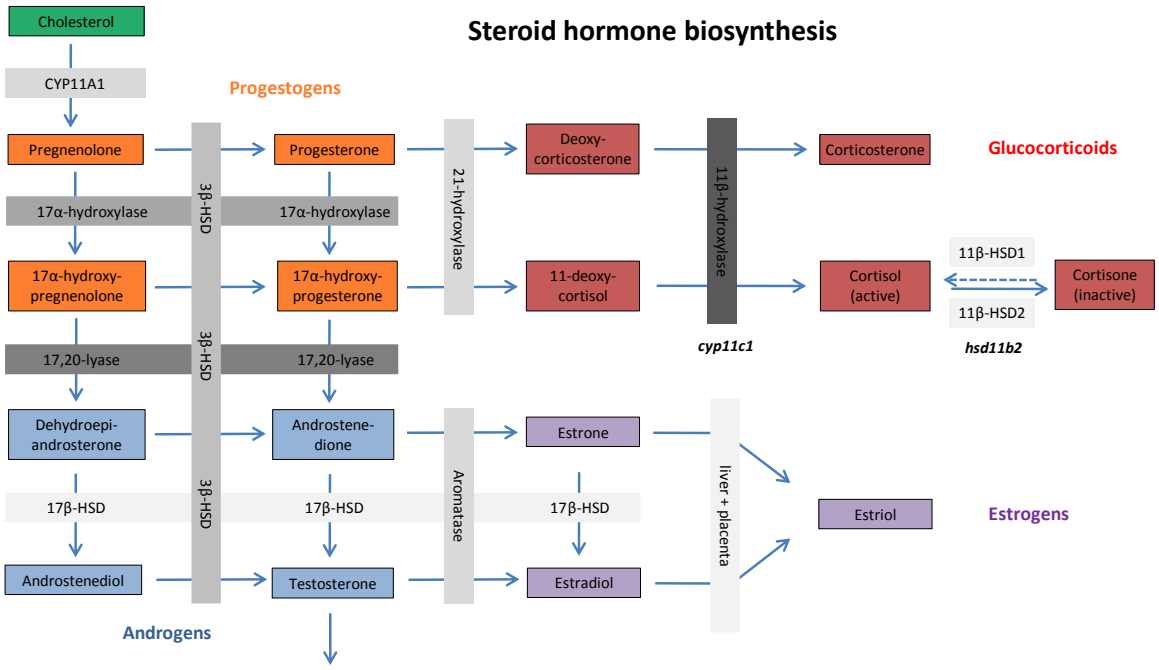


Figure 3: Steroid hormone biosynthesis pathway
Schematic visualization of progestogen (orange), GC (red), androgen (blue) and estrogen (purple) biosynthesis from the common precursor cholesterol (green). **CYP11A1** - cytochrome P450 side-chain cleavage; **17α-hydroxylase** - cytochrome P450 17α-hydroxylase (CYP17A1); **17,20-lyase** - cytochrome P450 17,20-lyase (CYP17A1); **3β-HSD** - 3β-hydroxysteroid dehydrogenase; **21-hydroxylase** - cytochrome P450 21-hydroxylase (CYP21A2); **Aromatase** - cytochrome P450 aromatase (CYP19A1); **11β-hydroxylase** - cytochrome P450 11β-hydroxylase (CYP11B1 in humans; CYP11C1 in zebrafish); **11β-HSD1** - 11β-hydroxysteroid dehydrogenase 1 (not identified in zebrafish), **11β-HSD2** - 11β-hydroxysteroid dehydrogenase 2; **17β-HSD** - 17β-hydroxysteroid-dehydrogenase (HSD17B). Gene names for 11β-hydroxylase and 11β-HSD2 of zebrafish in *italics*.

1.2.3 Glucocorticoid receptor (GR)

The best-studied mechanisms of Cort signaling involve regulation of gene expression via binding to the intracellular GR. The GR is ubiquitously expressed in organisms but its expression levels

are tissue- and cell-cycle specific (Africander et al. 2011; Lu et al. 2006). Like all steroid hormone receptors, the GR belongs to the nuclear receptor superfamily. This family consists, beside of the receptors for gonadal and adrenal steroids, also of receptors for nonsteroidal ligands (e.g., thyroid hormones, retinoic acid, vitamin D and fatty acids) and "orphan" receptors, for which the endogenous ligands are not yet completely identified (Mangelsdorf et al. 1995; Willson and Moore 2002). Nuclear receptors are ligand-inducible transcription factors. Accordingly, upon binding of an appropriate ligand, they mediate ligand-specific functions via regulation of target gene transcription.

1.2.3.1 GR structure and function

The GR shares a common structure with the other steroid hormone receptors, all being organized into several functional domains. These domains allow them to bind their respective ligands, recognize specific DNA-binding sequences on target genes and regulate mRNA transcription. The steroid receptor domain organization is illustrated in **Figure 4**.

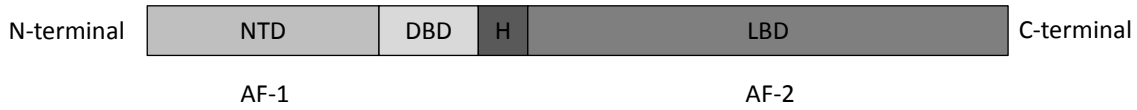


Figure 4: Scheme of the steroid hormone receptor domain organization
The N-terminal domain, where AF-1 is located, regulates transcription while the DNA-binding domain (DBD) recognizes specific DNA sequences. The hinge-region (H) allows structural flexibility during receptor binding and the ligand binding domain (LBD) enables binding to the respective ligands and ligand-dependent transcriptional activation via AF-2.

The amino (N)-terminal domain (NTD) has a variable sequence and length, which is unique to each steroid hormone receptor. The NTD carries an autonomous transcriptional activation function (AF-1), which is highly variable but rich in negatively charged acidic amino acids and weakly conserved within the superfamily of nuclear receptors. The AF-1 can be responsible for differential promotor regulation as it modulates transcription cell- and gene-specifically. It interacts directly with the basal transcriptional machinery, transcription factors and numerous cofactors, which enables certain transcriptional functions that are ligand-independent, but can also mediate transcriptional activation synergistically with another transcriptional activation function (AF-2) at the C-terminal end (Lavery and Mcewan 2005; McEwan et al. 1993; Weikum et al. 2017).
The zinc-finger DNA-binding domain (DBD) is highly conserved and centrally localized. It contains a nuclear localization signal (NLS), a nuclear export signal (NES) and is important for receptor dimerization, specificity of DNA binding and cofactor interactions. The DBD binds specific DNA sequences via a three dimensional configuration of the zinc fingers. While the N-terminal zinc finger recognizes the specific DNA response elements, the C-terminal zinc finger mediates receptor dimerization (Freedman and Luisi 1993; Griekspoor et al. 2007; Tang et al. 1998; Weikum et al. 2017).

The hinge region (H) also contains an NLS and serves as a flexible pivot, linking the DBD and the adjacent ligand binding domain (LBD) and allowing conformational changes of the receptor. The LBD at the carboxyl (C)-terminal end and the second transcriptional activation function AF-2 localized herein are highly conserved. The LBD allows binding of the corresponding ligands, contributes to the dimerization process of homologous receptors and interacts with heat shock protein 90 (HSP90). HSP90 is a general molecular chaperone that mediates folding of various proteins, including the steroid hormone receptors. In the absence of a ligand, it stabilizes the receptor and protects it from protease degradation. HSP90 also blocks the DBD and the NLSs, preventing receptor binding to DNA-response elements. Upon ligand binding, HSP90 dissociates from the receptor, enabling the NLSs to mediate translocation of the ligand-receptor complex from the cytoplasm via the nuclear pore into the nucleus (Africander et al. 2011; Beato et al. 1995; Bourguet, Germain, and Gronemeyer 2000; Heitzer et al. 2007; Savory et al. 1999; Weikum et al. 2017).

The receptor binds to the DNA as a homodimerized ligand-receptor complex and the second, ligand-dependent transcriptional activation function AF-2, located in the LDB, supports the initiation of transcriptional processes. AF-2 recruits transcriptional coactivators or corepressors, which interact with the transcriptional activation machinery (e.g. via chromatin remodeling proteins) and either induce or repress target gene transcription (Beato et al. 1995; Bourguet et al. 2000).

Although all steroid hormone receptors share these common domains, their individual structures within the NTD ensure a high specificity for their respective DNA-binding sites, which are denominated GC response element (GRE) for the GR. Also, individual sequences within the LBD increase the receptor affinity for the respective steroid hormone and their synthetic derivatives.

1.2.4 GR-mediated signaling

GR activity can be initiated by Cort or other natural and synthetic GC ligands. In the absence of ligands, the GR resides in the cytoplasm in a folded, inactive state, associated with protein complexes. Proteins involved include chaperones (dimerized HSP90, HSP70, p23) and immunophilins of the FK506-binding protein family (FKBPs; FKBP5 and FKBP52).

Steroids can enter the cell via passive diffusion across the plasma membrane, due to their lipophilic properties. Their binding to the LBD of the GR induces a specific conformational change of the receptor, which depends on the chemical structure of the ligand. Thereupon, the protein complex dissociates from the GR, enabling the NLSs to mediate translocation of the ligand-receptor complexfrom the cytoplasm into the nucleus, involving microtubules and passing through the nuclear pore. Within the nucleus, the activated ligand-receptor complex can be recruited to DNA-binding sites within hormone-regulated genes via different mechanisms. One major mechanism of GC signaling is direct DNA binding to specific GREs within the regulatory regions of target genes. Via this process, the ligand-receptor complex can either activate (transactivation) or reduce (transrepression) gene expression, depending on the type of GRE. However, the GR can also regulate target gene transcription without direct DNA binding, but via

tethering, a physical interaction with other transcription factors (Africander et al. 2011; Hammes and Levin 2007; Heitzer et al. 2007; Oakley and Cidlowski 2013; Pratt and Toft 1997; Schaaf and Cidlowski 2002).

1.2.4.1 Transactivation

Genes whose transcription is transactivated by the GR, among which are many genes involved in metabolic functions, frequently carry positive GREs. These GREs show high similarity with a palindromic consensus sequence that consists of two "half sites" with six base pairs each, separated by three spacer base pairs: AGAACAnnnTGTTCT. This consensus sequence can in principle be bound by GRs, MRs, ARs and PRs, due to their high conservation within the DBD. However, the specific GRE sequence and its environment defines the potential for binding of the GR or other steroid hormone receptors and influences the efficiency of receptor signaling activity.

For the attachment to the palindromic DNA sequences, head-to-head homodimerization of two ligand-receptor complexes, mediated via the C-terminal zinc finger of the DBD, is necessary (**Figure 5 a**). Homodimer binding initiates the recruitment of coactivators and transcriptional cofactors, which assemble as a macromolecular complex within the promotor region and stimulate chromatin remodeling, e.g. via histone acetylation. This engages the basal transcriptional machinery, and RNA polymerase II is enabled to bind within the opened chromatin structure and transcribes the target gene mRNA (Adcock et al. 2004; Africander et al. 2011; Bamberger et al. 1996; Beato 1989; Freedman and Luisi 1993; Griekspoor et al. 2007; Heitzer et al. 2007; Oakley and Cidlowski 2013; Schaaf and Cidlowski 2002; Tang et al. 1998; Weikum et al. 2017).

1.2.4.2 Transrepression

In case of transrepression, the GR binds to negative GREs (nGREs), which resemble an inverted-repeat of the consensus sequence and contain $CTCC(N)_{0\text{-}2}GGAGA$. This element cannot be bound by steroid hormone receptors other than the GR (Weikum et al. 2017). The GR can bind to nGREs either as one or two monomers, assembling on opposite sides of the DNA, and initiate the recruitment of corepressors and other transcriptional cofactors. Corepressors, such as nuclear receptor corepressor (NCoR) and silencing mediator or retinoid and thyroid receptors (SMRT), are proteins that repress target gene expression either in the absence of a ligand or if the GR is bound by an antagonist. Most cells express both corepressors and coactivators that interact with each other, but their ratio is crucial for the level of target gene expression. Thus, during transrepression, predominantly corepressors are recruited and mediate histone deacetylation, chromatin structure closure, prevention of RNA polymerase II binding, arrest of the basal transcription machinery and repression of target gene expression (**Figure 5 b**). One example for GR-mediated transrepression is the regulation of Cort production via a negative feedback loop of the HPA axis. Upon Cort activation of the GR, the ligand-receptor complex binds to nGREs within the promotor region of the proopiomelanocortin (POMC) gene, and in that way stopsthe production of ACTH and Cort biosynthesis (Beato 1989; Heitzer et al. 2007; Oakley and Cidlowski 2013; Schaaf and Cidlowski 2002; Weikum et al. 2017).

1.2.4.3 Tethering

Alternative to direct DNA binding, the GR can also trigger target gene expression via tethering to another DNA-bound transcription factor. By this protein-protein interaction, the DBD of the GR attaches to a transcription factor that in turn is attached to a specific recognition site of the target gene regulatory sequences (Adcock et al. 2004; Africander et al. 2011; Heitzer et al. 2007; Karin et al. 1998; Oakley and Cidlowski 2013; Weikum et al. 2017). Tethering of the GR can either induce or repress gene expression, but it is not known yet exactly if these mechanisms require a monomeric or a dimeric GR (Weikum et al. 2017).

Especially the anti-inflammatory properties of GCs are considered to be primarily mediated via tethering. For instance, in case of suppression of pro-inflammatory genes, the GR tethers to active, pro-inflammatory transcription factors within the regulatory regions of target genes (**Figure 5 c**). This induces the recruitment of transcriptional cofactors and corepressors which mediate histone deacetylation, closure of the chromatin structure and, thus, suppress gene expression (Adcock et al. 2004; Africander et al. 2011; Heitzer et al. 2007; Karin et al. 1998; Oakley and Cidlowski 2013; Schaaf and Cidlowski 2002; Weikum et al. 2017).

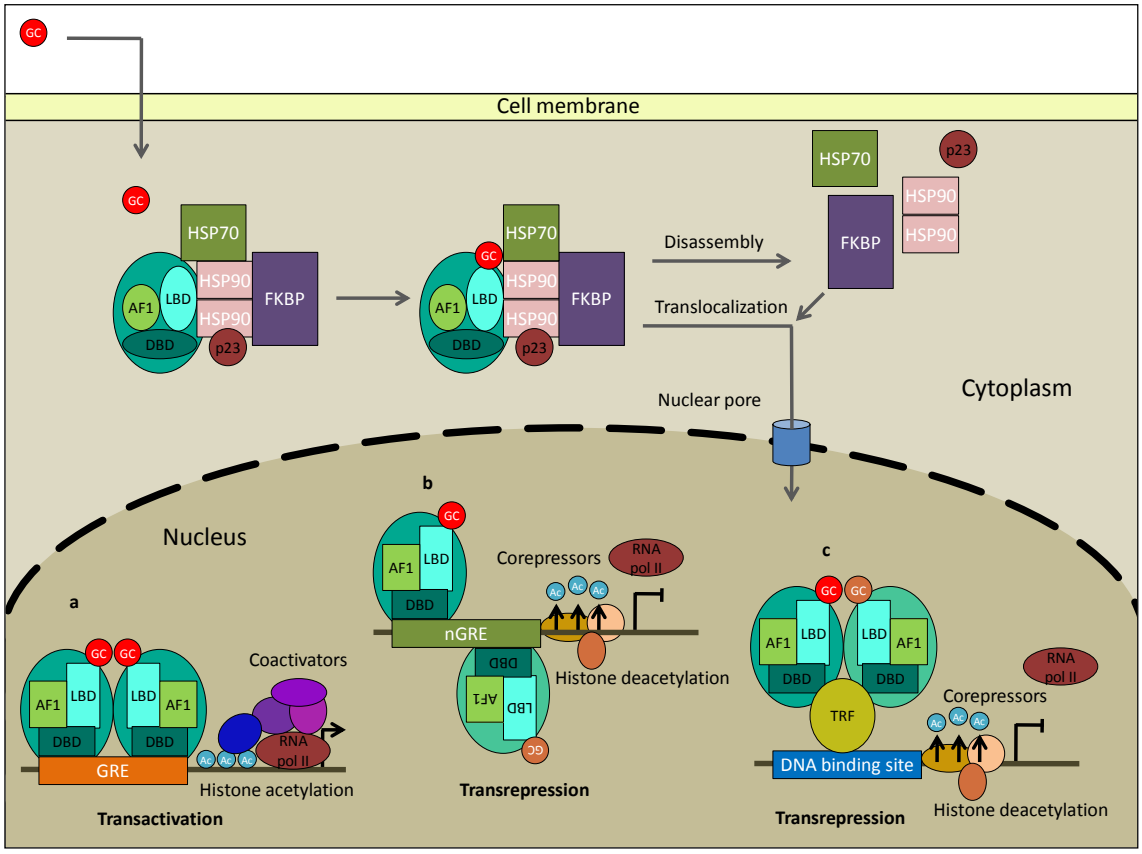


Figure 5: Glucocrticoid receptor-mediated signaling
The inactive GR resides in the cytoplasm, bound to a multimeric complex of a HSP90-dimer, HSP70, FKBPs, p23 and other proteins. GCs enter the cell and bind to the GR, which induces receptor activation, dissociation of the receptor-protein complex and receptor translocation. Within the nucleus, the GR can mediate GC signaling via three different major mechanisms. **a**) Transactivation requires binding of a GR homodimer to a specific positive GRE within the regulatory sequences of target gene DNA. Numerous coactivators and other transcriptional cofactors arerecruited, which induce histone acetylation, opening of the chromatin structure, RNA polymerase II binding and target gene expression. **b**) Transrepression is currently thought to involve either monomeric binding of the GR to a negative GRE or binding of two GRs on two sides

of the DNA-binding sequence (second GR faded, as not necessarily involved). GR binding initiates the recruitment of corepressors and other transcription cofactors that mediate histone deacetylation, closure of the chromatin structureandsuppression of target gene transcription. **c**) Tethering involves no direct GR binding to the DNA, but tethering to another DNA-bound transcription factor (TRF). This process may be mediated by a GR monomer or dimer (second GR faded, as not necessarily involved) and induce or prevent the transcription factor target gene expression. In case of gene expression inhibition, GR tethering to an active TRF induces corepressor recruitment, histone deacetylation and closure of the chromatin structure, which stops further gene transcription.

1.2.4.4 Context-specificity of glucocorticoid signaling

Whether GR binding results in activation or repression of gene transcription depends on the cofactors recruited by the ligand-receptor complex. Ligand binding to the GR may either induce an agonistic or an antagonistic effect, which in turn depends on the ligand itself and the presence of other competitive ligands. While an agonist activates a response on target gene transcription, an antagonist inhibits the effect mediated by an agonist, without inducing a response itself (Africander et al. 2011).

Agonistic effects are generally accompanied by hyper-phosphorylation of the receptor and a conformational change, which enables coactivator binding. An antagonist does not induce hyper-phosphorylation of the receptor and leads to a different conformational change, resulting in corepressor recruitment. (Africander et al. 2011; Nettles and Greene 2005; Schaaf and Cidlowski 2002).

Furthermore, in numerous vertebrate organisms including humans and fish, the GR is expressed in two isoforms, GRα and GRβ, which are generated from the primary transcript by alternative splicing. Although the expression of GRα is ubiquitous, the receptor levels can vary organ- and cell specifically. Some organs express no or only very low mRNA levels of GRβ under physiological conditions and within the tissues that generate both isoforms, GRβ expression is much weakerthan that of GRα. The comparably widespread distribution of GRα can be explained by the circumstance that exclusively this isoform is responsible for transactivation and transrepression of target gene transcription, while GRβ cannot bind ligands and is thought to act as a dominant negative inhibitor of GRα-mediated effects that may modulate the GC sensitivity in target organs (Bamberger et al. 1995; Lewis-Tuffin et al. 2007; Norbiato 2013; Oakley and Cidlowski 2013; Pujols et al. 2002; Pujols, Mullol, and Picado 2007; Schaaf and Cidlowski 2002; Weikum et al. 2017). The two GR isoforms can be expressed tissue-specifically, which leads to differential recruitment of coactivators and corepressors and can induce highly specific responses to GC binding, depending on the ligand characteristics and on the cellular context (Africander et al. 2011; Heitzer et al. 2007).

1.2.5 Anti-inflammatory effects of glucocorticoids

Due to their immunomodulatory properties GCs can suppress and stimulate immune reactions via transcription repression of inflammatory genes and induction of anti-inflammatory gene expression and protein production (Adcock et al. 2004; Besedovsky et al. 1975; Brown, Smith, and Blalock 1987; Gwosdow, Kumar, and Bode 1990; Silverman et al. 2005).It is considered that GCs mediate their anti-inflammatory properties primarily via tethering to proinflammatory

transcription factors and transcription suppression of their target genes (Adcock et al. 2004; Heitzer et al. 2007; Schaaf and Cidlowski 2002; Weikum et al. 2017).The glucocorticoid-induced leucine zipper (GILZ) protein is an important mediator of the anti-inflammatory effects of GCs and plays a major role in GC immunomodulation(Ayroldi and Riccardi 2009; Ronchetti, Migliorati, and Riccardi 2015; Scheschowitsch, Leite, and Assreuy 2017).

During immune challenges (e.g. infection, inflammation, injury, auto-immune responses), the immune system releases pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 or tumor necrosis factor (TNF)-α. These proteins mediate the immune response and recruit the transcription factors activator protein 1 (AP-1) or nuclear factor-κB (NF-κB) to bind to specific regulatory elements within the promotor region of pro-inflammatory genes in immune cells (Adcock et al. 2004; Epstein, Barnes, and Karin 1997).
AP-1 and NF-κB recruit other transcriptional cofactors, which assemble as a multi-protein complex within regulatory regions and enable the transcription of pro-inflammatory genes (Adcock et al. 2004; Heitzer et al. 2007; Schaaf and Cidlowski 2002; Weikum et al. 2017).
In order to suppress pro-inflammatory responses, cytokines can also induce the release of Cort by acting at all three levels of the HPA axis. Cort binds to the GR of immune cells, whereupon the ligand-receptor complex translocates to the regulatory regions of the transcriptionally active pro-inflammatory gene. The GR tethers to AP-1 or NF-κB, triggers histone deacetylation via cofactor and corepressor recruitment and thus suppresses the expression of the inflammatory gene (Adcock et al. 2004; Heitzer et al. 2007; Schaaf and Cidlowski 2002; Weikum et al. 2017).
In this way, Cort can suppress the production of more cytokines and their release via a negative feedback mechanism, and it maintains via the action of the HPA axis a critical balance between the beneficial and adverse effects of pro-inflammatory cytokines and the downstream immunomodulation. This prevents the organism from threatening effects of an overactive immune response (Kapcala, Chautard, and Eskay 1995; Munck, Guyre, and Holbrook 1984; Silverman et al. 2005; Silverman, Pearce, and Miller 2003).

1.2.5.1 Glucocorticoids in medical applications

Synthetic GCs are produced in large amounts and with broad diversity in order to mimic the immunosuppressive and anti-inflammatory properties of Cort for the treatment of inflammatory and autoimmune diseases. Today, they belong to the most widely prescribed drugs in the world. They were therapeutically applied over the last half century against numerous diseases including asthma, allergies, rheumatoid arthritis, sepsis, ulcerative colitis, multiple sclerosis and lymphatic cancer (Busillo and Cidlowski 2013; Miner, Hong, and Negro-Vilar 2005; Oakley and Cidlowski 2013; Rhen and Cidlowski 2005; Schaaf and Cidlowski 2002).Besides their anti-inflammatory applications, synthetic GCs are also used to treat individuals suffering from adrenal insufficiency, where the adrenal glands do not produce enough Cort endogenously (Oelkers 1996).

1.2.5.2 Side effects of glucocorticoids in medical applications

Unfortunately, long-term use of GC treatment can be accompanied by severe side effects, which limits their therapeutic benefits. Chronic intake of synthetic GCs may lead to diabetes,

abdominal obesity, skin atrophy, glaucoma, osteoporosis, hypertension and retarded growth in children (Gupta and Bhatia 2008; Louw-du Toit et al. 2017; Miner et al. 2005; Oakley and Cidlowski 2013; Rhen and Cidlowski 2005). Often these symptoms are a consequence of misguided homeostasis of endogenous hormones and their physiological functions. This ability of synthetic GCs to interfere with processes that are normally regulated by natural hormones is defined as endocrine disruption. For instance, treatment with the synthetic GCs Dexamethasone and Prednisolone was described to disrupt the biosynthesis of steroid hormones via suppression of the HPA axis(Gupta and Bhatia 2008; Louw-du Toit et al. 2017).
In order to develop novel, safer GCs with higher therapeutic benefits and lower side effects and to better understand beneficial mechanisms of action of GCs, medical and pharmacological research institutions undertake considerable efforts (Miner et al. 2005; Oakley and Cidlowski 2013; Rhen and Cidlowski 2005).

1.3 Endocrine disruption

Endocrine disruption per definition is induced by “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub)populations” (WHO 2002). Inherent in this definition is an extremely wide margin of possible effects of endocrine disruption, which may be mediated by numerous divergent pathways within an organism. Concern about possible health risks by EDCs is furthermore raised by studies in animal models and clinical observations in humans, which indicated EDC interference with metabolism, reproductive, neuroendocrine and cardiovascular systems, and their potential to trigger obesity and cancer development (Diamanti-Kandarakis et al. 2009).

Synthetic compounds that were designed to mimic the functions of natural steroid hormones are *per se* endocrine disruptors, as it is their main task to take over hormone-regulated effects. Drugs containing synthetic GCs for the applications described in section **1.2.5.1** belong to these intended endocrine disruptors, together with synthetic derivatives of progestogens, estrogens, androgens and mineralocorticoids that are applied in medications for contraception, hormone replacement, cancer therapy, adrenal insufficiency, heart failure, kidney disease, high blood pressure or low blood potassium (Africander et al. 2011; Gupta and Bhatia 2008; Louw-du Toit et al. 2017).
However, also agents without an intended direct function on the hormonal system can have endocrine disruptive effects, often mediated by similarities with steroid hormones in their chemical structures or characteristics. Among those are pesticides, plastics, plasticizers, parabens, industrial chemicals and fuels(Craig, Wang, and Flaws 2011; Diamanti-Kandarakis et al. 2009).

1.3.1 Mechanisms of glucocorticoid signaling disruption

According to the Environmental Protection Agency (EPA), endocrine disruptive chemicals (EDCs) can interfere with synthesis, secretion, transport, metabolism, receptor binding and elimination of endogenous hormones (Craig et al. 2011; Hotchkiss et al. 2008).

1.3.1.1 Direct interference with GR signaling

The best-studied mechanism of endocrine disruption is direct compound interference with steroid hormone receptor-mediated signaling. EDCs can bind to steroid hormone receptors and agonize or antagonize their transcriptional function on target gene expression, irrespective of whether the receptor acts via transactivation, transrepression, tethering or other mechanisms. Thus, all factors involved in GR mediated regulation of transcription are possible targets for EDCs.

The ligand-receptor binding affinity and nuclear localization can be affected by FKBPs, such as FKBP51 and FKBP52, which belong to the group of immunophilins. These immunosuppressive-drug-binding proteins can increase the GR response to ligands by inhibiting steroid export molecules and, thus, elevate the intracellular ligand concentration and GR activity. Furthermore, they can stimulate GR hormone binding affinity and translocation to the nucleus. Immunosuppressive ligands such as synthetic GCs can enhance the GR ligand-binding affinity and translocation via substitution of the negative regulator FKBP51 for the positive regulator FKBP52 within the receptor-protein heterocomplex (Davies, Ning, and Sánchez 2002, 2005; Mazaira et al. 2015).

The translocation of the ligand-receptor-complex into the nucleus can also be prevented by other mechanisms, for instance via the cytokines IL-2 and IL-4, which induce the p38 mitogen-activated protein kinase (MAPK) to phosphorylate the GR and reduce its nuclear import, a process which leads to GC resistance during chronic inflammatory disease (Irusen et al. 2002). Other MAPK proteins involved in nucleocytoplasmic GR shuttling can in turn enhance the nuclear export of the GR, which reduces the transcriptional efficiency and induces the hormone-dependent downregulation ofthe GR (Liu and DeFranco 2000).

Hormone-dependent GR downregulation can be mediated via autoregulatory transrepression of the GR gene. This mechanism often underlies GC resistance in response to long-term medication of chronic inflammations with synthetic GCs (Corrigan et al. 1991; Okret et al. 1986; Ramamoorthy and Cidlowski 2013; Rosewicz et al. 1988; van Rossum and Lamberts 2006; Schaaf and Cidlowski 2002). However, also other steroid hormone receptors can trigger GR downregulation. For instance, receptor crosstalk between the GR and the ER has been shown in human breast-cancer cell lines, where estrogen agonists provoked GR degradation (and inhibited GR target gene expression)(Kinyamu and Archer 2003).

Also the ligand-specific recruitment of coactivators and corepressors (e.g. SMRT) can affect the GR transcriptional efficiency and signaling response (Heitzer et al. 2007). For example, overexpression of SMRT can counteract coactivator effects on GR-mediated target gene transcription (Szapary, Huang, and Simons 1999).

1.3.1.2 Disruption of GC biosynthesis

EDCs can moreover prevent the production and release of all classes of steroid hormones via the HPA axis. Some EDCs (e.g. azole fungizides such as prochloraz or triazine herbizides such as atrazine), are capable to disrupt the steroid hormone biosynthesis by inhibition of steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase or 11β-hydroxylase (for an overview of the steroid hormone biosynthesis pathway see **Figure 3**) (Louw-du Toit et al. 2017; Sanderson 2006). Also other enzymes, regulating the availability of active and inactive forms of steroid hormones can be affected, for instance the enzyme 11β-hydroxysteroid dehydrogenase 2, which converts active Cort into inactive Cortisone (**Figure 3**)(Louw-du Toit et al. 2017; Miller 1988; Sanderson 2006).

1.3.1.3 Disruption of GC homeostasis via drug metabolism

Several enzymes involved in steroid hormone biosynthesis belong to the group of CYPs. While steroidogenic enzymes metabolize only one or a few endogenous substrates, other members of the CYP family are capable to catalyze reactions with multiple substrates of both endogenous and exogenous origin. These enzymes are responsible for the hormonal homeostasis and induce the deactivation and excretion of xenobiotics.The group of xenobiotics comprises all substances that either normally do not occur within the organism or are produced naturally in much lower concentrations. The most abundant CYPs involved in vertebrate drug and steroid metabolism belong to the CYP1, CYP2 and CYP3 families, which is the reason why numerous pharmaceuticals are designed to decrease the transcription and activity of these enzymes (Zanger and Schwab 2013).

Possible targets for such pharmaceutical actions are ligand activated transcription factors of the nuclear receptor superfamily, which regulate the expression of CYP enzymes upon xenobiotic and steroid binding. Predominantly, the pregnane x receptor (PXR), aryl hydrocarbon receptor (AHR) and constitutive androstane receptor (CAR) are involved in the transcriptional regulation of xenobiotic enzymes, but also other nuclear receptors such as the GR or the vitamin D receptor (VDR) may interfere with the expression of CYP genes (Bainy et al. 2013; Goodwin et al. 2001; Kliewer et al. 1998; Moore and Kliewer 2000; Pascussi et al. 2000).

Thus, it depends on the chemical properties of thepharmaceutical to which extent its uptake triggers the transcription and activity of xenobiotic enzymes. This may result in a fast breakdown and elimination of the causative substance, but also in alterations ofendogenous hormone homeostasis. It is therefore another pathway by which drugs may disrupt steroid hormone signaling (Creusot et al. 2015; Hotchkiss et al. 2008).

1.4 Endocrine disruptors in the environment

Most of the endocrine disruptive effect pathways are highly conserved among all vertebrate groups. Thus, EDCs can be threatening not only to humans, but also to wildlife. Especially aquatic organisms are affected, as EDCs can enter the aquatic environment via various entry paths. Municipal and hospital wastewaters, containing incompletely removed drug residues and their metabolites, constitute the major source for human pharmaceuticals in surface waters (Christen et al. 2010; Corcoran et al. 2010; Fent et al. 2006). Additional contributions to the environmental pollution of aquatic systems derive from discharges by chemical and pharmaceutical industries (Larsson 2014), from veterinary pharmaceuticals in aquaculture, and from manure produced by life stock, which is distributed on farmlands together with pesticides, causing both to enter surface waters via runoff and drainage (Christen et al. 2010; Colborn, vom Saal, and Soto 1993; Corcoran et al. 2010; Durhan et al. 2006; Fent 1996; Fent et al. 2006; Hotchkiss et al. 2008; Hutchinson et al. 2005; Jobling and Tyler 2003; Kugathas and Sumpter 2011; Larsson et al. 1999; Oehlmann et al. 2009; Runnalls et al. 2010; Tyler, Jobling, and Sumpter 1998).

Accordingly, human and veterinary pharmaceuticals represent a major part of environmental EDCs. Among the most commonly applied pharmaceuticals are anti-inflammatory drugs, antibiotics, steroids and lipid regulators (Daughton and Ternes 1999; Fent et al. 2006; Halling-Sørensen et al. 1998). Drug residues from these therapeutic classes were measured in effluents from sewage treatment plants in concentrations within a range of ng/L to µg/L, and concentrations in the range of ng/L were measured for fresh- and seawater systems (Christen et al. 2010; Daughton and Ternes 1999; Fent et al. 2006; Halling-Sørensen et al. 1998).

As most pharmaceuticals are designed for oral ingestion and have a nonpolar structure, they pass biological membranes easily via diffusion and are taken up by aquatic organisms via their dermal and gill surfaces. Therefore, both the designated pharmacological target pathways of drugs as well as their other, potentially not-yet characterized, side-effect mechanisms of action constitute health risks to aquatic organisms. Up to now, it is difficult to estimate which pharmaceuticals may pose a threat and which are of no environmental concern, but further elucidations of drug mechanisms can improve this understanding for environmental and human risk assessment.

1.4.1 Biotests for the assessment of endocrine disruption

For human risk assessment of pharmaceuticals, several *in vitro* bioassays are available, applying transfected cell lines in order to detect either steroid hormone receptor agonism and antagonism (e.g. OECD 455, OECD 457, Yeast Estrogen/Androgen Screening [YES/YAS] Tests) or the production of estrogens and androgens, as e.g. in the H295R Steroidogenesis Assay (OECD 456). However, *in vitro* models have a limited value for risk assessment as they represent an artificial, cell-specific scenario where drug effects may be completely different from their *invivo* responses in different organs, tissues and cells, due to varying availabilities and activities of ligands, receptors, enzymes or other proteins involved.

Hence, *in vivo* models constitute more reliable test systems, as they include all the above-mentioned variations and reflect realistic effects, resulting from the organism-specific metabolic and endocrine response to drug pharmacodynamics and kinetics. For human and veterinary pharmaceutical testing, rodents are the preferred model organism for many ethical and economical reasons. Several *in vivo* assays were therefore established for endocrine disruption testing in rodents, including for instance the Hershberger Bioassay in Rats (OECD 441), the Uterotrophic Bioassay in Rodents (OECD 440) and one- or two-generation studies (OECD 415 and OECD 416).
However, although metabolic and endocrine physiological pathways are highly conserved among all vertebrate groups, some endocrine processes and reproductive functions vary from one species to another and the extrapolation of EDC effects in animals to human risk assessment has to be considered carefully (Habert et al. 2014; Hult et al. 2004).

1.4.2 Environmental risk assessment for endocrine disruptors

Pharmaceuticals with endocrine active properties and other EDCs are also within the focus of environmental risk assessment. In the last three decades, numerous laboratory and field studies have been conducted to assess the effects of all kinds of EDCs on aquatic organisms (for details see reviews by Christen et al. 2010; Corcoran et al. 2010; Fent et al. 2006; Hotchkiss et al. 2008). However, as for human risk assessment, the applied test systems are primarily designed to detect direct disruption of sexual steroid hormones (estrogens, androgens, progestagens) and are based on endpoints related to hormone and protein levels, sexual development and reproduction of the organism (e.g. OECD 229, OECD 230, OECD 231, OECD 234).

1.4.3 Requirement for new testing strategies for endocrine disruption

The *in vivo* assessment of reproduction-related endpoints nowadays requires long-term studies with higher developmental stages of mammals and aquatic vertebrates (e.g. OECD 229, OECD 230, OECD 231, OECD 234, OECD 415, OECD 416, OECD 440, OECD 441). These studies are time-cost- and animal-intensive and disregard the 3Rs principles of reduction, refinement and replacement of animal testing (Russell and Burch 1959).
Furthermore, the evaluation of pharmaceutical interference with other groups of steroid hormones than the sex steroids, such as GCs, is badly covered by the regulations for human and environmental risk evaluation and the available assays. The assessment of the above mentioned reproduction-related endpoints may still ignore the potential of a drug to interrupt metabolic pathways and to indirectly affect sexual development and the fitness of organisms and populations. Certainly, the evaluation of such complex mechanisms further highlights the limitations of *in vitro* systems as appropriate options for alternative testing methods.
New *in vivo* testing strategies are therefore urgently needed for endocrine disruption risk assessment, which should enable to elucidate also metabolism- and steroidogenesis-based effects, but which would equally reduce and refine animal testing and contribute to the 3Rs principles.

1.4.4 Zebrafish as a potential model organism for endocrine disruption testing

Zerafish (*Danio rerio*) constitute a good model organism for endocrine disruption testing, as they offer numerous ethical and economical advantages compared to other model organisms. Furthermore, the zebrafish is an established test system for genetic modifications, as its genome has been completely sequenced and numerous genetic tools are available. The already established zebrafish reporter lines for hormone signaling of e.g. GC, estrogenic and thyroid hormone signaling are promising tools for the elucidation of endocrine effects, as they allow short-term assessment of hormonal disruptors already in larval stages (Benato et al. 2014, Krug et al. 2014, Lee et al. 2012, Weger et al. 2012, Gorelick and Halpern 2011, Terrien et al. 2011, Chen et al. 2010, Tong et al. 2009).

1.5 Zebrafish and their general advantages

Zebrafish are tropical freshwater fish that originate from the southeast of Asia (Bangladesh, India and Pakistan), where they live in stagnant or slow current water (e.g. paddy fields). They are easy to cultivate due to their small size (4-5 cm) and low aggressiveness. Moreover, the short generation cycles, year-round fecundity and large progeny numbers of zebrafish enable continuous and high-throughput test applications (Delvecchio, Tiefenbach, and Krause 2011; Rennekamp and Peterson 2015).
Already after 48-72 hours post fertilization (hpf) zebrafish larvae start to hatch, and their organogenesis is mostly completed at 120 hpf. This rapid, external development of zebrafish embryos and larvae makes them highly suitable for genetic and chemical manipulations, which can be monitored easily by live imaging techniques thanks to their transparency (Keller 2013, Kaufman, White, and Zon 2009, Tiso, Moro, and Argenton 2009).Because of these advantages, the zebrafish has become a well-established vertebrate model organism in numerous biological fields (Braunbeck et al. 2005; Scholz et al. 2008).

1.5.1 Zebrafish in environmental risk assessment

Within environmental risk assessment, several bioassays for the detection of toxic effects are conducted with zebrafish nowadays, instead of using larger fish species such as rainbow trout (*Oncorhynchus mykiss*) or common carp (*Cyprinus carpio*). These local fish species were for a long time un-replaceable especially for the risk evaluation of pesticides, but the economic advantages of zebrafish progressively have paved the way for the small fish into aquatic ecotoxicology testing (e.g. OECD 203 and OECD 215 with zebrafish have obtained regulatory approval).

Furthermore, toxicity assays for the detection of chemical impacts nowadays can be applied on zebrafish larvae and juveniles instead of requiring studies with adult fish (e.g. OECD 203 can be replaced by OECD 236 in several cases). As developing fish stages are more sensitive to chemical exposure than adults, application of such assays enables more conservative assessment of acute toxicity (Braunbeck and Lammer 2006). Moreover, the replacement of adult fish by embryonic

and larval stages contributes to the reduction of animal suffering, as their mental and physical concerns may be deemed less ethically problematic than those of full-grown fish.

Regarding the environmental risk assessment for endocrine disruptors, zebrafish studies with adult stages are used to investigate abnormalities of sexual development and reproduction (OECD 229, OECD 230, OECD 234). As these endpoints require sexual maturity, which is reached in zebrafish the earliest after 2 months, these conventional testing approaches cannot be conducted with larval stages.

1.5.2 Zebrafish larvae for endocrine disruption testing

Besides their use in the regulatory framework of environmental and EDC risk assessment, zebrafish have emerged as important model organisms in the field of endocrinology in recent years. They have been used successfully in studies of endocrine physiology for the elucidation of embryonic development of various endocrine glands (Lohr and Hammerschmidt 2011, Pogoda and Hammerschmidt 2009, Tiso, Moro, and Argenton 2009). Furthermore, many aspects of the hormone system and the impacts of diverse hormone groups could already be studied in larvae(Weger et al. 2012; Terrien et al. 2011; Alsop and Vijayan 2009; Dickmeis et al. 2007).

Substantial research has also been conducted with zebrafish larvae in order to assess endpoints related to sexual development. Changes in steroid hormone levels and the expression of enzymes and marker genes have been shown to be measurable upon EDC treatment already in zebrafish larvae (Lee et al. 2012; Menuet et al. 2005; Muncke and Eggen 2006; Schiller et al. 2013; Scholz et al. 2008). According to recent studies are endocrine markers already detectable in 48 hpf old embryos (Trant et al., 2001; Bardet et al., 2002; Lassiter et al., 2002; Lassiter and Linney, 2007; Kim et al., 2009; Sassi-Messai et al., 2009; Gibert et al., 2011).

1.5.3 Zebrafish larvae for the examination of GC signaling disruption

Zebrafish larvae represent an equally suitable tool for the evaluation of pharmaceutical interference with other groups of steroid hormones, i.e. GCs. Due to the phylogenetical conservation of the endocrine system among all vertebrate groups, zebrafish also possess an HPA axis, which is termed hypothalmic-pituitary-interrenal (HPI) axis in fish. As the human adrenal gland sits on top of the kidneys and fish do not develop kidneys in the way mammals do, their adrenal gland is more of a diffuse tissue which is spread out through the front end of the kidney and, thus, termed interrenal gland instead of adrenal.
Between 96-120 hpf, the HPI axis is fully developed in zebrafish larvae and all major hormonal pathways are already completely functional by this time (Pijanowski et al. 2015). This allows to elucidate functions of the GC system already at these developmental stages. Another advantage of zebrafish for GC signaling disruption testing is that they possess only one GR, like mammals, in contrast to many other teleost fish species which form two GRs due to a gene duplication within their genomes during evolution. Moreover, (zebra-) fish share with humans and most other mammals Cort as the main active GC, which is released in fish by the interrenal glands. In

rodents, however, corticosterone is the major endogenous ligand of the GR (Anacker et al. 2011). Considering the generally high conservation of steroidogenic and metabolic pathways in all vertebrates (McGonnell and Fowkes 2006) and the advantages of testing with zebrafish larvae compared to rodent studies, this close analogy of the fish and human GC system adds up one more argument for the application of zebrafish larvae for research regarding endocrine disruption of GC signaling.

For the assessment of potential compound effects on xenobiotic metabolism are zebrafish equally well suited. For instance, one of their major xenobiotic enzymes, which is expressed in the small intestinal and liver, is CYP3A65, the analog to human CYP3A4 (Bainy et al. 2013). Gene expression of these two members of the CYP3A subfamily is regulated by the same nuclear receptors (PXR, CAR, VDR, GR) and can be induced by the same ligands, e.g. by the synthetic GC Dexamethasone (Burk and Wojnowski 2004; Creusot et al. 2015; Goodwin et al. 2001; Moore and Kliewer 2000; Tseng et al. 2005).

Accordingly, zebrafish larvae possess all the endocrine and metabolic physiological requirements for the elucidation of complex disruptive effects of the GC system. They enable to detect compound interference with GR-mediated signaling, with the endogenous production of steroid hormones via the HPA axis and via steroidogenic enzymes, and with the xenobiotic metabolism. Therefore, zebrafish larvae constitute an excellent *in vivo* model for human and environmental risk assessment of GC signaling disruption.

Recently, research groups investigating the effects of synthetic GCs on zebrafish development showed morphological effects, which are similar to phenotypes observable in mammals with endogenous GC overproduction or after long-term administration of synthetic GCs.

For instance, adult zebrafish showed an osteoporosis phenotype in regenerating scalar bone when treated with 25 µM Prednisolone, a synthetic GC which has been repeatedly detected in the aquatic environment (de Vrieze et al. 2014).

At environmentally relevant concentrations (0.006-42 µg/l), in adult zebrafish, exposed for 21-daysto Fludrocortisone acetate (FLU), another commonly prescribed corticosteroid, alterations in plasma glucose levels and the circadian rhythm network, as well as reduced numbers of blood leukocytes (immune cells) were found even at 42 ng/l (Zhao, Zhang, and Fent 2016). The group showed furthermore that early markers for such morphological changes can already be found in zebrafish embryos. The progeny of GC-treated adults (F1 embryos) revealed significant behavioral and developmental changes, with increases in heartbeat, hatching success and swimming activity at 6 and 42 ng/l. These effects were also observed in eleuthero-embryos when exposed to FLU at 81 ng/l. Furthermore, significant changes in the transcription of biomarker genes involved in gluconeogenesis, circadian rhythm and immune response enabled to confirm the observed effects from adults already in embryonic stages.

Another group observed similar effects in zebrafish embryos when exposed to prednisolone [0.1-10 ng/l]. Treated embryos showed alterations in ontogeny and behavior, with a reduced frequency of spontaneous muscle contractions at 24 hpf and increased escape response in response to a mechanosensory stimulus at 48 hpf [0.1 µg/l]. Also, hatching success was

increased [1 and 10 µg/l] and a higher heart rate and oxygen consumption was measurable in zebrafish embryos (McNeil, Nebot, and Sloman 2016).
Furthermore, Hidasi (2016) observed that 5 dpf zebrafish larvae exposed to 0.1 nM of the highly potent synthetic GC Clobetasol propionate showed a significantly reduced inflammatory response when inflammation was induced by bacterial lipopolysaccharides (LPS). Also, the expression of several anti-inflammatory and GC-mechanism-related genes was significantly altered in these larvae when treated with Clobetasol propionate at 0.05 nM.

These studies highlight that synthetic GCs can alter the endocrine system of zebrafish, leading to morphological and behavioral effects which show analogies to those observable in humans. Accordingly, zebrafish are susceptible to GC signaling disruption and already in embryonic stages, showing behavioral abnormalities and developmental changes on the morphological, as well as on the genetical level upon GC treatment in environmental relevant concentrations. This constitutes zebrafish larvae as a suitable model to investigate disruption of the GC system also by other compounds.

1.6 A new bioassay for the detection of glucocorticoid activity

In order to develop a new testing strategy for the detection of GC signaling disruption, the zebrafish was selected as model organism due to its numerous advantages compared to other model organisms. Especially the broad spectrum of tools for genetic modification in zebrafish, which easily enables the generation of transgenic reporter lines, constitutes an opportunity to develop new *in vivo* testing strategies with straightforward endpoints and readouts.

1.6.1 Transgenic *Tg(GRE:Luc)* zebrafish line

Therefore, our working group has developed a transgenic zebrafish line which is able to detect GC signaling (Weger et al. 2012). The *Tg(GRE:Luc)* zebrafish line (**Figure 6**) carries a transgenic construct that expresses a luciferase reporter gene under the control of four concatenated GREs and a minimal TATA-box promotor (P_{min}). A poly(A) tail (pA) within the transgenic construct enhances transcriptional efficiency of the reporter gene and the whole reporter construct was stably integrated into the zebrafish genome via the Tol2 transposon (Tol2) system. Natural and synthetic GR agonists can activate the endogenous GRs in zebrafish and induce ligand-receptor binding to the 4xGREs. Thereupon, reporter gene transcription and expression of luciferase is induced. Luciferase catalyzes the oxidation of luciferin (added to the medium) that leads to the emission of a bioluminescent signal, which can be captured by a bioluminescence reader.

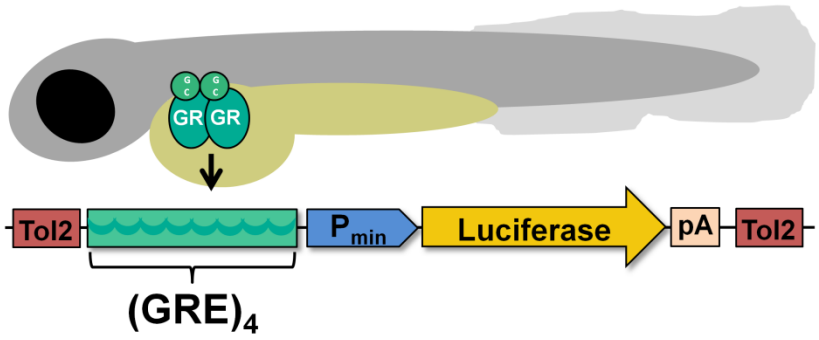


Figure 6: Transgenic zebrafish line *Tg(GRE:Luc)* for the detection of GC activity
A luciferase reporter gene is under the control of four concatenated GREs [$(GRE)_4$] and a minimal TATA-box promotor (P_{min}). (pA), poly (A) tail The whole transgenic construct is flanked by Tol2 sites (Tol2) for integration into the zebrafish genome mediated by the Tol2 transposase. GR agonists activate endogenous GRs and initiate ligand-receptor binding to the $(GRE)_4$. Luciferase expression is induced and light-emitting enzymatic activity of luciferase can be captured by a bioluminescent reader.

1.6.2 Transgenic zebrafish cell line AB.9-*GRE:Luc*

In order to obtain also a cell-based reporter, which carries the same reporter cassette as the *Tg(GRE:Luc)* zebrafish line, zebrafish AB.9 cells, originally derived from fin fibroblast tissue, were stably transfected with the *GRE:Luc* reporter (Weger et al. 2012). Parallel measurement of a compound in transgenic larvae and in AB.9-*GRE:Luc* cells enables comparison of *in vivo* with *in vitro* effects, which may allow to distinguish cell autonomous from whole animal effects (e.g. organ, tissue or cell type specific responses, metabolism or cross talk between organs).

1.6.3 The Glucocorticoid Responsive *In vivo* Zebrafish Luciferase activitY (GRIZLY) assay

Based on these two established reporter systems an assay principle was developed which facilitates high-throughput screening and allows comparison of *in vivo* with *in vitro* effects. As shown in **Figure 7,** transgenic larvae and cells are distributed into 96-well plates and incubated in luciferin-containing medium before they are treated with a compound library. Immediately after the start of the treatment, the 96-well plates are placed into the bioluminescent reader and the emitted bioluminescent signal is traced for a certain time period (e.g. 24 h). The GRIZLY assay has been shown in a former study to specifically re-identify known (*bonafide*) activators of GCsignaling from an FDA approved drug library and to detect Pregnenolone induced Cort production *in vivo* (Weger et al. 2012).

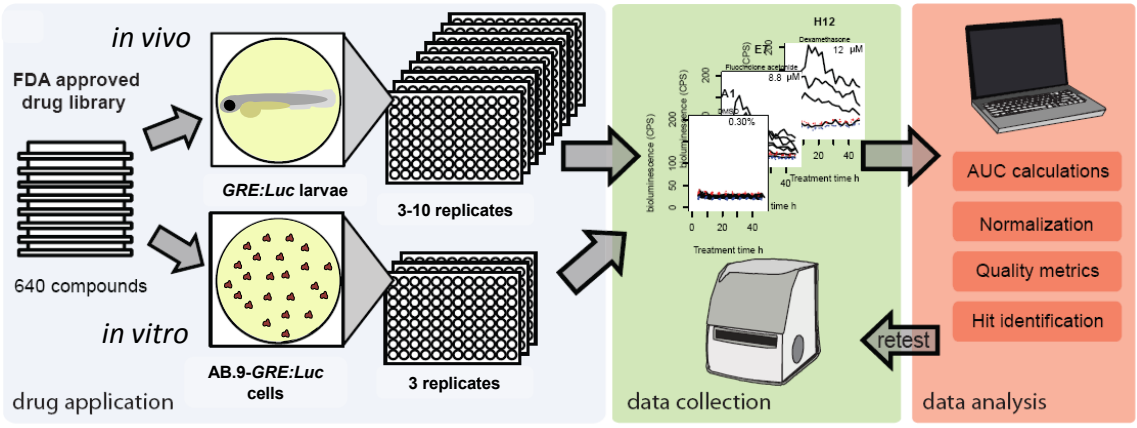


Figure 7: Principle of the GRIZLY assay for screening procedures
Application of *Tg(GRE:Luc)* zebrafish and AB.9-*GRE:Luc* cells allows comparison of *in vivo* with *in vitro* effect. Larvae and cells are distributed into 96-well plates, incubated in luciferin-containing medium and treated with a drug library for high-throughput screening. A bioluminescence reader measures the reporter bioluminescence over time. Conversion of the data into area under the curve (AUC) values enables straightforward data analysis and statistical evaluation.

1.7 Aim of the study

The GC system has a broad physiological role as it regulates various important processes within the body such as cell cycle, development, metabolism, immune- and stress-response. Pharmaceuticals are likely to interfere with the GC system, which may lead to disruption of GC signaling pathways and can be accompanied by side-effects, which are often not well-elucidated. As disruption of the GC system is badly covered by human and environmental risk assessment and no appropriate *in vivo* biotests are available so far to target these mechanisms, it is the major objective of this project to establish a suitable test system for the detection of GC signaling disruption. This system will extend research regarding chemical interference with GC signaling and help to investigate the mechanisms underlying endocrine disruption.

For this purpose, the test design of the GRIZLY assay will be modified in order to detect inhibitors of GC signaling. Validation of this testing approach will further verify the specificity and reliability of the GRIZLY assay and its suitability to detect chemical interference with the GC system at diverse levels. Once validated, the assay will be applied to identify GC signaling disruptors from drug libraries. Confirmed hits will be further investigated for the mechanisms underlying their disruptive effects on GC signaling. By conducting different modes of the GRIZLY assay, it will be possible to pre-categorize compound inhibitory mechanisms into either direct interference with GR-mediated signaling or indirect disruption via inhibition of GC biosynthesis. In the latter case, putative inhibition of GC production will be followed-up by chemical analysis (UHPLC-MS/MS) of endogenous steroid levels. Furthermore, gene expression analysis (real-time qPCR experiments) of GC and xenobiotic targets will provide valuable information about the molecular mechanisms of identified compounds and may indicate the mechanistic origin of GC signaling disruption. This will help to verify hits with - so far - unknown potential to interfere with GC signaling and improve the understanding of drug non-target mechanisms of action and their side-effects on the GC system.

In summary, the aims of this study are,

1) to assess the suitability of the GRIZLY assay for the detection of GC signaling inhibitors,
2) to identify disruptors of the GC biosynthesis pathway and
3) to identify compounds with - so far - unknown potential to interfere with GC signaling or with - so far - unknown mechanisms of action,

with the long-term objective to establish with this reporter an alternative testing method for endocrine disruption of the GC system, which could contribute to the reduction, refinement and replacement (3Rs) of animal testing (Russell and Burch 1959).

Chapter 2

2. Material and Methods

2.1 Material

2.1.1 Instruments

96-channel pipette	LIQUIDATOR96®, Steinbrenner Laborsysteme GmbH
Analysis scale	ES 220A, 0.01-220 g (e = 1 mg; d = 0.1 mg), Precisa Gravimetrics AG
Analytical column	Kinetex 2.6u XB-C18 100 A, 100 x 2.1 mm, Phenomenex
Bioluminescence multilabel plate reader	Wallac EnVision™ 2104 (with stacker 2101-1010), PerkinElmer
Centrifuge (5-50 ml)	ZK 380, HermleLabortechnik GmbH
Centrifuge (1.5-2.0 ml)	Microcentrifuge 5417R, Eppendorf®
Counting chamber	Neubauer ZK06, A. Hartenstein GmbH
ELISA Microplate reader	VERSAmax, tunable, Molecular Devices
Evaporator	Vapotherm basis mobil II, Barkey GmbH & Co. KG
Fluorescence reader	Lambda Fluor 320 Plus, BioTek Instruments, Inc.
Microinjector	Femtojet express, Eppendorf
Micropipette puller	Sutter Instrument Company
RT-qPCR system	StepOnePlus system, Applied Biosystems
Spectrophotometer	NanoDrop™ 1000,Thermo Scientific
Stereo microscope	SMZ 645, Nikon
Tissue homogenizer	ULTRA-TURRAX®, IKA Works
UPLC-MS/MS	UHPLC: ExionLC™ AD; LC-MS/MS: API 4000™ System, both AB Sciex
Vortex	Genie 2, Scientific industries, Inc.

2.1.2 Software

Analyst® Software	version 1.6.3, AB Sciex
GraphPad Prism 6	GraphPad Software, Inc.
LAMBDA KC4	Kineticalc for Windows, MWG-Biotech A
NanoDrop™ Software	version 3.7.0, Thermo Scientific™
StepOne™ Software	version 2.3, Applied Biosystems
Wallac EnVision™ Manager	version 1.12, PerkinElmer

2.1.3 Consumables

6-well plates CELLSTAR®	# M8562	Greiner Bio-One
96-well PCR plate	# SL-AM0910C	Steinbrenner Laborsysteme GmbH
96-well plate CELLSTAR®, flat bottom	# M0812	Greiner Bio-One
96-well CulturPlate-96 (white)	# 6005680	PerkinElmer
96-well OptiPlate-96 (white)	# 6005299	PerkinElmer
96-well storage plates Abgene 0.8 ml	# AB0765	Thermo Fischer Scientific
adhesive seals TopSeal-A PLUS	# 6050185	PerkinElmer
borosilicate glass capillaries (GC100-10)	# 30-0016	Warner Instruments
brown glass vials1.5 ml, screw-thread	# VT1101211	Dr. R. ForcheChromatographie
cell culture flasks CELLSTAR® 25 cm^2	# 690170	Greiner Bio-One
cell culture flasks CELLSTAR® 75 cm^2	# 658170	Greiner Bio-One
Microloader	# 5242956.003	Eppendorf AG
needlesSterican® 0.45 x 25 mm G26	# 4657683	B. Braun Melsungen AG
PCR strips, 0.2 ml/well, clear	# SL-PSF08	Steinbrenner Laborsysteme GmbH
petri dishes 94/16 mm	# 633180	Greiner Bio-One
pipette tips 0.1-10 µl, barrier	# 70803	Corning
pipette tips 1-200 µl, barrier	# 70833	Corning
pipette tips 100-1000 µl, barrier	# 70853	Corning
polypropylene tube CELLSTAR® 50 ml	# 227261	Greiner Bio-One
polypropylene tube CELLSTAR® 15 ml	# 188261	Greiner Bio-One
qPCR plate seal, clear	# SL-AM0560	Steinbrenner Laborsysteme GmbH
reaction tubes 1.5 ml, colorless	# 0030120086	Eppendorf AG
reaction tubes 2.0 ml, colorless	# 0030120094	Eppendorf AG
reaction tubes 5.0 ml, colorless	# 0030119401	Eppendorf AG
silicone/PTFE caps	# CT11S3015	Dr. R. ForcheChromatographie
syringes Injekt® 2 ml	# 4606027V	B. Braun Melsungen AG

2.1.4 Solvents

The solvents used to dissolve chemicals for single compound testing, for molecular biological techniques and for UPLC-MS/MS experiments were purchased from different suppliers as indicated in Table 1.

Table 1: Solvents and liquid chemicals

Name	*CAS no.*	*Order no.*	*Supplier*
Acetonitril HiPerSolv CHROMANORM®	75-05-8	83.640.320	VWR International
Chloroform for analysis EMSURE®	67-66-3	102445	Merck
Dimethylsulfoxid	67-68-5	A994.2	Carl Roth GmbH & Co. KG
Ethanol absolute ≥99.8%	64-17-5	83672.290	VWR International
Ethyl acetate for analysis EMSURE®	141-78-6	1096231000	Merck
Formic acid for analysis 98-100% EMSURE®	64-18-6	100264	Merck
Methanol HiPerSolv CHROMANORM®	67-56-1	83.638.320	VWR International
2-Propanol, ROTIPURAN® ≥99,8%	67-63-0	6752.1	Carl Roth GmbH & Co. KG

2.1.5 Chemicals

The FDA approved drug library was purchased from ENZO Life Science (# BML-2841) and supplied in 96-well plates, each plate containing 80 compounds, dissolved in Dimethylsulfoxid (DMSO) at a concentration of 2 mg/ml. For screening procedures, the chemicals were diluted 1:50 with E3 medium, obtaining a concentration of 0.04 mg/ml and total volume of 2% v/v DMSO.

The compounds used for concentration range testing were purchased from different suppliers as indicated in **Table 2**. Stocks of 100 mM were prepared in DMSO and stored at –20°C.

Table 2: Compounds for concentration range testing

Name	*CAS no.*	*Order no.*	*Supplier*
10-Hydroxycamptothecin	64439-81-2	LKT-C0155.25	biomol
11-Ketotestosterone	564-35-2	K8250-5MG	Sigma Aldrich
Acitretin	55079-83-9	LKT-A0933.25	biomol
Amorolfine	78613-38-4	SML0283-10MG	Sigma Aldrich
Corticosterone	50-22-6	27840-100MG	Sigma Aldrich
Cortisone	53-06-5	C2755-250MG	Sigma Aldrich
Dehydroepiandrosterone	53-43-0	Cay-15728	biomol
Dexamethasone	50-02-2	D1756-100MG	Sigma Aldrich
DL-Aminoglutethimide	125-84-8	A9657-100MG	Sigma Aldrich
Erlotinib	183321-74-6	Cay10483-250	biomol
Estriol	50-27-1	E1253-100MG	Sigma Aldrich
Estrone	53-16-7	E9750-500MG	Sigma Aldrich
Ethisterone	434-03-7	46272-250MG	Omnilab
Etomidate	33125-97-2	E6530-10MG	Sigma Aldrich
Fenbufen	36330-85-5	F8755-5G	Sigma Aldrich
Flutamide	13311-84-7	F9397-1G	Sigma Aldrich
Galanthaminehydrobromide	1953-04-4	G1660-2MG	Sigma Aldrich
Gestrinone	16320-04-0	16320-04-0	Cayman Chem.
Hydrocortisone	50-23-7	H4001-1G	Sigma Aldrich
Ibudilast	50847-11-5	I0157-10MG	Sigma Aldrich
Ketoconazol	65277-42-1	K1003-100MG	Sigma Aldrich
Leflunomide	75706-12-6	Cay-14860	biomol

Levonorgestrel	797-63-7	L0551000	Sigma Aldrich
Melengestrolacetate	2919-66-6	33998-100MG-R	Sigma Aldrich
Methyltestosterone	58-18-4	69240-5G	Sigma Aldrich
Metyrapone	54-36-4	M2696-10MG	Sigma Aldrich
Mifepristone	84371-65-3	M8046-100MG	Sigma Aldrich
Nabumetone	42924-53-8	N0020000	Sigma Aldrich
Nisoldipine	63675-72-9	N0165-10MG	Sigma Aldrich
Norethindrone	68-22-4	N4128-50MG	Sigma Aldrich
Paroxetine	110429-35-1	LKT-P0297.25	biomol
Pioglitazone	MFCD00865504	CDS021593-50MG	Sigma Aldrich
Prednisolone	50-24-8	P6004-100MG	Sigma Aldrich
Prednisone	53-03-2	P6254-1G	Sigma Aldrich
Pregnenolone	145-13-1	P9129-1G	Sigma Aldrich
Progesterone	57-83-0	P0130-25G	Sigma Aldrich
RetinoicAcid	302-79-4	R2625-50MG	Sigma Aldrich
Spironolactone	52-01-7	S3378-1G	Sigma Aldrich
Stanozolol	10418-03-8	S7132-1G	Sigma Aldrich
Tranilast	53902-12-8	T0318-10MG	Sigma Aldrich
Trenbolone	10161-33-8	T3925-250MG	Sigma Aldrich
Vitamin A acetate	127-47-9	PHR1236-1G	Sigma Aldrich

2.1.6 Buffers, solutions and media

Buffers and solutions were purchased from different suppliers or prepared from powders as indicated in Table 3. Media were prepared with the ingredients listed in **Table 4** according to the given recipes.

Table 3: Buffers and solutions

Name	*Order no.*	*Supplier*
DPBS (1x), no calcium, no magnesium (PBS)	14190094	Gibco™
HBSS, calcium, magnesium, no phenol red	14025050	Gibco™
D-Luciferin stock, 50 mM		
Firefly, potassium salt dissolved in H_2O, at –80°C	L-8220	Biosynth
Methylen blue solution 1 g/l (0.1% w/v)		
Methylen blue dissolved in H_2O, at 4°C	M9140-100G	Sigma Aldrich
Triton™ X-100	X100-100ML	Sigma-Aldrich
Trypan blue	T8154	Sigma-Aldrich
Trypsin-EDTA (0.25%), phenol red	25200056	Gibco™
Phenol red solution	P0290-100ML	Sigma Aldrich

Table 4: Media

Name	*Composition*	*Order no.*	*Supplier*
E3 medium	5 mM NaCl	P029.2	Carl Roth GmbH + Co. KG
(60x)	0.17 mMKCl	6781.1	Carl Roth GmbH + Co. KG
	0.33 mM $CaCl_2$	CN93.2	Carl Roth GmbH + Co. KG
	0.33 mM $MgSO_4$ x H_2O	0261.1	Carl Roth GmbH + Co. KG
AB.9 cell	500 ml Leibovitz's L-15 Medium (red)	11415056	Gibco™
maintenance	100 ml (17% (v/v)) FBS	S0115	Biochrom AG
medium	100 U/ml Penicillin	15140148	Gibco™
	100 µg/ml Streptomycin	15140148	Gibco™
	50 µg/ml Gentamicin	15750045	Gibco™
AB.9-*GRE:Luc*	100 ml AB.9 cell maintenance medium		
medium	250 µg/ml Geneticin™ (G418 Sulfate)	11811064	Gibco™
E3-luciferin medium (larvae)	Luciferin stock diluted to 500 µM in E3 medium		
L-15-luciferin medium (cells)	Luciferin stock diluted to 500 µM in Leibovitz's L-15 Medium (clear)	21083027	Gibco™

2.1.7 Kits and reagents

All kits and reagents used for molecular- and cell-biological techniques are listed in Table 5.

Table 5: Kits and reagents

Name	*Order no.*	*Supplier*
alamarBlue® reagent	BUF012B	Bio-Rad Lab. Inc.
Cytotoxicity Detection Kit (LDH)	11 644 793 001	Roche Diagnostics
DNase I, RNase-free	M6101	Promega
FuGENE® HD Transfection reagent	E2311	Promega
GoTaq® qPCR Master Mix	A6002	Promega
mMESSAGE mMACHINE™ SP6 Transcription Kit	AM1340	Invitrogen™
pGL3-Control Vector	E174A	Promega
Random Hexamer Primer	SO142	Thermo Scientific™
RNAse free water	P1193	Promega
RNasin® Ribonuclease Inhibitor	N2511	Promega
SuperScript™ III Reverse Transcriptase	18080044	Invitrogen™
TRIzol™ Reagent	15596026	Invitrogen™

2.2 Methods

2.2.1 Zebrafish handling and general techniques

2.2.1.1 Zebrafish stock and maintenance

Wild-type zebrafish originate from the AB strain (University of Oregon, Eugen) and were bred for several years in the European Zebrafish Research Center (EZRC) in Karlsruhe. The transgenic line *Tg(GRE:Luc)*^{sb6} was generated in this genetic background. At the EZRC, zebrafish were maintained in glass aquaria in a recirculating system under flowthrough conditions, with a 14 : 10 light : dark photoperiod. Fish were kept in groups of up to 50 fish and cultured in fishwater that was pre-heated to 26 ± 2°C, UV-sterilized and activated charcoal and particle-filtered. They were fed twice a day with flake food, pellets and on-site hatched, live artemia.

2.2.1.2 Zebrafish breeding

Fish were bred and raised according to the procedures as described by Westerfield (1995). On the day before mating, groups of four male and four female zebrafish were transferred into small, two-compartment mousecages that were separated by a mesh. The next morning, immediately after the onset of light, male and female fish were put together into the upper compartment. Spawned eggs sank through the mesh and were collected from the lower compartment.

2.2.1.3 Culture and staging of embryos

The harvested eggs were cleaned from dirt and unfertilized or dead eggs in order to avoid contamination of the embryo culture. Groups of 30-50 eggs were transferred into 10 cm petri dishes (# 633180, Greiner Bio-One) containing E3 medium with methylene blue (approximately 1 mg/l) to prevent fungal infections. The petri dishes were kept in an incubator at 28°C, and each day dead embryos were removed and E3 medium was replaced until the start of the experiment. Larvae that were bred for raising were moved into fish tanks in the EZRC between 2-5 days post fertilization (dpf). Embryo developmental stages were determined on a stereo microscope according to Kimmel et al. (1995).

2.2.1.4 Generation of a new stable *Tg(GRE:Luc)* reporter line

The *Tg(GRE:Luc)*^{sb6} zebrafish reporter line was generated by Weger et al. (2012) and since then maintained in the EZRC. However, several generations of outcrossing probably weakened the transgenic construct, therefore, I injected the reporter plasmid anew in wild-type zebrafish eggs.

The *pT2-GRE:Luc* plasmid

The *pT2-GRE:Luc* plasmid was cloned by Weger et al. (2012). The vector carries two Tol2 transposable elements, which enable Tol2 transposase binding (Kawakami 2004). The Tol2 transposon system has been shown to facilitate genome integration of the reporter construct during zebrafish transgenesis (Suster et al. 2009).

Tol2 transposase RNA

For a successful integration of the *pT2-GRE:Luc* plasmid into the zebrafish genome via the Tol2 transposon system, Tol2 transposase RNA has to be supplemented to the injection mix. Capped Tol2 RNA was synthesized by digesting the pCS-TP plasmid (Kawakami et al. 2004) with NotI and transcribing it with the mMESSAGE mMACHINE™ SP6 Transcription Kit (# AM1340, Invitrogen™) according to the manufacturer's protocol. RNA quality was verified by agarose gel electrophoresis (gel: 1% agarose in TAE; 50 x TAE: 2 M Tris, 1 M glacial acetic acid, 0.05 M EDTA). Only RNA of high quality was used for microinjection.

Microinjection

Microinjection was conducted as described by Müller et al. (1999). Injection needles were produced from borosilicate glass capillaries (GC100-10, # 30-0016, Warner Instruments) with a micropipette puller (Sutter Instrument Company). Injection mixes containing 30 ng/µl of the *pT2-GRE:Luc* vector and 20 ng capped Tol2 transposase RNA were prepared and supplemented by 0.1% (v/v) phenol red, enabling the tracing of injection success and progress. The mix was injected with a microinjector (Femtojet express, Eppendorf) into one-cell stage zebrafish eggs. Embryos were raised in E3 medium supplemented with methylene blue until they were identified for transient expression of the *GRE:Luc* reporter construct.

Breeding

The injected larvae (4 dpf) were transferred into 96-well plates and luciferase reporter activity was assessed with a bioluminescence reader (Wallac EnVision™ 2104, PerkinElmer) according to the procedure described below (section **2.2.3** and **2.2.3.1**). Individuals showing transient reporter activity were raised and outcrossed with wild-type zebrafish. F1 larvae were screened for stable integration of the reporter gene construct and founders were isolated. Progeny of the founder showing the strongest reporter activity (Founder 1) was used to breed a new generation of *Tg(GRE:Luc)* zebrafish by in- and out-crossing them with wild-type. Notably, as before, when the transgenic line was first established by Weger et al. (2012), *Tg(GRE:Luc)* larvae showed no bioluminescence from endogenous GC levels, but responded specifically to GC treatment. Apparently, basal GC levels are not sufficient to induce the luciferase reporter gene transcription.

2.2.2 Cell culture

2.2.2.1 Cell lines and their maintenance

The AB.9 (ATCC, CRL-2298) zebrafish cells, originally derived from fibroblast tissue, were a kind gift of N. S. Foulkes. Stable AB.9-*GRE:Luc* cells were generated by Weger et al. (2012) according to the protocol of Vallone et al. (2007). The cell lines were cultured in their specific AB.9 and AB.9-*GRE:Luc* maintenance media, according to the descriptions by Vallone et al. (2007). Cells were kept in 75 cm² cell culture flasks (CELLSTAR®, # 658170, Greiner Bio-One) at 28°C and were passaged at a ratio of 1:10 once per week.

2.2.2.2 Generation of the AB.9-*pGL3-Control* cell line

As a control for compound interference with the luciferase reporter activity, AB.9 cells were transiently transfected with the pGL3-Control vector (# E174A, Promega), which drives constitutive luciferase expression. Cells were transfected with the FuGENE® HD Transfection reagent (# E2311, Promega) according to the manufacturer´s protocol. Briefly, cells were washed once with 1 x DPBS (PBS) and trypsinized (Trypsin-EDTA [0.25%], phenol red, # 25200056, Gibco™) for approximately 5 min at 27°C (day 1). Detached cells were transferred into a 50 ml polypropylene tube (CELLSTAR® # 227261, Greiner Bio-One) and the cell number was determined with the Trypan blue (# T8154, Sigma-Aldrich) exclusion method in a Neubauer counting chamber (ZK06, A. Hartenstein GmbH). The density was adjusted to 200 000 cells per ml by adding AB.9 medium to the culture. Cells were seeded into 6-well plates (CELLSTAR®, # M8562, Greiner Bio-One) with 800 000 cells per well in order to obtain 80% cell confluency after overnight incubationat 28°C. The next day (day 2), cells were washed with PBS and the AB.9 medium was replaced by culture medium free of antibiotics (L-15 red medium + FBS). The transfection mix was prepared with 100 µl L-15 medium, 1 µg vector DNA (1 µl) and 4 µl FuGENE® HD Transfection reagent. The reagent mixture was incubated for 15 - 20 min at RT to enable the formation of the transfection complex before it was added drop-wise to the cells. After overnight incubation at 28°C, the cells were washed with PBS, trypsinized and transferred into white 96-well plates (CulturPlate-96, # 6005680, PerkinElmer) with AB.9 medium (day 3). Cell attachment was allowed overnight before cells were measured for luciferase activity (day 4) as described below (in section **2.2.3.2**).

2.2.3 Bioluminescence measurements

Luciferase-expressing transgenic cells and zebrafish larvae, which are exposed to luciferin medium, can convert D-luciferin into oxyluciferin in a light-emitting reaction. A bioluminescent reader can capture the light and thereby quantify the luciferase activity. The EnVision bioluminescence multilabel plate reader, equipped with an enhanced luminescence sensitivity sensor (Wallac EnVision™, # 2104-0010A, PerkinElmer) and a stacker unit for measurements up to 50 plates (# 2101-1010, PerkinElmer), was used for *in vivo* luciferase activity measurements. The reader was setup in a separate dark room with a controlled temperature of 28°C. The 96-well plates carrying transgenic cells or larvae were covered with adhesive seals sealed (TopSeal-A PLUS, # 6050185, PerkinElmer) to prevent medium evaporation, and *in vivo* luciferase activity was monitored for 24 h using the Wallac EnVision™ Manager software (version 1.12, PerkinElmer).

2.2.3.1 Detection of glucocorticoid signaling in *Tg(GRE:Luc)* larvae

At 4 dpf, *Tg(GRE:Luc)* larvae were transferred into 96-well plates and incubated in E3-luciferin medium [0.5 mM] for 24 h. At 5 dpf, the larvae were treated with Dex [20 µM] and luciferase reporter activity was measured on the EnVision bioluminescence reader.

2.2.3.2 Detection of glucocorticoid signaling in AB.9-*GRE:Luc* cells

Stable AB.9-*GRE:Luc* cells were washed with PBS, trypsinized and the cell number was adjusted to 200 000 cells/ml as described in section **2.2.2.2**. In each well of a 96-well plate, 22 500 cells were seeded with 250 µl AB.9-*GRE:Luc* maintenance medium and allowed to attach over night. The next day, the cells were washed with PBS and incubated for 1 h at 28°C in 200 µl L-15-luciferin medium. Chemical treatments were applied according to the experimental setups as specified for screening procedures in **Figure 11** and for single compound testing in **Figure 12**. Subsequently, *in vivo* luciferase activity was monitored with the EnVision bioluminescence reader.

2.2.3.3 Compound effects on luciferase activity in AB.9-*pGL3-Control* cells

Transfected AB.9-*pGL3-Control* cells obtained as specified in section **2.2.2.2** were washed with PBS before 200 µl L-15-luciferin medium were added to each well of a 96-well plate. Cells were incubated at 28°C for 1 h before 50 µl of treatment solution were supplemented in order to achieve exposure conditions according to the scheme in **Figure 8**. Immediately after exposure start, bioluminescence was measured.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO		Mife [1 µM] + Dex [20 µM]		10-HC [20 µM] + Dex [20 µM]		Trani [50 µM] + Dex [20 µM]		Aci [20 µM] + Dex [20 µM]		Ret. acid [2 µM] + Dex [20 µM]	DMSO
B												
C												
D												
E	Dex [20 µM]											
F												
G												
H												

transient AB.9-*pGL3-Control* cells [0.2 % DMSO]

AB.9 cells [0.2 % DMSO]

Figure 8: Treatment scheme for the assessment of compound effects on luciferase activity
Compound effects on transiently transfected AB.9-*pGL3-Control* cells with constitutively active luciferase expression (orange) and on non-transfected AB.9 cells (grey) were assessed. Negative control treatments consisted of DMSO or Dex [20 µM]. The compound effects were assessed by exposing the cells to a treatment combination of Dex [20 µM] with one concentration each of Mifepristone (Mife [1 µM]), 10-Hydroxycamptothecin (10-HC [20 µM]), Tranilast (Trani [50 µM]), Acitretin (Aci [20 µM]), Retinoic acid (Ret. acid [2 µM]), which led to a strong decrease of luciferase activity in AB.9-*pGL3-Control* cells. The no-transfection control cells were treated with DMSO. The amount of DMSO was 0.2% (v/v) in all wells.

2.2.4. The GRIZLY assay

2.2.4.1 Assay principle

The FDA library was screened for disruptors of GC signaling with three different variations of the GRIZLY assay (**Figure 7**) as shown in **Figure 9**:

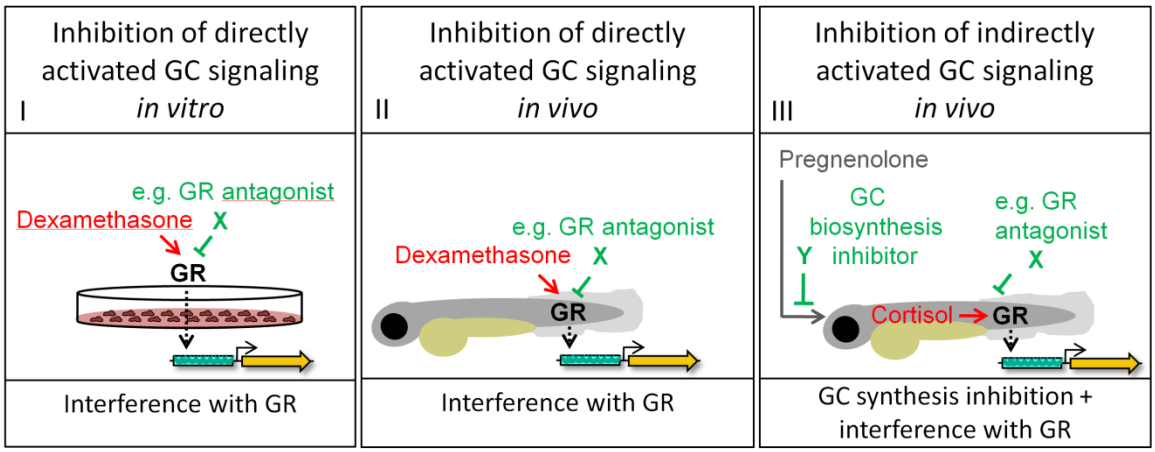


Figure 9: Three different approaches of the GRIZLY assay
I) GRIZLY 1: Direct activation of GC signaling in AB.9-*GRE:Luc* cells with Dex [20 µM]. Mife [1 µM] serves as a control inhibitor, acting via GR antagonism. **II**) GRIZLY 2: Direct activation of GC signaling in *Tg(GRE:Luc)* larvae with Dex [20 µM]. Mife [1 µM] serves as a control inhibitor, acting via GR antagonism. **III**) GRIZLY 3: Indirect activation of GC signaling in *Tg(GRE:Luc)* larvae with Preg [5 µM] as control activator and Mety [40 µM] as reference inhibitor.

GRIZLY 1 - Inhibition of directly activated GC signaling (with Dex) in AB.9-*GRE:Luc* cells (**I**)
GRIZLY 2 - Inhibition of directly activated GC signaling (with Dex) in *Tg(GRE:Luc)* larvae (**II**)
GRIZLY 3 - Inhibition of indirectly activated GC signaling (with Preg) in *Tg(GRE:Luc)* larvae (**III**)

2.2.4.2 Experimental setup for FDA drug library screening

The bioluminescence measurements with AB.9-*GRE:Luc* cells and *Tg(GRE:Luc)* larvae were conducted as described above (sections **2.2.3** - **2.2.3.2**). Deep-well plates with chemical stocks diluted in E3 medium were prepared for the screening procedures and treatment was carried out with a 96-channel pipette (**Figure 10**). All test plates were loaded with eight replicate wells of the positive (DMSO + Dex/ Preg) and the negative (Mife/ Mety + Dex/ Preg) control and one well per library compound. Each of the eight library plates was measured in three replicates in GRIZLY 1 and in three to ten replicates in GRIZLY 2 and GRIZLY 3, with a maximum of twelve test plates per screen run.

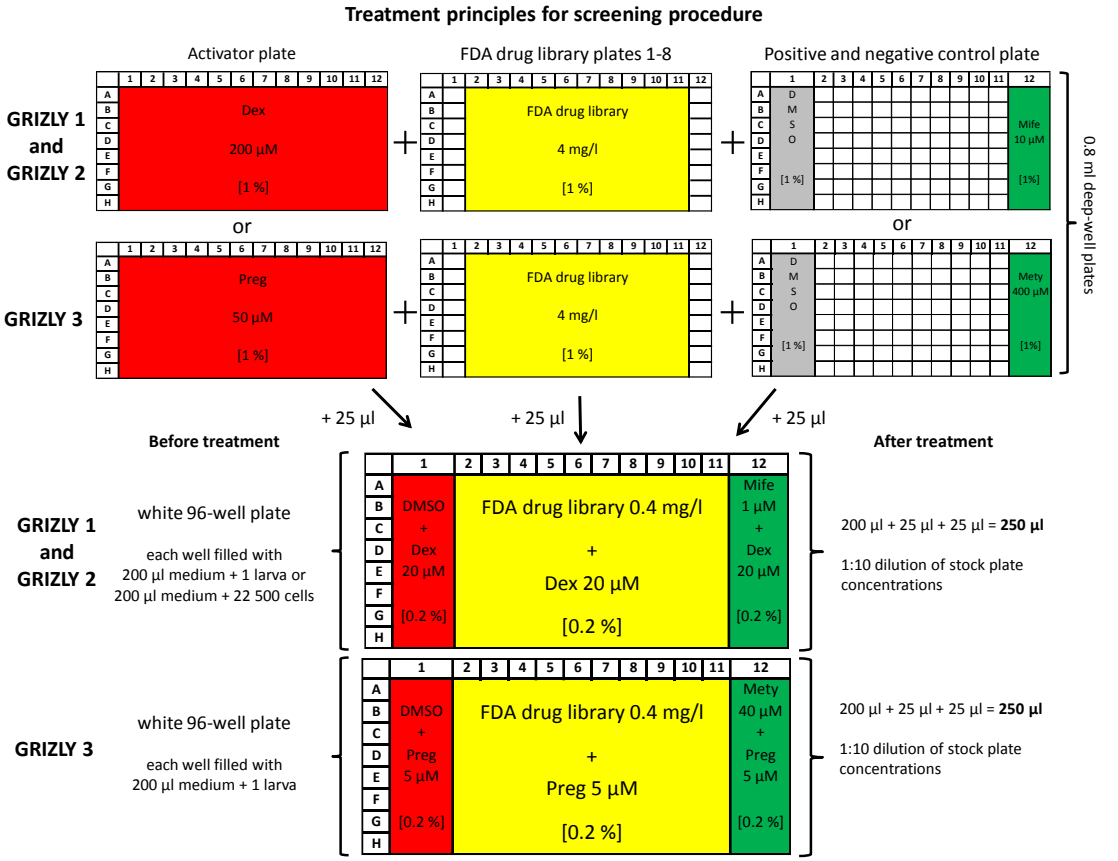


Figure 10: Treatment schemes for the screening procedure with three different GRIZLY assay approaches
Larvae and cells are distributed with 200 µl luciferin medium into the wells of a 96-well plate. For activation of GC signaling in GRIZLY 1 and GRIZLY 2, 25 µl of Dex are pipetted to each well and immediately, 25 µl of the negative control (DMSO; n = 8) and the positive control (Mife; n = 8) for inhibition of GC signaling are added to the wells of column 1 and 12 and 25 µl of the FDA drug library (n = 1) are supplemented to the wells of column 2 - 11. The final volume of each well is 250 µl, with 0.2% DMSO and 20 µM Dex in each well. The test concentration of Mife is 1 µM and that of the library compounds 0.4 mg/l. In GRIZLY 3, GC signaling in larvae is activated by the addition of 25 µl Preg to all wells. To columns 1 and 12, 25 µl of the negative control (DMSO; n = 8) and the positive control (Mety; n = 8) are added and columns 2 - 11 are treated with 25 µl of the FDA drug library (n = 1). The test concentrations in total volumes of 250 µl are 5 µM Preg, 40 µM Mety and 0.4 mg/l FDA drug library compounds. Each test plate was measured in 3-10 replicates.

2.2.4.3 Experimental setup for single compound testing

The bioluminescence measurements were performed as described above. Deep-well stock plates were prepared and treatment was applied with a 96-channel pipette according to **Figure 11**. All test plates were loaded with eight replicate wells of the positive (DMSO + Dex/ Preg) and the negative (Mife/ Mety + Dex/ Preg) control and 16 wells for each of the five concentrations of the compound.

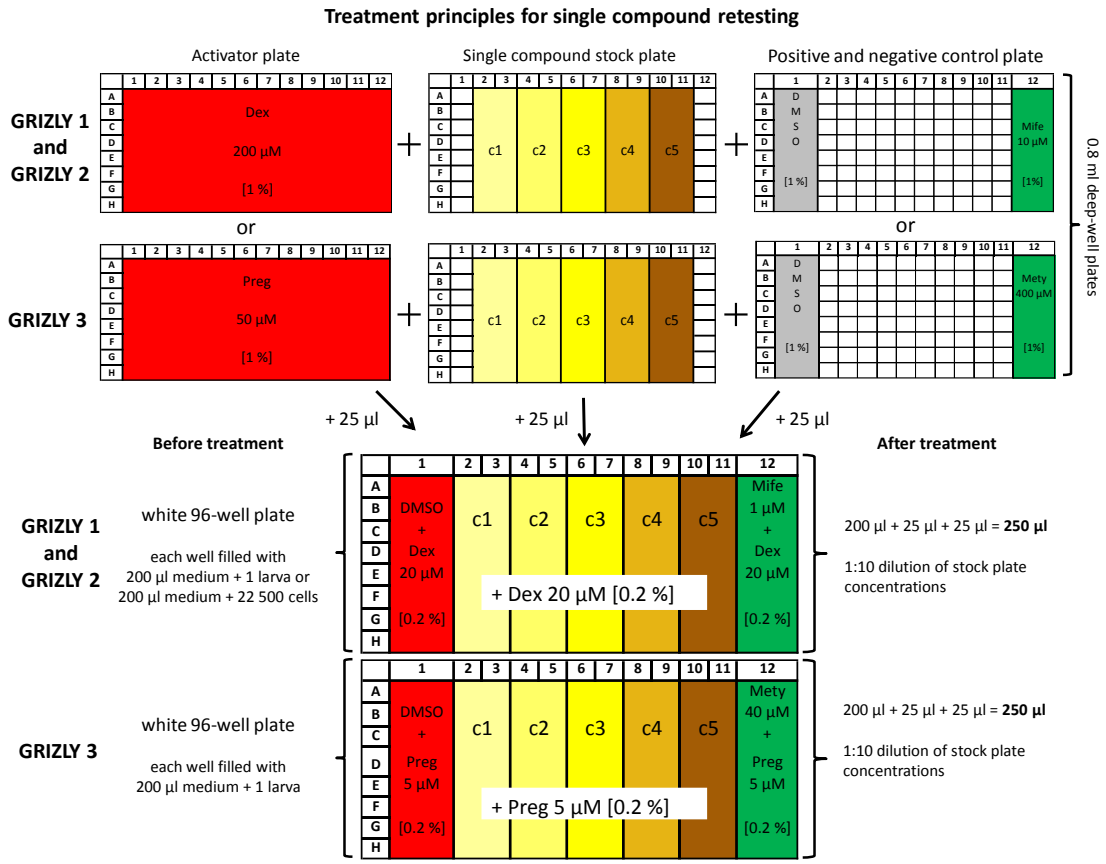


Figure 11: Treatment schemes for single compound testing with three different GRIZLY assay approaches
Larvae and cells are distributed with 200 µl luciferin medium into the wells of a 96-well plate. For activation of GC signaling in GRIZLY 1 and GRIZLY 2, 25 µl of Dex are pipetted to each well. 25 µl of the negative control (DMSO; n = 8) and the positive control (Mife; n = 8) for inhibition of GC signaling are added to the wells of column 1 and 12 and 25 µl of the single compound stock plate are supplemented to the wells of column 2 - 11, with five different concentrations of the compound (n = 16 per concentration). The final volume of each well is 250 µl, with 0.2% DMSO and 20 µM Dex in each well. The test concentration of Mife is 1 µM. In GRIZLY 3, GC signaling in larvae is activated by the addition of 25 µl Preg to all wells. To columns 1 and 12, 25 µl of the negative control (DMSO; n = 8) and the positve control (Mety; n = 8) are added and columns 2 - 11 are treated with 25 µl of the single compound stock plate, with five different concentrations of the compound (n = 16 per concentration). The test concentrations in total volumes of 250 µl are 5 µM Preg and 40 µM Mety. Each test plate was measured in 3-10 replicates.

2.2.4.4 Data processing

For the GRIZLY assay as for all other bioluminescence measurements (section **2.2.3**), data files are were imported to Excel for further processing. For each well, area under the curve (AUC) values were calculated from the bioluminescent traces over time and normalized relative to the respective negative and positive control. Mean relative AUC values ± SEM were subjected to GraphPad Prism 6 for statistical evaluation.

2.2.5 Larvae toxicity

Larvae were checked for toxic treatment effects immediately at the end of 24 h bioluminescence measurements. Larval movement upon a tactile stimulus was monitored and immotile individuals were considered dead. Toxicity for larvae was calculated according to the following formula:

$$Larvae\ toxicity\ [\%] = \frac{x\ (non - motile\ larvae\ per\ compound)}{n\ (replicate\ larvae\ per\ compound)} * 100$$

To achieve a high stringency of the toxicity evaluation, compounds were considered as potentially toxic *in vivo* if the number of immobile larvae exceeded 10%.

2.2.6 Cell toxicity

In vitro toxicity of the FDA drug library compounds was assessed in a separate screen as medium-requirements were divergent for the cytotoxicity assays and for the GRIZLY setup. Two different cytotoxicity tests, the AlamarBlue® cell proliferation (AlamarBlue®) and the Lactate Dehydrogenase (LDH) assay, were conducted in parallel and each was repeated in triplicates.

2.2.6.1 Experimental setup for cytotoxicity screens of the FDA drug library

For preparation, cells were seeded into clear, 96-well plates (CELLSTAR®, flat bottom, # M0812, Greiner Bio-One) with 60 000 cells per well and incubated in AB.9-*GRE:Luc* medium over night at 28°C to allow cell attachment. The following day, cells were washed with PBS and incubated in 200 µl L-15 medium for 1 h. Deep-well plates with chemical stocks diluted in E3 medium were prepared for the controls and library compounds as shown in Figure 12 and treatment was carried out accordingly with a 96-channel pipette. All test plates were loaded with four replicate wells of cytotoxicity positive control (Triton 0.1%; wells 1 A - D) (Triton™ X-100, # X100-100ML, Sigma-Aldrich), negative control for cytotoxicity and for GC signaling inhibition in the GRIZLY assay (Dex + DMSO, wells 1 E - H), blank (L-15 + E3 medium without cells, wells 12 A - D) and positive control for GC signaling inhibition in the GRIZLY assay (Mife + Dex, wells 12 E - H) and with one well per library compound + Dex. All wells were treated for 4h (except the positive control, where 0.5 h of Triton X - 100 exposure were sufficient to induce 100% cytotoxicity). This treatment duration was selected accordig to the GRIZLY 1 measurements, where the uninhibited bioluminescent signal peaks after 4 h and is, thus, the timepoint of interest for the assessment of compound toxicity. As an onset of compound cytotoxicity later than 4h of exposure time was not expected to have a remarkable effect on the signal intensity of the GRIZLY response at the peak timepoint, but a longer exposure duration may instead have led to a false-positive assignment of cytotoxicity in case of real inhibitors of GC signaling (between 0 and 4h) of exposure, the shorter treatment period of 4h appeared to provide the more relevant information for this testing approach than the exposure duration of 24 h, as in the GRIZLY assay. Each of the eight library plates was measured in three replicates with both cytotoxicity assays. The procedures for the alamarBlue® and the LDH assay will be described in the next chapters.

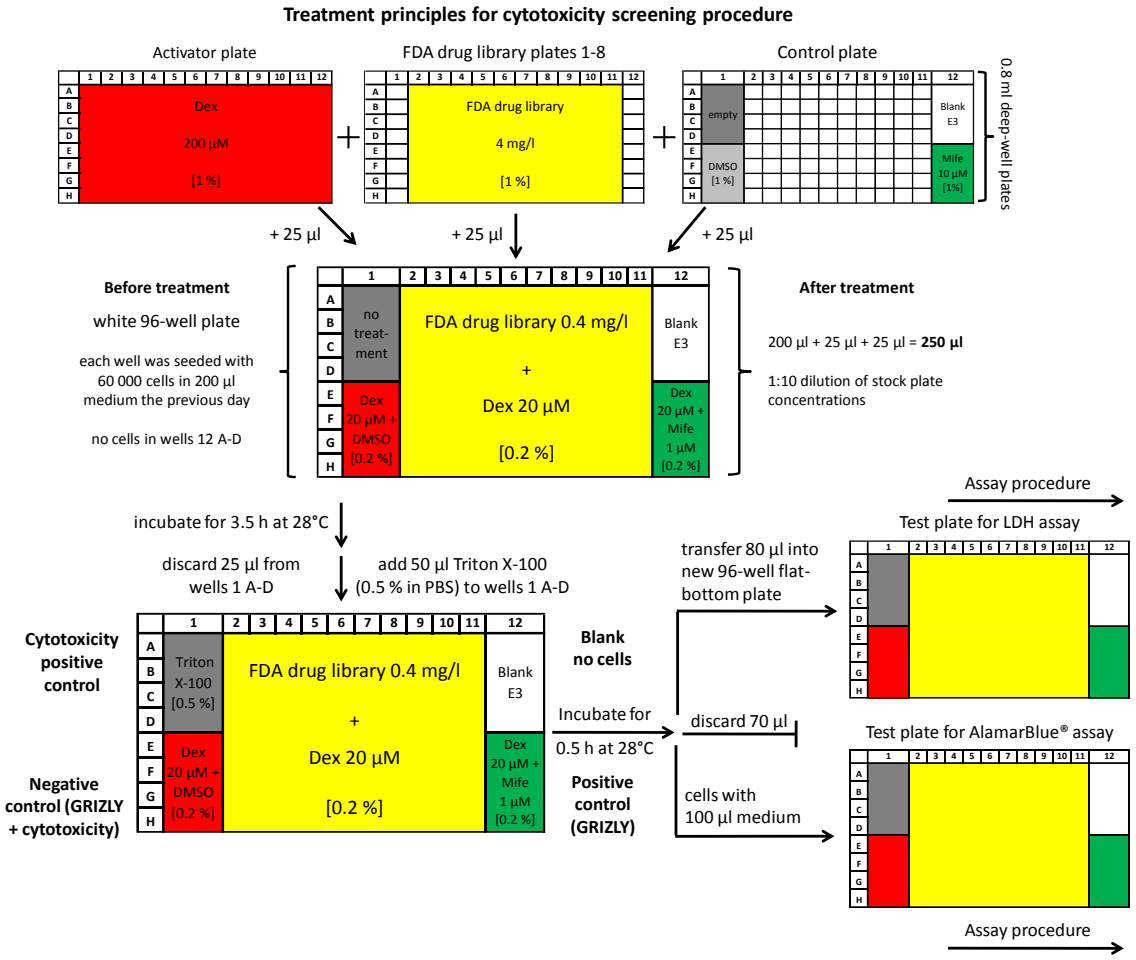


Figure 12: Cytotoxicity screen of the FDA drug library
A 96-well plate seeded with cells (except wells 12 A - D) in 200 µl L-15 medium for 1 h. Controls and library compounds were applied from deep-well stock plates with a 96-channel pipette. All test plates containedfour replicate wells each of no treatment (wells 1 A - D), negative control for cytotoxicity and for GC signaling inhibition in the GRIZLLY assay (Dex + DMSO, wells 1 E-H), blank (L-15 + E3 medium without cells, wells 12 A - D) and positive control for GC signaling inhibition in the GRIZLY assay (Mife + Dex, wells 12 E-H) and one well per library compound + Dex when the cytotoxicity screen started. Plates were incubated at 28°C for 3.5 h before 25 µl medium were removed from wells 1 A - D where 50 µl [Triton X - 100 0.5%] were added as cytotoxicity positive control (Triton X - 100, 0.1%). Plates were incubated for another 0.5 h to let the Triton destroy the cells. From each well of the test plate, 80 µl of cell-free medium were transferred into a new flat-bottom, 96-well plate for LDH assay procedures. Another 70 µl cell-free medium were discarded from the test plate in order to keep the cells with 100 µl of medium for the AlamarBlue® cell proliferation assay. Each of the eight library plates was measured in three replicates with both cytotoxicity assays.

2.2.6.2 AlamarBlue® cell proliferation assay

Assay principle

The alamarBlue® reagent was used to indicate cell viability. It contains non-fluorescent Resazurin, a cell permeable reagent that is continuously reduced by living cells into the fluorescent molecule Resorufin (**Figure 13**). This irreversible reaction enables the quantification of cell viability.

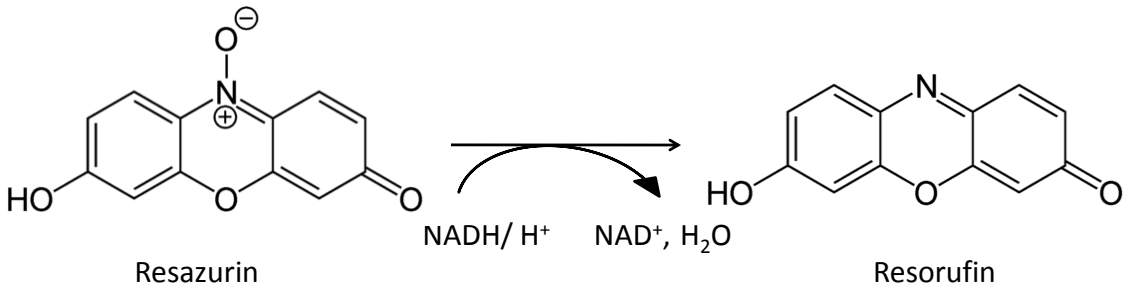


Figure 13: Measurement of cell viability with the AlamarBlue® cell proliferation assay Resazurin permeates the cell and is reduced by living cells into Resorufin, which can be quantified colorimetrically. NADH + H^+ is reduced to NAD^+ + H_2O in this reaction step.

Assay procedure

The alamarBlue® assay was conducted according to the manufacturer´s protocol (BioRad; QIFUBUF012 alamarBlue® Technical Datasheet IFU, Issue no.3, 3.9.2013). Briefly, the alamarBlue® mix (alamarBlue® reagent, # BUF012B, Bio-Rad Lab. Inc.) was diluted 1:10 in Hank's Balanced Salt Solution (HBSS) buffer (# 14025050, Gibco™) and 100 µl of the alamarBlue® mix were pipetted to all wells containing 100 µl cells+medium (1:1). The plates were incubated overnight (19-21 h) at 28°C. The next day, 180 µl of each well were transferred into a new clear, flat-bottom 96-well plate. Cell viability was quantified by the colorimetrical measurement of resorufin produced by the cells with a flourescence reader (Lambda Fluor 320 Plus, BioTek Instruments, Inc.), at Ex/Em: 560/620 nm. Data were exported as txt.files from the software (LAMBDA KC4, Kineticalc for Windows, MWG-Biotech AG) and processed in Excel for statistical analysis.

2.2.6.3 Lactate Dehydrogenase (LDH) assay

Assay principle

Lactate Dehydrogenase (LDH) is a stable enzyme which is present in the cytoplasm of all cells. Upon plasma membrane damage, it is rapidly released into the cell culture medium. Incubation of the cell-free supernatant with the substrate mixture of the Cytotoxicity Detection Kit (LDH) enables quantification of LDH activity. In a coupled enzymatic reaction, LDH oxidizes lactate, which is provided by the substrate mix, into pyruvate. In this step, NAD^+ is reduced to NADH + H^+, whereby the free H^+ subsequently allows the reduction of tetrazolium salt INT (a dye in the substrate mixture) into the red dye formazan salt (**Figure 14**). The red dye can then be quantified colorimetrically.

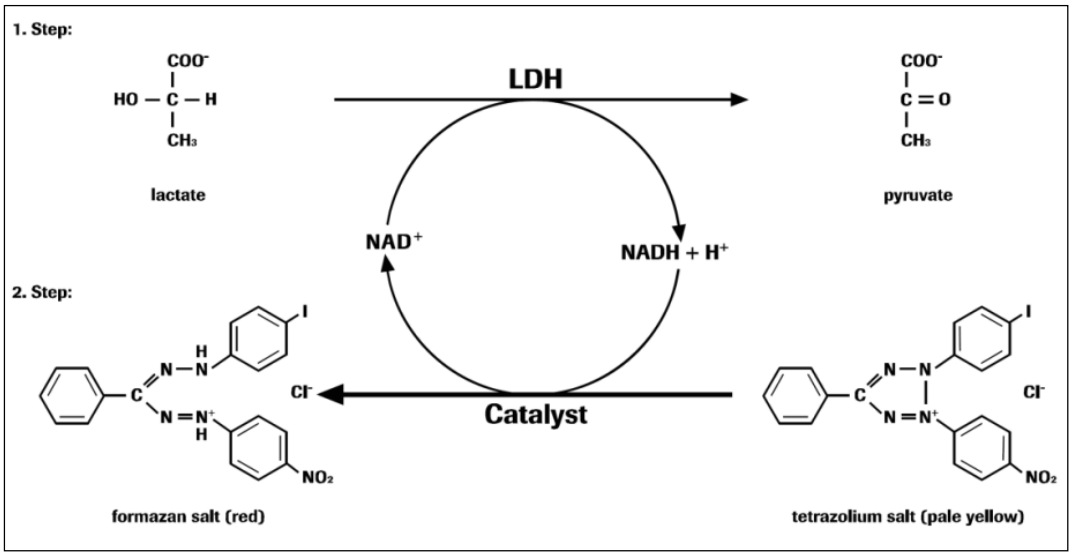


Figure 14: Measurement of cell plasma membrane damage with the lactate dehydrogenase (LDH) assay
Upon plasma membrane damage, LDH is released from cells into the cell culture medium. When the cell-free supernatant is incubated in darkness with the substrate mix from the Cytotoxicity Detection Kit (LDH), LDH oxidizes lactate, which is provided by the substrate mix, into pyruvate in a first reaction. In this step, NAD^+ is reduced to $NADH + H^+$, whereby the free H^+ enables the second reaction step, the reduction of tetrazolium salt (a dye in the substrate mixture) into formazan salt (red dye). Formazan salt can quantified colorimetrically. (source: Figure 5 from the Cytotoxicity Detection Kit (LDH) protocol, version 11 from march 2016, Roche Diagnostics)

Assay procedure

The LDH assay was conducted according to the manufacturer´s protocol. (Cytotoxicity Detection Kit [LDH], version 11 from march 2016, Roche Diagnostics). Shortly, the LDH mix (Cytotoxicity Detection Kit (LDH), # 11 644 793 001, Roche Diagnostics) was prepared protected from light, with 0.125 ml solution 1 + 5.6 ml solution 2 + 5.6 ml PBS per 100 reactions. Immediately, 80 µl of the mix were added to the 80 µl cell-free supernatant (1 : 1) in each well of the 96-well plate that was prepared according to **Figure 12**. The plates were incubated at RT in darkness for 1.5 - 2.5 h, until the cytotoxicity positive control wells (Triton X - 100 0.1%) changed their color to pinkish. The plate was placed into the ELISA plate reader (VERSAmax tunable microplate reader, Molecular Devices) and LDH activity was measured spectrophotometrically via the quantification of formazan salt production, at Ex/Em:495/650 nm. Data were exported as txt.files from the software (SOFTmax Pro Software, Molecular Devices) and processed with Excel for subsequent statistical evaluation.

2.2.6.4 Data processing and cytotoxicity calculations

Means were calculated from the four replicate wells for each of the controls. The blank mean was substracted from all data before cytotoxicity was calculated according to the following formula (as also described by (Chan, Moriwaki, and De Rosa 2013):

$$Cytotoxicity\ [\%] = \frac{sample\ -\ negative\ control\ (DMSO)}{positive\ control\ (Triton)\ -negative\ control\ (DMSO)} * 100$$

Mean ± SEM values were calculated and subjected to Graph Pad Prism 6 for statistics.

2.2.7 Chemical analysis of steroid hormone levels in zebrafish larvae

2.2.7.1 Larvae exposure

For chemical analysis experiments, wild-type zebrafish larvae were bred as previously described (2.2.1.3). At 3 dpf, each 100 zebrafish larvae were distributed with 20 ml E3 medium into 25cm^2 cell culture flasks. At 4 dpf, the larvae were co-treated with 2.5 ml activator and 2.5 ml inhibitor solution (total volume 25 ml, 0.2% DMSO, no solvent in E3 medium control) according to Table 6.

Timecourse experiments were performed in triplicates and compound tests in four replicates. For the timecourse experiments, larvae were exposed for 0, 1, 2, 4, 8 and 24 h and compound tests were conducted with 24 h treatment durations.

At the end of the exposure time, the larvae were poured into a small net and the cell culture flasks were rinsed twice with E3, in order to ascertain that all larvae were collected. Remaining steroid-containing treatment medium was removed by rinsing the larvae carefully with E3 medium, followed by three successive washing steps in PBS before the larvae were transferred with blunt pipette tipsinto autoclaved 1.5 ml reaction tubes (# 0030120086, Eppendorf AG). All liquid was removed from the tubes prior to flash-freezing the larvae in liquid nitrogen. Samples were stored at –20°C until homogenization.

Table 6: Treatment scheme for chemical analysis experiments

Replicates for each experiment per treatment duration						Experiment	Direct activation of GC signaling	Indirect activation of GC signaling		Inhibition of directly or indirectly activated GC signaling
0 h	1 h	2 h	4 h	8 h	24 h	100 larvae in 20 ml E3	Activator / (2.5 ml)	Activator (2.5 ml)	+	Inhibitor (2.5 ml)
3							E3	E3	+	E3
	3	3	3	3	3	Timecourse	DMSO	DMSO	+	DMSO
	3	3	3	3	3	Preg	Dex [20 µM]	Preg [5 µM]	+	DMSO
	3	3	3	3	3		Dex [20 µM]	Preg [5 µM]	+	Mety [40 µM]
					4		DMSO /	DMSO	+	DMSO
					4		Dex [20 µM] /	Preg [5 µM]	+	DMSO
					4		Dex [20 µM] /	Preg [5 µM]	+	Retinoic acid [2 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Spironolactone [2 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Estrone [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Norethindrone [10 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Gestrinone [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Mifepristone [1 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	10-Hydroxycamptothecin [4 µM]
					4	Compound test	Dex [20 µM] /	Preg [5 µM]	+	Nabumetone [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Tranilast [10 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Stanozolol [12 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Estriol [12 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Ethisterone [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Leflunomide [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Dehydroepiandrosterone [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Fenbufen [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Acitretin [4 µM]
					4		Dex [20 µM] /	Preg [5 µM]	+	Metyrapone [40 µM]

2.2.7.2 Homogenization of zebrafish larvae and sample spiking

Frozen samples were put on ice and 0.5 ml PBS were added to each tube containing 100 larvae. The samples were homogenized (tissue homogenizer, ULTRA-TURRAX®, IKA Works) for 2 x 20 sec and put back on ice. The stainless-steel blades of the homogenizer were cleaned after each sample by rinsing them once with isopropanol (ROTIPURAN® ≥99,8%, # 6752.1, Carl Roth GmbH & Co. KG), once with PBS and washing them for 5 sec in a 15 ml tube filled with 10 ml PBS in order to avoid carry-over of steroid residues.
The samples were spiked with internal standards (ISTDs). To all larval homogenates 250 ng (10 µl) deuterated pregnenolone ([500 ng/ml]; Preg-D4 = Pregnenolone 17α,21,21,21-D4, 98%, # 61574-54-7 D5341, CNN Isotopes Inc.) which was dissolved in methanol (MeOH; # 83.638.320, VWR International) were added. The samples were stored at –20°C until steroid extraction.

2.2.7.3 Preparation of external calibration standards

External calibration standards were prepared in triplicates by spiking 0.5 ml PBS with Hydrocortisone (Cort), Corticosterone (Cortico), Progesterone (Prog), Dexamethasone (Dex) [25 ng each, 50 ng/ml, in MeOH] and Pregnenolone (Preg) [500, 1000 ng/ml; in MeOH]. The standards were diluted 1:1 with PBS in 4 serial steps in order to obtain standard ranges of 50, 25, 12.5, 6.25, and 3.125 ng/ml for Cort, Cortico, Prog and Dex, and a range of 1000, 500, 250, 125 and 62.5 ng/ml for Preg. The ISTD Preg-D4 was added to each sample of the calibration series [250 ng, 500 ng/ml] and samples were vortexed (Genie 2, Scientific industries, Inc.) before they were stored at –20°C for steroid extraction.

2.2.7.4 Liquid-liquid extraction (LLE) of steroid hormones

The larvae homogenates were vortexed (Vortex-Genie 2, Scientific industries, Inc.) for 20 sec and transferred into 5 ml reaction tubes (# 0030119401, Eppendorf AG). For the extraction of steroid hormones, 2 ml Ethyl acetate (# 1096231000, Merck) were added and samples were vortexed thoroughly for 2 x 20 sec and centrifuged (ZK 380, Hermle Labortechnik GmbH) for 1 min at 5000 rpm for phase separation. After centrifugation, 1.8 ml of the organic phase, containing the steroid fraction, were transferred into new 5 ml reaction tubes and dried under a liquid nitrogen stream at 45°C on an evaporator (Vapotherm basis mobil II, Barkey GmbH & Co. KG). The dry samples were stored at -20°C for further processing. The frozen samples were reconstituted in 0.5 ml MeOH and vortexed for 20 seconds. Then, they were transferred into 1.5 ml screw-thread brown glass vials (art. no.: VT1101211, Dr. R. Forche Chromatographie), closed with silicone/ PTFE caps (art. no.: CT11S3015, Dr. R. Forche Chromatographie) and subjected to UPLC-MS/MS analysis as described in the following section.

2.2.7.5 UPLC-MS/MS

Detection and quantification of steroid hormones in zebrafish larvae samples was conducted by Ultra-performance liquid chromatography tandem mass spectrometry UPLC-MS/MS. Chromatographic separation was performed by an ExionLC™ AD system (AB Sciex). From each sample, a volume of 4 µl was injected and fractionated by a Kinetex XB-C18 2.6 µm, 100 mm x

2.1 mm, column (Phenomenex). In direct analyses, mobile phase were A, 0.1% formic acid (FA;, # 100264, Merck) in acetonitrile (# 83.640.320, VWR International) and B, 0.1% FA in water. The gradient condition 0 - 0.10 min, 60% B; 0.10 - 0.65 min, 60 - 20% B; 0.65 - 1.20 min; 20% B; 1.20 - 1.25 min, 20 - 60% B; 1.25 - 2.00 min, 60% B was applied for the elution of steroids from the column. The flow rate was 0.80 ml/min, and the column was maintained at 45°C using a column oven. Mass spectrometry was conducted using an API 4000™ tandem mass spectrometer with a TurboIonSpray source (AB Sciex) in the positive mode. Mass detection was achieved operating in the multiple reaction monitoring (MRM) mode analyzing precursor ion/product ion mass transitions. The optimized MS parameters and the MRM mass transitions for each compound are given in the appendix as **Supplementary Table A**.

2.2.7.6 Data aquisition and processing

The Analyst software (version 1.6.3, AB Sciex) was used for data acquisition and analysis. The samples were normalized based on their internal standards (ISTDs) and quantified by comparison with the standard calibration series. Data were exported to Excel. "No Peak"= limit of detection (LOD) was replaced manually by 0. Values smaller than the smallest standard of the external standard-curve (62.5 ng/ml for Preg and 3.125 ng/ml for the other standards) were below the limit of quantification (LOQ). For the timecourse experiments, mean, SD and SEM were calculated for each steroid from the raw data of replicate measurements and subjected to statistical analysis. For compound testing, ratios were calculated by dividing the raw data from each test compound by the mean of the respective negative control (Dex + DMSO for direct activation of GC signaling, Preg + DMSO for indirect activation of GC signaling). In case of 0-values for raw data and 0-values for the mean of the negative control, 0 would be divided by 0, thus, the occurring error ("#DIV/0!") was replaced by a ratio value of 0 manually. For each steroid, mean, SD and SEM were calculated from the replicate ratio values and subjected to statistical analysis.

2.2.8 Gene expression analysis

2.2.8.1 Larvae exposure

For real-time quantitative polymerase chain reaction (qPCR) experiments, wild-type zebrafish larvae were bred as described in section 2.2.1.3. At 4 dpf, they were distributed into small cell culture flasks (CELLSTAR® 25 cm^2 # 690170, Greiner Bio-One), with 30 larvae in 6 ml E3 medium per flask. The next day, the larvae were co-treated with 0.75 ml activator and 0.75 ml inhibitor solution (total volume 7.5 ml, 0.2% DMSO, no solvent in E3 medium control) according to **Table 7**.

Table 7: Treatment scheme for qPCR analysis

Replicates for each experiment per treatment duration						Experiment	Direct activation of GC signaling	Indirect activation of GC signaling		Inhibition of directly or indirectly activated GC signaling
0 h	1 h	2 h	4 h	12 h	24 h	30 larvae in 6 ml E3	Activator / (0.75 ml)	Activator (0.75 ml)	+	Inhibitor (0.75 ml)
3							-	E3	+	E3
	3	3	3	3	3	Timecourse	-	DMSO	+	DMSO
	3	3	3	3	3	Preg	-	Preg [5 µM]	+	DMSO
	3	3	3	3	3		-	Preg [5 µM]	+	Mety [40 µM]
			3				DMSO /	DMSO	+	DMSO
			3				Dex [20 µM] /	Preg [5 µM]	+	DMSO
			3				Dex [20 µM] /	Preg [5 µM]	+	Retinoic acid [2 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Spironolactone [2 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Estrone [4 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Norethindrone [10 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Gestrinone [4 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Mifepristone [1 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	10-Hydroxycamptothecin [4 µM]
			3			Compound	Dex [20 µM] /	Preg [5 µM]	+	Nabumetone [4 µM]
			3			test	Dex [20 µM] /	Preg [5 µM]	+	Tranilast [10 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Stanozolol [12 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Estriol [12 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Ethisterone [4 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Leflunomide [4 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Dehydroepiandrosterone [4 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Fenbufen [4 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Acitretin [4 µM]
			3				Dex [20 µM] /	Preg [5 µM]	+	Metyrapone [40 µM]

2.2.8.2 Cell exposure

The cell experiments for qPCR analysis were carried out in triplicates. The first day, 400 000 AB.9-*GRE:Luc* cells per well were seeded with 4 ml AB.9-*GRE:Luc* medium into 6-well plates. Cells were allowed to attach over night at 28°C before they were washed with 4 ml PBS and treated either with DMSO or with DMSO + Dex [20 µM] (L-15, end volume 5 ml, 0.2% DMSO) for 4 h at 28°C. At the end of the exposure duration, the treatment solution was completely removed prior to RNA extraction.

2.2.8.3 Total RNA extraction

To the snap-frozen larvae and the cells, 1 ml of TRIzol™ Reagent (# 15596026, Invitrogen™) were added. The cells were transferred into 1.5 ml reaction tubes. Cell membranes were disrupted with a tissue homogenizer for 2 x 20 sec before the samples were stored at –80°C for at least 12 h. Total RNA was isolated following the manufacturer's instructions of the TRIzol™ Reagent. RNA quantity and integrity were determined spectrophotometrically (NanoDrop™ 1000, using NanoDrop™ Software, version 3.7.0, both Thermo Scientific™) and only samples with a 260/280 ratio above 1.7 were carried forward for cDNA synthesis. The RNA concentration was adjusted to 200 ng/µl.

2.2.8.4 cDNA synthesis

Any genomic DNA was removed from the samples by treatment with 1 U DNAse I (# M6101, Promega) at 37°C for 30 min and cDNA was synthetized with Random Hexamer Primer (# SO142, Thermo Scientific™), RNasin® Ribonuclease Inhibitor (# N2511, Promega), SuperScript™ III Reverse Transcriptase (# 18080044, Invitrogen™) and RNAse free water (# P1193, Promega) according to the manufacturer´s protocols. The concentration of the obtained cDNA was adjusted to 10 ng/µl.

2.2.8.5 Primer selection

For real-time quantitative PCR measurements, the two housekeeping genes ribosomal protein L13 (*rpl13*) and elongation factor 1-alpha (*ef1α*) were selected for data normalization. Genes of interest were GR (NR3C1, *gr*) with the two splice variants GR α (*grα*) and GR β (*grβ*), 11β-hydroxysteroid dehydrogenase 2 (*hsd11b2*), cytochrome P450 11β-hydroxylase (*cyp11c1*), cytochrome P450, family 3 subfamily A polypeptide 65 (*cyp3a65*), cytochrome P450 family 1 subfamily A member 1 (*cyp1a1*), progesterone receptor (NR3C3, *pgr*), pregnane x receptor (NR1I2, *pxr*), FK506 binding protein 5 (*fkbp5*), GC-induced leucine zipper (*gilz*), proopiomelanocortin (*pomc*), androgen receptor (NR3C4, *ar*) and mineralocorticoid receptor (NR3C2, *mr*).

Primers for *hsd11b2* (Wilson et al. 2013), *cyp11c1* (Wilson et al. 2013), *cyp3a65* (Bainy et al. 2013), *cyp1a1* (Brammell 2010; Lister et al. 2009), *gr* (both splice variants; Alsop & Vijayan 2008), *grα* (Manuel et al. 2014), *pgr* (Bainy et al. 2013; Chen et al. 2010) were used as previously reported and for *rpl13*, *ef1α*, *fkbp5*, *gilz*, *pomca*, *grβ*, *ar* and *mr*, appropriate primers were selected and designed as follows: The transcript sequences and accession numbers for the genes of interest were obtained by searching the zebrafish database of the NCBI webpage. The Refseq mRNA was then used to design appropriate oligonucleotide primer pairs in application of the Primer-BLAST tool from the ncbi webpage (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). For primer selection, it was considered that oligonucleotides should have a content of 40 - 60% of the nucleotide bases guanine (G) or cytosine (C), with minimal clusters of the two bases together and 20 - 40 base pairs in length. It was aimed to pick primers with melting temperatures between 57°C and 63°C and with a maximum difference of 3°C between the primer pair. If possible, primers were designed to span an exon/exon junction to prevent genomic DNA amplification and self-complementarity was avoided in order to decrease the possibility of primer-dimer formation.

All primers were purchased as lyophilized powders from Invitrogen by Thermo Fischer Scientific and were reconstituted in RNAse free water as 100 µM stocks for storage at –20°C. For all utilized primers, the gene names, accession numbers, forward and reverse sequences, as well as their product sizes are listed in **Table 8**. Italicized primer names correspond to the official zebrafish gene symbol.

Table 8: Primers used for real-time quantitative PCR measurements

Primer name	Accession number	Forward sequence	Reverse sequence	Product length
rpl13	NM_212784.1	CCATGAAGTTGGCTGGAAGT	GAAGAGAAAGGAAAAGGCCAA	69
ef1α	NM_131263.1	ACCTACCCTCCTCTTGGTCG	GGAACGGTGTGATTGAGGGA	164
hsd11b2	NM_212720.2	GGGGGTCAAAGTTTCCACTAT	GTACTCTGCGTTACTGCTCTGC	67
cyp11c1	NM_001080204.1	ACGCAGGATAGCAGAGAACG	TCAGCTTGAAGGTCCTCAGAA	65
fkbp5	NM_213149.1	TTCCACACTCGTGTTCGAGA	ACGATCCCACCATCTTCTGT	72
gilz	NM_200569.2	CAAGATCGAACAAGCAATGG	CCTTGATCTGCTCCTTCAAGA	89
cyp3a65	NM_001037438.1	ATGGTGCCGACCTACGCCCTC	GGGCCCAGACCGAACGGCAT	135
cyp1a1	NM_131879.2	CTGGACGAAAACTCCAACCTG	GATAGTGTCGAAACCGGCTCC	87
pomca	NM_181438.3	GAAGAGGAATCCGCCGAAA	CCAGTGGGTTTAAAGGCATCTC	98
gr (α+β)	NM_001020711.3	ACAGCTTCTTCCAGCCTCAG	CCGGTGTTCTCCTGTTTGAT	116
gr α	EF436284.1	ACTCCATGCACGACTTGGTG	GCATTTCGGGAAACTCCACG	90
gr β	EF436285.1	TCCAAAGCCCTAGTGAGCTG	AGCGGAATCACTATGACGCA	135
pgr	NM_001166335.1.1	GGGCCACTCATGTCTCGTCTA	TCTCCACTCTGAAAATATGTGGACTTT	95
ar	NM_001083123.1.	TACGGCCGAAGTACTGCTCTG	AAATGTCACTCCTCCCTCCGT	181
mr	NM_001100403.1	TGCCACTACGGGGTTGTTAC	GTGCCCCAAGATTCATCCCA	181
pxr	NM_001098617.1	GCATTCGCGTCCATATCACAGAG	CTAACTAGGGCTCCACTTCCTGG	101

2.2.8.6 Real-time quantitative PCR measurements

The qPCR measurements were carried out using the qPCR Master Mix kit (GoTaq® qPCR Master Mix , # A6002, Promega). Each sample was measured in technical duplicates and in biological triplicates. According to the manufacturer's instructions, the 20 µl reaction volumes were composed of 10 µl 2 x PCR Master Mix, 2 µl of primer mix (forward and reverse primer, 2 µM each), 0.2 µl reference dye and 3.8 µl H_2O and 4 µl cDNA (10 ng/µl). Master-mixes were prepared and distributed on a translucent 96-well plate. A template control without cDNA (NTC) for each primer pair and a transcription control without the SuperScript™ III Reverse Transcriptase (-RT) for each cDNA was also included. The plate was sealed with an adhesive film (qPCR plate seal, # SL-AM0560, Steinbrenner Laborsysteme GmbH) and placed into the Real-Time PCR reader (StepOnePlus™ System, # 4376600, Applied Biosystems). The following parameters were used for product amplification: 5 min at 94°C for denaturation, followed by 40 annealing cycles at 95°C for 15 sec and 60°C for 30 sec. The melting curves of the products were determined at the end of the amplification phase and C_t values were determined by the StepOne™ Software (version 2.3, Applied Biosystems). Data were exported as txt. files from the software and processed in Excel for statistical analysis.

2.2.8.7 Data processing

The relative mRNA expression of each target gene was normalized to that of the two housekeeping genes *rpl13a* and *ef1α*. Ratios of mRNA expression relative to the negative control (Dex/Preg + DMSO) were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). For the assessment of nuclear receptor mRNA expression in untreated (DMSO) AB.9-*GRE:Luc* cells, ratios were calculated as follows: $1/(C_t$ target - C_t *ef1α*). For all experiments, mean ratios ± SEM were calculated and subjected to GraphPad Prism 6 for statistical evaluation.

2.2.9 Statistics

Statistical analysis was conducted with the GraphPad Prism 6 software (GraphPad Software, Inc.). With all mean ± SEM data obtained from GRIZLY, cytotoxicity, UPLC-MS/MS and qPCR experiments, a grouped analysis was conducted with a multiple t-test (α = 0.05) where each condition (and each steroid in case of UPLC-MS/MS) was individually tested against the negative control. The obtained data were corrected for multiple comparisons using the Holm-Šídák method, where the significance level is adjusted according to this formula: $adjusted\ \alpha = 1 - (1 - \alpha)^{(1/i)}$(Holm 1979), with i = 640 for GRIZLY and cytotoxicity screens, i = 7 for concentration-dependent retests and single compound tests with the GRIZLY assay and i = 19 for UPLC-MS/MS and qPCR experiments.

Chapter 3

3. Results

In order to pursue the first objective of this study, to assess the suitability of the GRIZLY assay for the detection of GC signaling inhibition, the testing conditions needed to be adjusted accordingly. The two generated transgenic reporter lines, *Tg*(*GRE:Luc*) zebrafish larvae and the AB.9-*GRE:Luc* zebrafish cells, were applied under comparable testing conditions to enable the discrimination between *in vivo* and *in vitro* compound effects. An FDA-approved drug library (FDA library), consisting of 640 compounds, was selected to be screened with the reporter lines. The application of a library of known compounds should enable the identification of already established inhibitors and, moreover, facilitate the substance classification in cases where unknown inhibitory effects on GC signaling are detected.

3.1 Preparation: Validation experiments for an inhibitory GRIZLY assay

Inhibition of directly activated glucocorticoid signaling

Before the FDA library could be screened for GC signaling inhibitors, a control activator of GC signaling was needed, which is functional *in vivo* and *in vitro*. The synthetic GC Dexamethasone (Dex) is a reference compound for specific GR agonism and was identified to induce GC signaling in both reporter lines within a previous screen for activation of GC signaling (Weger et al. 2012). For the present study, these findings were validated by retesting, where Dex showed concentration-dependent induction of GC signaling in transgenic zebrafish larvae (**Figure 15 a**). Statistically significant activation was induced by 5, 10 and 20 µM, with 20 µM Dex showing the strongest induction.

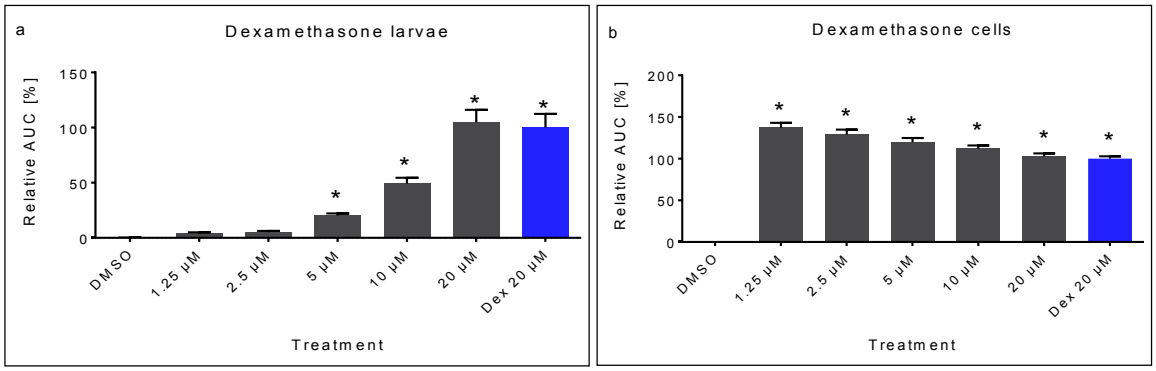


Figure 15: Direct activation of glucocorticoid signaling with Dexamethasone
Glucocorticoid signaling was assessed *in vivo* (**a**) and *in vitro* (**b**). Relative AUC values are shown for a range of five different concentrations of Dex (grey, n = 16). The data are normalized relative to the negative control (blue, n = 8) and the positive control (blue, n = 8, Dex [20 µM]). The amount of DMSO was 0.1% in all treatment conditions. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in

a multiple t-test, α = 0.01. After multiple testing correction with the Holm-Šídák method, significance is defined as p < α < 0.002= *.

In vitro, the same concentrations of Dex were applied in order to ensure comparable exposure conditions between the two systems. The transgenic cells have a higher sensitivity for Dex than the *Tg(GRE:Luc)* zebrafish larvae (Weger et al. 2012) . This possibly relies on their more direct exposition to Dex and the higher number of reporter constructs in a multitude of single cells, filling the entire bottom of the testing plate well, compared to the exposure to one larva, where Dex has to pass the skin barrier and metabolic clearance mechanisms first. Accordingly, no concentration-dependent signal increase was observable with the selected range from 1.25-20 µM (**Figure 15 b**). Instead, all concentrations significantly activated GC signaling. Albeit lower concentrations of Dex [2.5-10 µM] could have been equally or more suitable for cell exposure, the concentration of 20 µM was determined for the control activation concentration, as it showed the strongest activation in larvae and was still appropriate for the *in vitro* system.

With Dex as a reference activator for GC signaling, a control inhibitor had to be selected next. Due to the direct GR agonism of Dex, the reference GR antagonist Mifepristone (Mife) was the prior choice, in order to have an inhibitor which acts via the same mechanism as the activator. Further, Mife has already been shown to antagonize GC signaling in zebrafish (Krug et al. 2014; Weger et al. 2012). In the experiments, Mife concentration-dependently inhibited Dex-induced GC signaling in both, larvae (**Figure 16 a**) and cells (**Figure 16 b**). All tested Mife concentrations significantly reduced the Dex-activated signal, when compared to the negative control DMSO + Dex. Consistent with previous results (Weger et al. 2012), the highest concentration of Mife [1 µM] resulted in the strongest inhibition and was therefore selected as the control inhibitor concentration for the inhibitory GRIZLY screening applications.

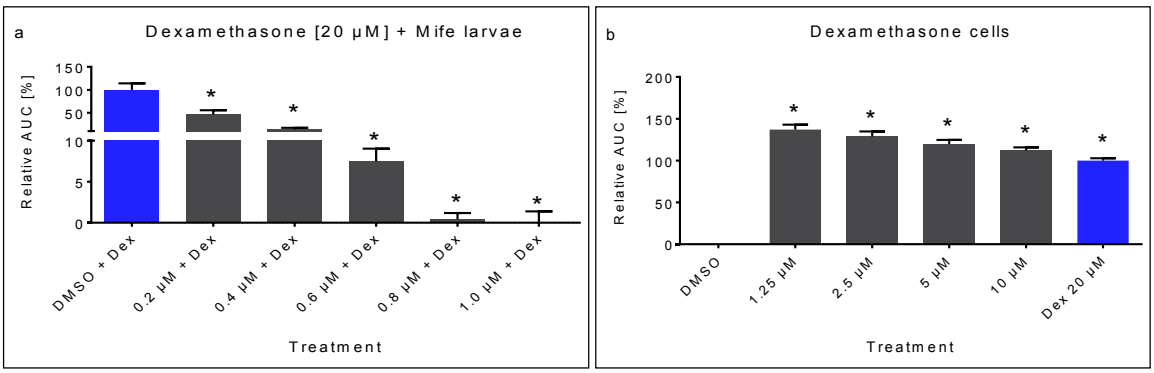


Figure 16: Inhibition of Dexamethasone-induced glucocorticoid signaling with Mifepristone
Effects on glucocorticoid signaling were assessed *in vivo* (**a**) and *in vitro* (**b**). Relative AUC values are shown for a range of five different concentrations of Mife + Dex [20 µM] (grey, n = 16, all 0.2% DMSO). The data are normalized relative to the negative control (blue, n = 8, DMSO + Dex [20 µM]) and the positive control (blue, n = 8, Mife [1 µM] + Dex [20 µM). The amount of DMSO was 0.2% in all treatment conditions. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, α = 0.01. After multiple testing correction with the Holm-Šídák method, significance is defined as p < α < 0.002= *.

Inhibition of indirectly activated glucocorticoid signaling

Beside the GC signaling activation by synthetic GR agonists like Dex, also signal induction by natural GCs can be detected with the *GRE:Luc* reporter (Weger et al. 2012). However, the bioluminescent signal coming from basal levels of endogenous GCs is relatively low in larvae and cannot be distinguished from background noise. Stimulation of endogenous GC over-production, in contrast, results in an enhanced bioluminescent signal. This possibility, to detect indirect induction of GC signaling, represents another attractive application of the GRIZLY assay, based on which a second screening approach was elaborated.

For the second screen, the endogenous production of cortisol (Cort), the main active GC in fish and humans, is induced. This indirect stimulation of Cort biosynthesis and thereby GC signaling not only targets to identify candidates which directly interfere with GR-mediated signaling, it enables furthermore the identification of compounds inhibiting GC signaling via disruption of Cort production. One possibility to induce Cort over-production in larvae is their exposure to natural precursors of the GC synthesis pathway, which then are endogenously metabolized into Cort. Pregnenolone (Preg), a natural prohormone which itself is not known to activate the GR, is a suitable candidate for this approach. Preg was identified within the previous screen to activate the *GRE:Luc* reporter as a substrate, that induced Cort biosynthesis (Weger et al. 2012). This observation was confirmed by concentration-depentent retesting, which validated Preg as a suitable positive control for indirect activation of GC signaling.

As shown in **Figure 17 a**, a constant signal increase was observable for the concentrations of 1.25, 2.5 and 5 µM. At 10 and 20 µM, Preg started to show toxic effects in larvae and the signal decreased. Therefore, 5 µM, showing the highest signal induction, was determined as control concentration for indirect GC signaling activation in further applications. In contrast to the *in vivo* response, no signal activation could be detected when AB.9-*GRE:Luc* cells were treated with a range of Preg concentrations (**Figure 17 b**). The lack of GC signaling activation *in vitro* was not surprising, as the metabolization of Preg into Cort requires a whole cascade of adrenal enzymes, a circumstance which is unlikely to be available in cultured fibroblast-like fish cells.

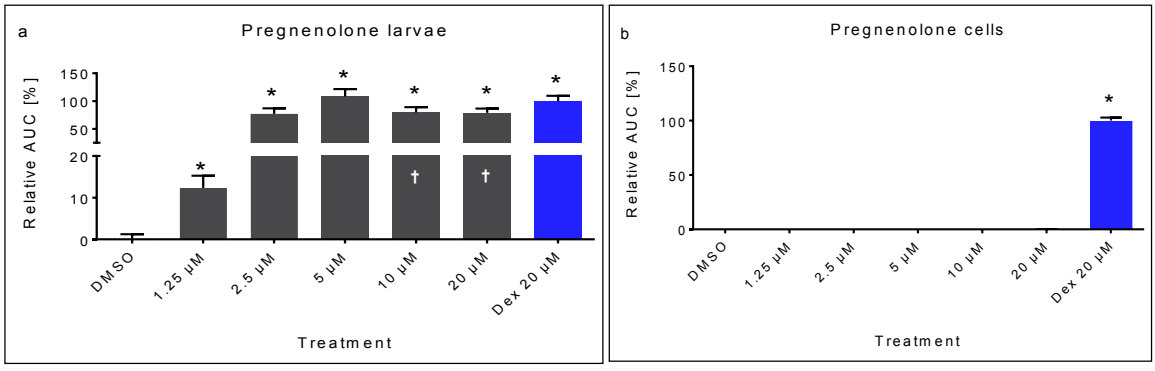


Figure 17: Indirect activation of glucocorticoid signaling with Pregnenolone
Pregnenolone activated GC signaling *in vivo* (**a**) but not *in vitro* (**b**). Relative AUC values are shown for a range of five different concentrations of Preg (grey, n = 16). The data are normalized relative to the negative control (blue, n = 8) and the positive control (blue, n = 8, Dex [20 µM]). The amount of DMSO was 0.2% in all treatment conditions. Crosses indicate toxicity in the tested concentration. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, α=0.01. After multiple testing correction with the Holm-Šídák method, significance is defined as $p < \alpha < 0.002$= *.

However, as Preg could possibly induce GC signaling directly via binding to the PR due to structural similarities of GR and PR and then unspecifically activate the *GRE:Luc* reporter in larvae, it appeared necessary to proof that not a lack of PR expression in cells might be the reason for the failed signal induction. I conducted qPCR experiments in order to ascertain that the PR gene (*pgr*) was expressed in AB.9-*GRE:Luc* cells. I also assessed the presence and abundance of the two housekeeping genes (hkgs) ribosomal protein L13 (*rpl13*) and elongation factor 1-alpha (*ef1α*), of the genes for the two GR isoforms GRα (*grα*) and GRβ (*grβ*), and of the nuclear receptors MR (*mr*), AR (*ar*) and PXR (*pxr*).

In **Figure 18 a**, the expression ratios are shown for each target gene relative to *ef1α.* The *pgr* gene was indeed expressed in DMSO (0.1%) -treated cells, which further substantiates the assumption that the lack of signal induction in Preg-treated AB.9-*GRE:Luc* cells underlies the inability of the cells to synthesize Cort from Preg. Although a significant difference between *ef1α* and *rpl13* was detected by statistical analysis, both hkgs were expressed in nearly equal abundance in the cells. The gene expression for all nuclear receptors except *GRβ* was significantly lower than for *ef1α*, with *GRα* mRNA being the most and *PXR* the least abundant.

Furthermore, I assessed if Dex treatment changed the expression of *grα*, *grβ*, *mr*, *ar*, *pxr* in AB.9-*GRE:Luc* cells. As shown in **Figure 18b**, the levels of *grα*, *G grβ* and *mr* significantly decreased when cells were treated with Dex ([20 µM], 0.1% DMSO), compared to the control treatment (DMSO 0.1%). Apparently, a cell-autonomous negative feedback loop down-regulated the mRNA expression of these three transcripts, probably to prevent or counteract overactivation of the corticosteroid receptors by Dex.

Interestingly, albeit not significant due to variations between the test replicates, *pxr* expression was strongly induced by Dex, suggesting increased, xenobiotic clearance activity within the cells. Although Dex is a known PXR ligand, the nuclear receptor majorly mediates xenometabolism by regulating the expression of CYP enzymes in the liver and intestine (phase I metabolic enzymes), which are not expected to be available in these cultured cells. However, the PXR can regulate xenobiotic and endobiotic clearance via various mechanisms, which may induce the cellular export of drugs (here Dex) via enzymes other than those involved in phase I xenobiotic metabolism in liver and intestine (Ihunnah, Jiang, and Xie 2011; Xu, Li, and Kong 2005). For instance via phase II metabolic enzymes, which can be located in numerous tissues, or via xenobiotic transportert proteins (Ihunnah et al. 2011), may the PXR may have induced xenobiotic clearance activity in the cells.

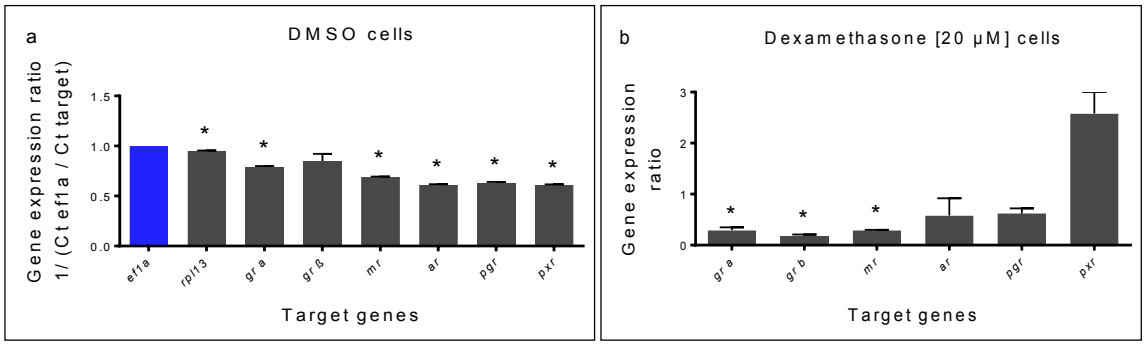


Figure 18: Expression of nuclear receptor and housekeeping genes in AB.9-*GRE:Luc* cells
AB.9-*GRE:Luc* cells were exposed for 4h to (**a**) DMSO or (**b**) Dex [20 µM]. The amount of DMSO was 0.1% in all treatment conditions. (**a**) Mean gene expression ratios of three biological replicates + SEM are shown for *rpl13*, *grα*, *grß*, *mr, ar* and *pxr* (all grey, n = 3), relative to *ef1α* (blue, n = 3) mean CT values. (**b**) Mean gene expression ratios of three biological replicates + SEM are visualized for the nuclear receptors *grα*, *grß*, *mr, ar* and *pxr* measured in cells exposed for 4h to Dex (grey, n = 3), relative to 4h of DMSO treatment (not shown, n = 3). Statistic evaluation of gene expression data was conducted with the 2△CT method, measured with two technical replicates per plate and two housekeeping genes, *rpl13α* and *ef1α*. (**a** + **b**) Statistical significance is determined by comparison of the mean ratio of the control (**a**: *ef1α*; **b**: DMSO) with the mean ratio of the target genes in a multiple t-test, α=0.05. After multiple testing correction with the Holm-Šídák method, significance is defined as p<α<0.002=*.

Now, with Preg as a control activator of endogenous GC production *in vivo*, an appropriate reference inhibitor was needed for the second screening approach, which enables the identification of substances specifically inhibiting Preg-induced GC signaling. Such inhibitors can either interact directly with GR-mediated signaling or indirectly, independent from the GR, e.g. by disrupting the GC biosynthesis pathway. For direct interaction with GR signaling, Mife serves also within this testing approach as a good positive control. The antagonistic effect of Mife on GR signaling, induced by either directly administered Cort (**a**) or endogenously produced Cort (**b**), is shown in **Figure 19**. In both cases, Mife concentration-dependently inhibited GC signaling.

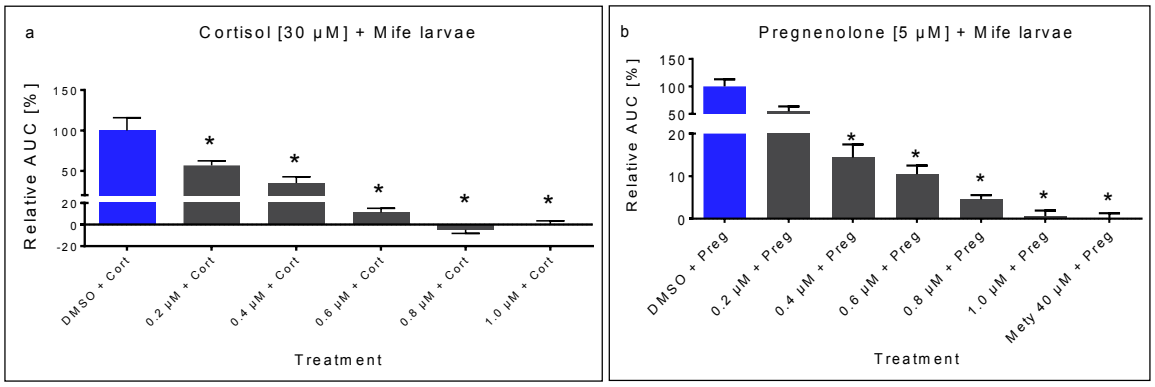


Figure 19: Inhibition of Cortisol- and Pregnenolone-induced glucocorticoid signaling with Mifepristone *in vivo*
Cort- (**a**) and Preg- (**b**) induced GC signaling was inhibited concentration-dependently in larvae with Mife. Relative AUC values are shown for a range of five different concentrations of Mife + Cortisol [30 µM] (**a**)/ Preg [5 µM] (**b**)(grey, n = 16). The data are normalized relative to the negative control DMSO + Cort [30 µM] (**a**)/ DMSO + Preg [5 µM] (**b**) (blue, n = 8) and the positive control Mife [1 µM] + Cort [30 µM] (**a**)/ Mety [40 µM] + Preg [5 µM] (**b**) (blue, n = 8). The amount of DMSO was 0.2% in all treatment conditions. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, α = 0.01. After multiple testing correction with the Holm-Šídák method, significance is defined as p < α < 0.002= *.

Selection of a suitable control for glucocorticoid biosynthesis disruption

In need of an appropriate control for indirect inhibition of Preg-induced GC signaling in the second screening approach, certain requirements had to be fulfilled by the inhibitor. A suitable candidate was expected to specifically and significantly reduce Preg-induced GC signaling. Further, unlike Mife, it had to be ineffective on signaling induced by the synthetic GC Dex, because otherwise interference of the candidate with the GR might rather be the mechanism of inhibition than disruption of GC biosynthesis. In the literature, several disruptors of the GC synthesis have been described, mostly acting on diverse enzymes within the GC biosynthesis pathway. Three of them, Ketoconazole (Keto), Etomidate (Eto) and Metyrapone (Mety), were tested for their suitability as a control for glucocorticoid biosynthesis disruption in the GRIZLY screening approach (**Figure 20**).

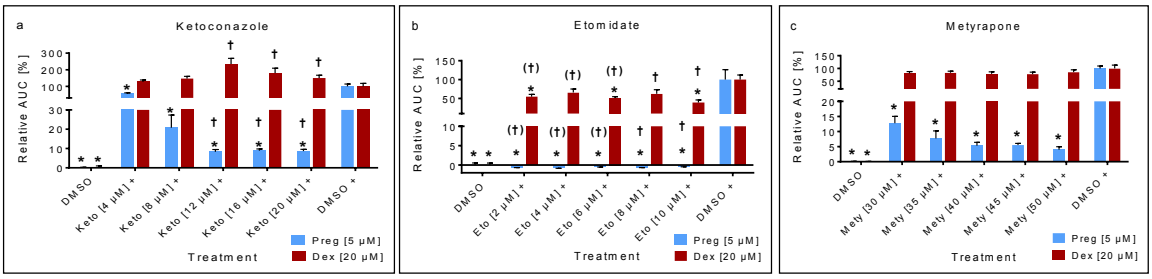


Figure 20: Glucocorticoid biosynthesis disruptors
Keto (**I**),Eto (**II**) and Mety (**III**) were co-exposed with Preg ([5 µM], blue) and Dex ([20 µM], red) to *Tg*(*GRE:Luc*) larvae. Relative AUC values are shown for a range of five different concentrations (n = 16). The data are normalized relative to the DMSO control (n=8) and the negative control DMSO + Preg [5 µM]/ DMSO + Dex [20 µM] (n=8). The amount of DMSO was 0.2% in all treatment conditions. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, α=0.01. After multiple testing correction with the Holm-Šídák method, significance is defined as p<α<0.002=*. Sublethal toxic effects = (†), lethality = † (**a**). Bioluminescent traces [Mean RLUs] of the treatments for Preg + Keto (**I**)/ Eto (**II**)/ Mety (**III**) (**b**) and for Dex + Keto (**I**)/ Eto (**II**)/ Mety (**III**) (**c**) are monitored over the exposure duration of 24 h.

Keto is a synthetic imidazole drug, which is used primarily to treat fungal infections but also finds application in the treatment of hypercortisolism in Cushing´s syndrome, as it suppresses the biosynthesis of Cort (Sonino et al. 1991; Loli et al. 1986; Graybill et al. 1983). It has been shown to inhibit the cholesterol side-chain cleavage enzyme (P450scc, CYP11A), which converts cholesterol into Preg, and also cytochrome P450 (CYP)17A1 (CYP17A1), an enzyme that has 17α-hydroxylase (production of GCs) and 17,20-lyase (production of estrogens and androgens) activity whereby it catalyzes diverse steps downstream of Preg in the steroid biosynthesis pathway (Ankley et al. 2012; DeVore and Scott 2012; Hilscherova et al. 2004). Moreover, it inhibits 11β-hydoxylase (CYP11B1 in human, CYP11C1 in fish), the enzyme which converts 11-deoxycortisol into Cort (Bergström et al. 1998; Yin et al. 2012; Zhu et al. 2016). In addition to its broad potency in disrupting the GC biosynthesis directly, Keto is furthermore a reference inhibitor of diverse xenobiotic enzymes, including the CYP3A family and CYP1A1 (Eagling, Tjia, and Back 1998; Greenblatt et al. 2011; Paine, Schmiedlin-Ren, and Watkins 1999). Thus, Keto has the potency to disrupt the GC biosynthesis directly, via inhibition of several steroidogenic enzymes, but also indirectly, by affecting the xenobiotic clearance, which may alter the

availability of GCs and their precursors in the organism. Therefore, its suitability as a specific control inhibitor of GC biosynthesis is questionable.

When exposed to larvae, sufficient inhibition of the Preg-induced signal was only detectable when the concentrations of Keto started to induce also toxic effects [> 8 µM] (**Figure 20 a**). Accordingly, no specific inhibition but toxicity was observable. In co-treatment with Dex, the higher concentrations of Keto [> 8 µM] also led to larvae toxicity. However, at sub-toxic concentrations [4 and 8 µM], Keto showed a tendency to co-induce GC signaling with Dex. Although these effects were not significant when compared to the negative control (Dex + DMSO), this observation might be related to the above mentioned inhibitory effect of Keto on xenobiotic enzymes. Potentially, Keto reduced the xenobiotic clearance of Dex, which is a known substrate of enzymes involved in phase I xenobiotic metabolism such as CYP3A (Lanzarotti and Rossi 2013; McDonagh et al. 2012; Natale et al. 2016). In this case, increased Dex activity in the larvae might be expectable due to compound accumulation. To further pursue this consideration and for comparison of *in vivo* and *in vitro* effects of Keto, I co-exposed the substance with Dex to AB.9-*GRE:Luc* cells. As xenobiotic CYP enzymes are primarily located in liver and intestine, their inhibition requires a whole-organism environment and thus, I expected no such co-inductive effect in cultured fibroblast-like fish cells. The cell experiment (**Figure 21**) was conducted with lower concentrations of Keto [1-5 µM] than before with larvae [4-20 µM] due to the observed *in vivo* toxicity at ≥ 2 µM. Still, in case Keto had a disruptive effect on cell xenometabolism, a signal increase could have been expected in this concentration range, as the cells are normally more sensitive to compound treatment than the larvae. However, no increase of the Dex-induced signal by Keto treatment was observable *in vitro*. This result supports the possibility that co-induction *in vivo* may have underlain xenobiotic clearance, which led to a higher Dex level in larvae. Furthermore, the highest Keto concentration [5 µM] showed reduced activation compared to the negative control (DMSO + Dex). This signal decrease may have been caused by weak cytotoxic effects of Keto, occurring at this concentration, but was not further assessed for this thesis work.

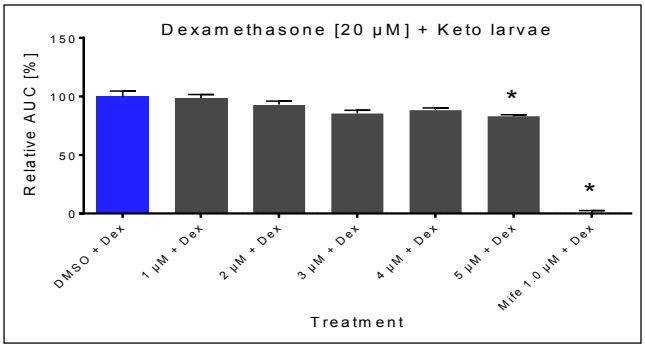


Figure 21: Effect of Ketoconazole on Dexamethasone-induced glucocorticoid signaling in *vitro*
Relative AUC values are shown for a range of five different concentrations of Keto + Dex [20 µM] (grey, n = 16) measured in AB.9-*GRE:Luc* cells. The data are normalized relative to the negative control DMSO + Dex [20 µM] (blue, n = 8) and the DMSO control (blue, n = 8). The amount of DMSO was 0.2% in all treatment conditions. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, α=0.05. After multiple testing correction with the Holm-Šídák method, significance is defined as $p < \alpha < 0.005$= *.

Eto is an anesthetic, used for operations of short durations under sedation (Forman 2011; Giese and Stanley 1983). Furthermore, it suppresses the corticosteroid synthesis by inhibiting only one enzyme, 11β-hydroxylase, for which reason it is also therapeutically used to treat Cushing´s syndrome (Bergström et al. 1998; Greening et al. 2005; Preda et al. 2012), and which is why I considered it generally suitable as a control inhibitor for the current testing approach.
As shown in **Figure 20 b**, Eto inhibited the Preg-activated GC signaling, but it also appears to decrease the Dex-activated signal. Furthermore, and probably being involved in the signalk decreases, Eto induced toxic effects in larvae at all tested concentrations, with both activators. Lower concentrations [2 - 6 µM] showed mostly sub-lethal effects, including malformations of heart and yolk, reduced to no bloodflow and enlarged eyes (data not shown). At 8 µM, these effects aggravated and several larvae had no heartbeat and thus, were considered dead. At 10 µM most larvae were dead. These toxic effects in combination with the primary medical application of Eto as an anesthetic suggest insufficient effect specificity of the compound, which makes it unsuitable as a reference inhibitor of GC biosynthesis.

Mety is a drug used for the diagnosis of adrenal insufficiency and, like the former two candidates, for the treatment of Cushing's syndrome (Paek et al. 2015; Sosenko, Lewis, and Frank 1986). It is mechanistically suited as a control inhibitor, as it also blocks the biosynthesis of Cort by reversibly inhibiting steroid 11β-hydroxylase (Bergström et al. 1998; Gold et al. 1960). Inactivation of this enzyme reduces the negative feedback mechanism on adrenocorticotropic hormone (ACTH) production, which induces the secretion of ACTH and thereby increases the plasma levels of 11-deoxycortisol, the direct precursor of Cort.
As shown in **Figure 20 c**, Mety concentration-dependently inhibited the Preg-induced GRIZLY signal, but had no effect on Dex-induced signaling. This is consistent with the expected effect mechanism of Mety to not directly interfere with the GR activity (as Mife does) in the transgenic larvae, but to inhibit the biosynthesis of endogenous Cort. Moreover, in contrast to the two other candidates, Mety showed no indications of toxicity or harm for the larvae at any concentration tested. These results qualify Mety as a suitable control inhibitor of Preg-induced Cort biosynthesis. The concentration of 40 µM was selected for control applications in the GRIZLY assay. In **Figure 22**, a broader range [10 - 50 µM] of test concentrations (than in **Figure 20 c**; 30 - 50 µM) is shown for Mety in co-exposure with Preg (**a**) and Dex (**b**) in order to emphasize a clear concentration-dependency in the inhibitory effect of Mety.

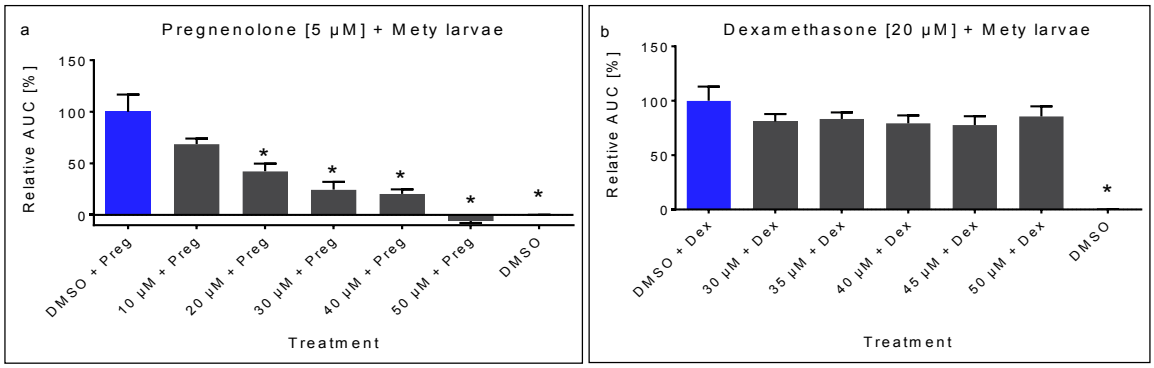


Figure 22: Metyrapone inhibits Pregnenolone-, but not Dexamethasone-induced glucocorticoid signaling *in vivo*

Metyrapone inhibited concentration-dependently Preg- (**a**), but not Dex-induced (**b**) GC signaling in larvae. Relative AUC values are shown for a range of five different concentrations of Mety + Preg [5 µM] (**a**)/ Dex [20 µM] (**b**)(grey, n = 16). The data are normalized relative to the negative control DMSO + Preg [5 µM] (**a**)/ Dex [20 µM] (**b**) (blue, n = 8) and the DMSO control (blue, n = 8). The amount of DMSO was 0.2% in all treatment conditions. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, $\alpha = 0.01$. After multiple testing correction with the Holm-Šídák method, significance is defined as $p < \alpha < 0.002$= *.

Combined compound analysis with different variations of the GRIZLY

The validation experiments with the selected controls show in summary that the combined application of two different GRIZLY testing approaches does indeed represent a revealing tool to investigate diverse mechanisms of GC inhibition. While both setups detect inhibitors that interfere directly with GR signaling in larvae, exclusively the first approach also allows GC signaling induction in cultured cells and, thus, enables the comparison of compound effects *in vivo* and *in vitro*. In return, the second approach additionally permits the identification of upstream inhibitors and disruptors of the GC biosynthesis pathway *in vivo*. Therefore, the comparative analysis of a compound screen conducted with three variations of the GRIZLY assay (**Figure 9**) will provide first hints of compound-specific mechanisms of action. The three variants of the GRIZLY assay and their endpoints are:

GRIZLY 1 - Inhibition of directly activated GC signaling (with Dex) in AB.9-*GRE:Luc* cells
GRIZLY 2 - Inhibition of directly activated GC signaling (with Dex) in *Tg(GRE:Luc)* larvae
GRIZLY 3 - Inhibition of indirectly activated GC signaling (with Preg) in *Tg(GRE:Luc)* larvae

3. 2 Testing strategy - A tiered approach

The FDA drug library should be screened with these three variations of the GRIZLY assay in order to target the three aims of my study, 1) to assess the suitability of the GRIZLY assay for the detection of GC signaling inhibitors, 2) to identify disruptors of the GC biosynthesis pathway and 3) to identify compounds with - so far - unknown potential to interfere with GC signaling or with unknown mechanisms of action. The outcomes of the screens with the three GRIZLY assay variations should allow me to pre-categorize compounds, for instance as putative GR antagonists or as disruptors of the Cort biosynthesis. In order to validate the screen results and to proof the GRIZLY assay suitability for the detection of GC signaling inhibitors, the identified hits will have to

be confirmed by retesting. For further characterization of the compound effects, also suitable follow-up studies will have to be conducted.
Therefore, I planned my experiments according to the principle of a tiered testing approach (**Figure 23**), whereby I aimed to isolate real candidates of interest out of a library of compounds. With increasing testing complexity and endpoint specificity, substances which are beyond the focus of the study should successively be excluded.

Tier 1 is designated to detect GC signaling disruptors from the drug library. The three GRIZLY screens are performed and the FDA library compounds are measured for their potential to inhibit directly activated GC signaling in cells (GRIZLY 1), directly activated GC signaling in larvae (GRIZLY 2) and indirectly activated GC signaling in larvae (GRIZLY 3). Furthermore, compound toxicity is assessed in this step, in order to distinguish between specific inhibitors and false-positive hits, where compounds decrease the GC signal because they are toxic to cells or larvae. Statistically significant, non-toxic hits are then transferred to the next level.

In tier 2, the screen hits are validated. The respective substances are purchased from a different supplier in order to exclude false-positive screen hits that derived e.g. from contaminated library compounds. Test solutions in at least five nominal concentrations are prepared for each substance and they are exposed to cells and larvae in the same three testing setups as before in the screen. Compounds that are confirmed during retesting are considered verified disruptors of GC signaling. With a high correlation between the detected screen hits and the confirmed GC signaling disruptors, a good assay reliability is indicated, whereby the first aim of this study, to assess the suitability of the GRIZLY assay for the detection of GC signaling inhibitors, would be achieved. Confirmed disruptors of GC signaling are then considered in a combined analysis of compound effects in all three GRIZLY approaches. This will provide more information about the compound effect dynamics and may help to pre-categorize their potential mechanisms of action. Validated compounds with disruptive effects on GC signaling are subjected to selected follow-up experiments.

Tier 3 serves a more detailed characterization of compound effects in order to address the two other aims of this study, to identify 2) disruptors of the GC biosynthesis pathway and 3) compounds with - so far - unknown potential to interfere with GC signaling and/ or with unknown mechanisms of action.
In tier 3a, confirmed *in vivo* disruptors of GC signaling are examined for their effects on endogenous steroid hormone levels. For this, larvae are exposed to reasonable concentrations of the selected compounds in co-exposure with Dex and Preg, respectively. UPLC-MS/MS experiments are conducted with larvae homogenates and endogenous concentrations of Preg, Prog, Cort and Dex (only in Dex-exposed larvae) are measured. Altered steroid hormone levels identified by chemical analysis provides valuable information about inhibitory compound effects on the steroid hormone biosynthesis pathway, drug uptake and -metabolism.

Tier 3b consists of gene expression analysis with validated *in vivo* GC signaling disruptors. The same treatments as for UPLC-MS/MS experiments are applied to the larvae. Real-time qPCR experiments are performed and a set of selected target genes is analyzed for compound effects on mRNA expression. The obtained gene expression patterns provide more insight into the compound mechanisms of action.

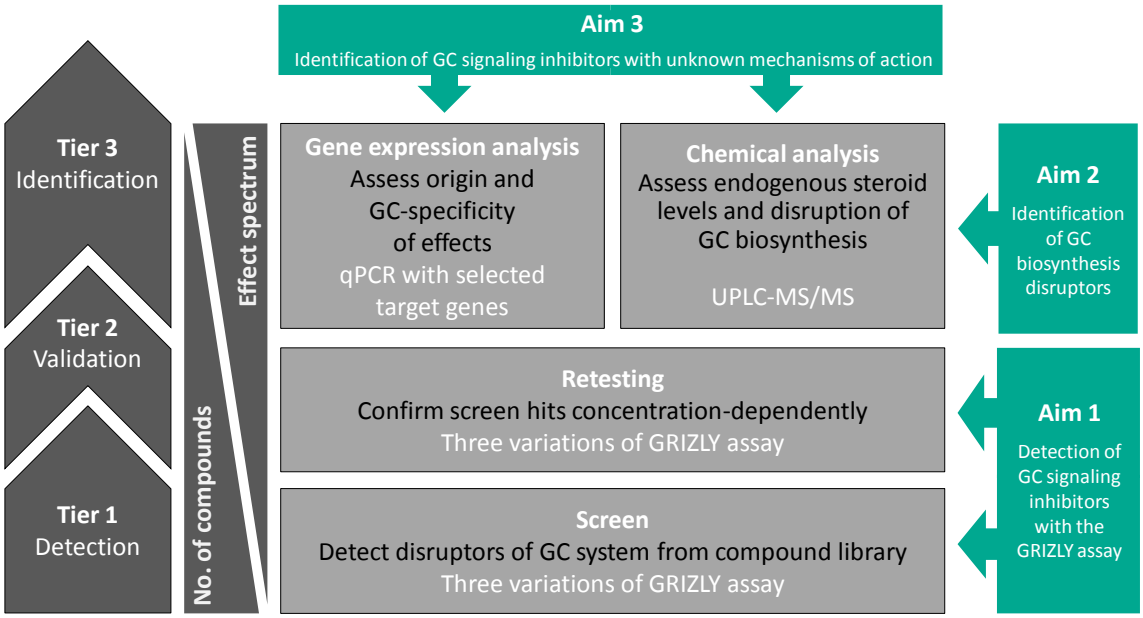


Figure 23: Schematic structure of the tiered testing approach
With increasing endpoint specificity, the number of library compounds is successively reduced until the candidates of interest are isolated from a library of compounds. From each level, only statistically significant and relevant hits are carried forward. Tier 1 consists of the drug library screen with three variations of the GRIZLY assay in order to detect inhibitors of GC signaling. In tier 2, screen hits are retested concentration-dependently in all three GRIZLY variants. Tier 3 serves to gain more information about the compound effect mechanisms in order to identify disruptors of GC biosynthesis and GC signaling inhibitors with unknown mechanisms of action. In tier 3a, UPLC-MS/MS experiments are conducted with validated *in vivo* disruptors of GC signaling. Compound effects on endogenous steroid hormone levels and on Dex uptake and metabolism are assessed in chemical analysis. In tier 3b, qPCR experiments are performed with validated *in vivo* disruptors of GC signaling. A set of target genes is analyzed for expression changes mediated by the compounds. The obtained gene expression patterns provide more information about the compound mechanisms of action.

3.3 Tier 1: Screening of the FDA library

The FDA library was screened with the three GRIZLY variants. In GRIZLY 1, GC signaling was directly activated with Dex [20 µM] in AB.9-*GRE:Luc* cells in order to detect compounds that interfere with GR signaling in a cell autonomous fashion. Mife [1 µM] served as a control inhibitor, as it directly antagonizes the GR and prevents GC target gene transcription. The *in vitro* approach specifically enables the detection of substances which disrupt GC signaling without prior metabolic activation or tissue-specific interactions.

In GRIZLY 2, the same controls were applied as in GRIZLY 1, but to *Tg*(*GRE:Luc*) larvae. This setup targets to detect disruptors of GR signaling *in vivo*, taking into account whole-organism effects of the compounds. GRIZLY 3 was also conducted *in vivo*, but with Preg [5 µM] as control activator and Mety [40 µM] as reference inhibitor. In this approach, GC signaling is indirectly stimulated,

as the *Tg*(*GRE:Luc*) larvae metabolize Preg into Cort, which then agonizes the GR. In addition to GC signaling inhibition by mechanisms also targeted in GRIZLY2, indirect effects, in especially disruptions of the Cort biosynthesis pathway can be detected in this assay mode. The same testing conditions were applied in order to assess toxicity in the three screening setups.

3.3.1 Screens for toxicity assessment

For the *in vivo* assays, larvae toxicity was assessed after 24 h exposure. Immediately at the end of each GRIZLY screen, the cover films of the 96-well plates were opened and immobile and dead larvae were scored. Compounds were considered as potentially toxic *in vivo* if the amount of immobile larvae was ≥ 10%. Out of 640 library compounds, 133 were regarded as toxic in at least one of the two *in vivo* assays.
In vitro, toxicity was assessed in a separate screen. Two different cytotoxicity testing methods were applied simultaneously, the AlamarBlue® and the LDH assay. Compounds were considered as cytotoxic when statistically significant reduction of cell viability (AlamarBlue®) or induction of cell death (LDH) was confirmed in one of the two assays. By means of the AlamarBlue® cell proliferation assay, 12 compounds were identified as cytotoxic. Assessment with the LDH assay did not lead to any significant results.

In total, toxicity was detected in 140 out of 640 compounds. The numbers of toxicants and their overlap between the different screening approaches are shown in the Venn diagram in **Figure 24**. In GRIZLY 1, 12 substances were toxic, 77 in GRIZLY 2 and 97 in GRIZLY 3. According to the low number of substances that were toxic *in vitro*, only two compounds were identified as toxic in all three screens. Two other compounds were found in both the larvae and the cell culture screen for inhibition of directly activated GC signaling. Between GRIZLY 1 and GRIZLY 3, only one substance was found to be toxic in common. Seven compounds showed toxicity in the cell assay while they were not harmful for larvae. The overlap of toxicants found between the two *in vivo* screens was 50% for GRIZLY 2 (39 out of 77) and 40% for GRIZLY 3 (39 out of 97).

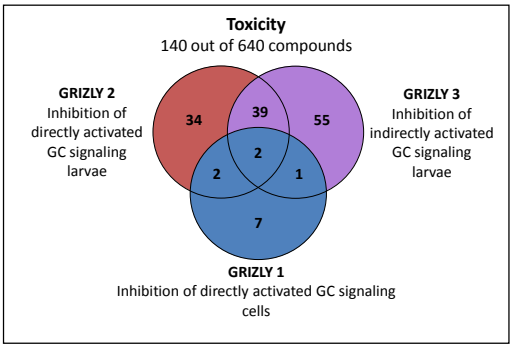


Figure 24: Venn diagram showing toxicity in the FDA library screens
FDA ibrary compounds were defined as toxic *in vivo*, if ≥ 10% of larvae were immobile, or *in vitro*, if cytotoxicity was statistically significant with $p < \alpha <$ 0.0001 in at least one out of the AlamarBlue® and the LDH assay. In 140 out of 640 screening compounds, toxicity was found. The numbers of toxic compounds identified in each screen are indicated in colored circles. GRIZLY 1 - inhibition of directly activated GC signaling in cells: blue; GRIZLY 2 - inhibition of directly activated GC signaling in larvae: orange; GRIZLY 3 - inhibition of indirectly activated GC signaling in larvae: pink. Numbers assigned to overlapping areas between the circles indicate the number of compounds with common toxicity in two or three screens.

3.3.2 Screens for inhibitors of GC signaling in three GRIZLY assay modes

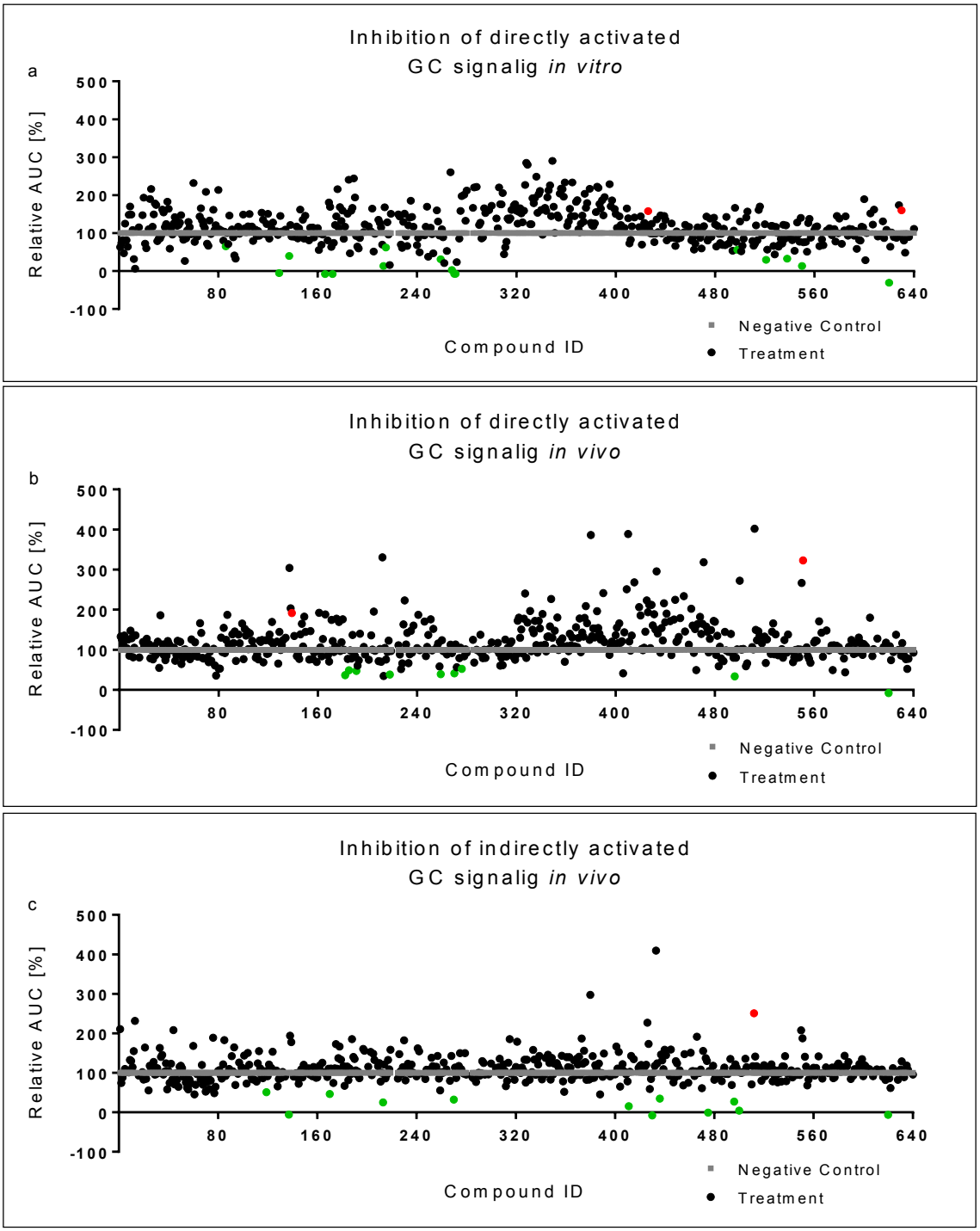


Figure 25: Result overview of the FDA approved drug library screen in the three GRIZLY screen modes

Data are normalized relative to the negative control (100%) and the positive control (0%), mean relative AUC values are shown (DMSO in all treatments 0.2%). **a**) Inhibition of directly activated GC signaling *in vitro* (n = 3 - 4). **b**) Inhibition of directly activated GC signaling *in vivo* (n = 5 - 10). **a + b**) Treatment conditions: Dex [20 µM] + library compounds (black), negative control: Dex [20 µM] + DMSO (grey); positive control Dex [20 µM] + Mife [1 µM] (not shown). **c**) Inhibition of indirectly activated GC signaling *in vivo* (n = 3 - 10). Treatment: Preg [5 µM] + library compounds (black), negative control: Preg [5 µM] + DMSO (grey); positive control Preg [5 µM] + Mety [40 µM] (not shown). The amount of DMSO was 0.2% in all treatment conditions. Statistical significance is determined by a multiple t-test ($\alpha = 0.05$) and corrected for multiple comparisons according to the Holm- Šídák method with $p < \alpha < 0.0001$. Significant inhibitors (green) and superactivators (red) are highlighted.

Overview of screen results

The FDA library was screened with the three variations of the GRIZLY assay. Statistical analysis was conducted for all 640 compounds, but the 140 potentially toxic candidates were excluded from data visualization, in order to highlight only real (non-toxic) significant hits. In **Figure 25**, the outcomes of GRIZLY 1 (**a**), GRIZLY 2 (**b**) and GRIZLY 3 (**c**) are shown. Mean relative AUC values are indicated for 500 library compounds (black), normalized on their controls (negative control: grey, positive control not shown).

The data distribution is diverse between the three screens. The majority of values is scattered closely around the negative control, but in each screen, several significant hits were identified. These mostly reduced GC signaling, (green) but in some cases, also superactivation of GC signaling was detected (red).

Altogether, 16 inhibitors and two superactivators were identified with GRIZLY 1, nine inhibitors and two superactivators with GRIZLY 2 and 12 inhibitors plus one superactivator in GRIZLY 3. The total numbers of identified compounds and their overlaps between the different screening approaches are visualized as Venn diagrams in **Figure 26**. Among the 29 inhibitors (**a**), two compounds were identified as significant hits in all three screens and four candidates were inhibitors in two screens each, but the majority was uniquely detected within the respective setup. For the five identified superactivators (**b**), no overlap occurred between the single screens.

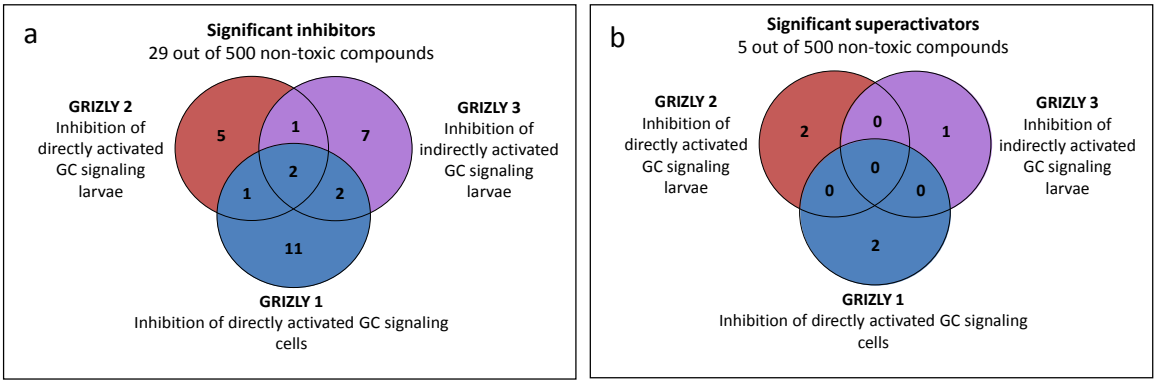


Figure 26: Venn diagrams - inhibitors and superactivators of glucocorticoid signaling
The Venn diagrams show the distribution of significant inhibitors (**a**) and superactivators (**b**) of glucocorticoid signaling according to the three GRIZLY assay variants. The numbers of significant inhibitors and superactivators identified in each screen are indicated in colored circles. GRIZLY 1 - inhibition of directly activated GC signaling in cells: blue; GRIZLY 2 - inhibition of directly activated GC signaling in larvae: orange; GRIZLY 3 - inhibition of indirectly activated GC signaling in larvae: pink. Numbers assigned to overlapping areas between the circles indicate the number of compounds with common inhibitory or superativating effects in two or three screens.

Detailed screen results

The detailed screen results are shown as color code profiles in **Figure 27**. Color code profiles are shown for controls and library compounds. No difference of the substance effect compared to the negative control (Dex [20 µM]/ Preg [5 µM] + DMSO) is indicated in yellow. A significantly increased or decreased signal intensity compared to the negative control is indicated in green

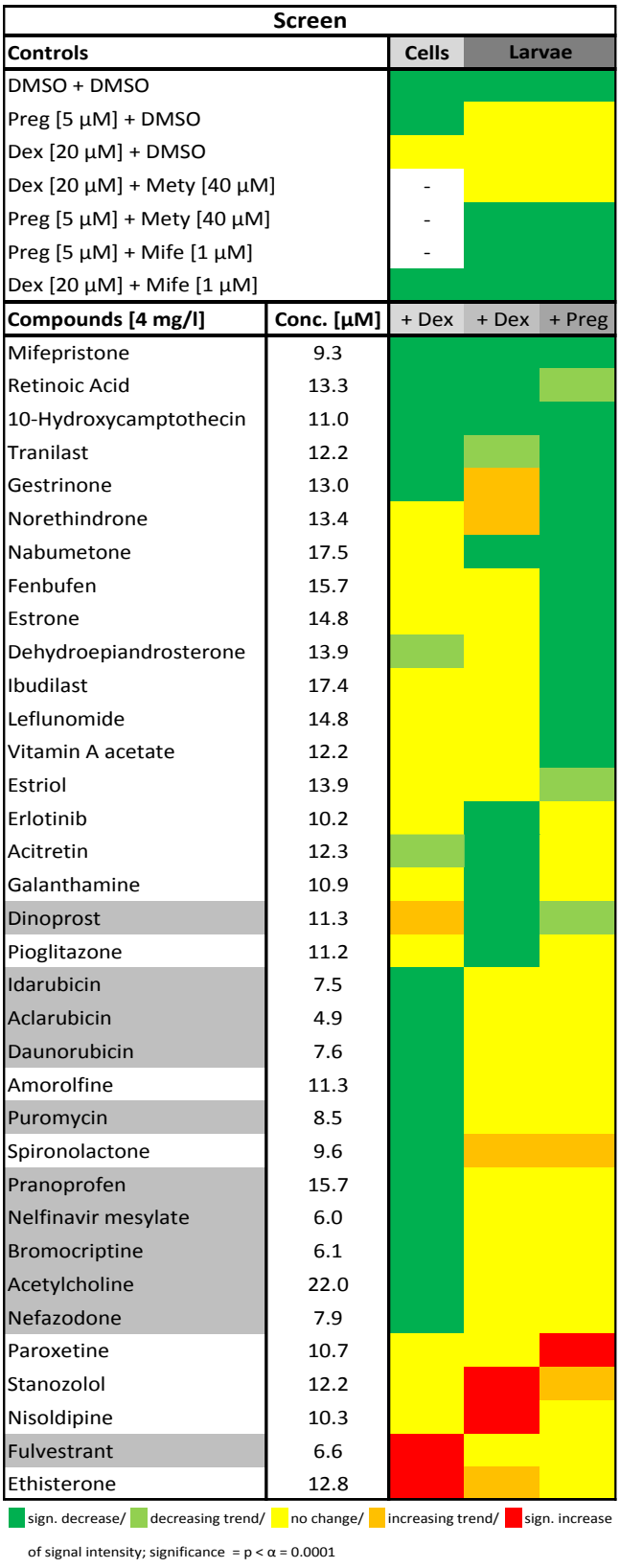


Figure 27: Significant hits identified in three GRIZLY screening approaches

Color code profiles are shown for controls and library compounds. No difference of the substance effect compared to the negative control (Dex [20 µM]/ Preg [5 µM] + DMSO) is indicated in yellow. A significantly increased or decreased signal intensity compared to the negative control is indicated in green and red, respectively. Non-significant trends ($p < \alpha < 0.05$) for increase or decrease are shown in orange and light green, respectively. Significance is defined as $p < \alpha < 0.0001$.

and red, respectively. The FDA library screen concentration was 4 mg/l, the corresponding concentrations in [µM] are indicated for each compound.
In addition to the 34 compounds with significant effects in at least one of the three GRIZLY approaches, Estriol is included in the figure. Although it had no significant effect, it showed a strong trend to inhibit Preg-induced signaling, which was confirmed by concentration-dependent retesting (see below, chapter **3.2.2**). Mife, which was applied as positive control inhibitor of directly activated GC signaling was identified as a library compound. It was one of the two substances in the FDA library which inhibited GC signaling in all three GRIZLY screens. The recovery of this reference GR antagonist from a library of 640 compounds within all setups serves as a proof of principle for the suitability and reliability of the GRIZLY assay screening approach.

Selection of compounds for tier 2

During the screening procedures, several inhibitors and also superactivators of GC signaling were detected. However, these findings had to be confirmed by retesting procedures, in order to show the reproducibility and reliability of the results, and to qualify the GRIZLY assay as a suitable test system for the detection of GC signaling disruptors.
For retesting, the total number of candidates should be reduced due to feasibility issues. According to the second aim of the study, to identify potential disruptors of the GC biosynthesis pathway, all compounds that inhibited Preg-, but not Dex-induced signaling were most promising and thus selected for the next testing step.
Furthermore, covering the third aim of the study, to identify disruptors of GC signaling with unknown mechanisms of action, all candidates which significantly inhibited in two or three GRIZLY setups were selected, as well as several inhibitors of Dex-activated signaling only, four from the larvae- and two from the cell screen. Of the five superactivators, all except one (Fulvestrant) were retested, as Fulvestrant was the only compound which exclusively superactivated *in vitro* without any effect trend *in vivo*, and thus was aside the focus of this study. Altogether, 11 compounds were excluded from retesting (highlighted in grey in **Figure 27**) and 24 substances were selected for tier 2.

3.4 Tier 2: Retesting of screen hits

In order to validate the findings from the chemical screens, the selected compounds were retested concentration-dependently. For this, they were ordered from a different supplier in order to eliminate potential unspecific effects due to e.g. impure synthesis procedures or contaminations during library preparation. Then, these compounds were retested in all three GRIZLY setups, in at least five different nominal concentrations each. The range of concentrations was selected surrounding the applied screening concentration. In cases where none of these concentrations appropriately reproduced the screening results, the range was adjusted accordingly.

3.4.1 Assessment of screen reliability

For the assessment of the assay reliability, the screening outcomes are compared with the retesting results in **Figure 28**. The compounds are grouped according to their effect patterns in the retest. Color code profiles are shown for controls and library compounds. No difference of the substance effect compared to the negative control (Dex [20 µM]/ Preg [5 µM] + DMSO) is indicated in yellow. A significantly increased or decreased signal intensity compared to the negative control is indicated in green and red, respectively. The FDA library screen concentration was 4 mg/l, the corresponding concentrations in [µM] are indicated for each compound. Validated inhibitors or superactivators from retesting are indicated with their lowest observed effect concentration (LOEC) in the respective GRIZLY assay. Assay reliability is surveyed by direct comparison of a screen result with the respective retesting outcome. Confirmed screen hits or trends are noted with a check. Confirmation was given when at least one of the retesting concentrations significantly reflected the screen result. Diverging results are indicated with a cross.

In general, the retesting results largely confirmed the screening outcomes. From 72 screen results, 16 could not be confirmed, which accounts for 22%. Thereof, nine compounds (12.5%) were false-negatively not detected by the screen, but identified during concentration-dependent retesting. In some cases, the LOEC in the retest was higher than the screen concentration, which explains the lack of identification within the screen. For the compounds where the screen concentration was as high as or higher than in retesting, a high variance within screen replicates was potentially the reason why they were not detected as significant inhibitors or superactivators in the first place.

From the total 22% of non-confirmed results, the other seven compounds (9.5%) were false-positively identified in the screen. In these cases, lacking reproducibility by concentration-dependent retesting with newly ordered compounds might indicate that single wells on the library stock plates were possibly contaminated (which may have bben the case for instance Nisoldipine, which showed superactivation in GRIZLY 2 in the screen). Potentially, carry-over from other library compounds may have induced the effects within screening conditions.

Altogether, with a recovery rate of 78%, the screen reliably detected disruptors of GC signaling. Not only significant hits but also observed trends in the screen (e.g. reduced GC signaling in GRIZLY 3 by Estriol) were reproduced and confirmed during retesting. Accordingly, the first aim of this study was achieved. The GRIZLY assay is a suitable tool to detect inhibitors of GC signaling. Moreover, it enabled the detection of superactivators, which constitutes an additional useful application of the assay.

Controls		Screen			Retest LOEC [µM]			Confirmed?	
		Cells	Larvae		Cells	Larvae			
DMSO + DMSO								✔✔	
Preg [5 µM] + DMSO								✔✔	
Dex [20 µM] + DMSO								✔✔	
Dex [20 µM] + Mety [40 µM]		-			-			✔✔	
Preg [5 µM] + Mety [40 µM]		-			-			✔✔	
Compounds [4 mg/l]	**Conc. [µM]**	+ Dex	+ Dex	+ Preg	+ Dex	+ Dex	+ Preg		**Pattern group**
Mifepristone	**9.3**				0.2	0.4	0.2	✔ ✔ ✔	
10-Hydroxycamptothecin	**11.0**				4	4	4	✔ ✔ ✔	
Retinoic Acid	**13.3**				0.4	1.2	1.6	✔ ✔ ✔	1
Tranilast	**12.2**				10	10	10	✔ ✔ ✔	
Acitretin	**12.3**				4	8	4	✔ ✔ X	
Gestrinone	**13.0**				4	4	4	✔ ✔ ✔	
Norethindrone	**13.4**				40	10	10	X ✔ ✔	
Ethisterone	**12.8**					4		X ✔ ✔	2
Stanozolol	**12.2**				12	12	8	X ✔ ✔	
Spironolactone	**9.6**				6	2	2	✔ ✔ ✔	
Nabumetone	**17.5**					4	4	✔ ✔ ✔	3
Fenbufen	**15.7**					8	4	✔ X ✔	
Estrone	**14.8**				8		4	X ✔ ✔	
Dehydroepiandrosterone	**13.9**				20		4	✔ ✔ ✔	4
Ibudilast	**17.4**				4		8	X ✔ ✔	
Galanthamine	**10.9**				4		4	X X X	
Leflunomide	**14.8**						4	✔ ✔ ✔	5
Estriol	**13.9**						12	✔ ✔ ✔	
Amorolfine	**11.3**				8			✔ ✔ ✔	6
Nisoldipine	**10.3**				1.0			X X ✔	
Erlotinib	**10.2**							✔ X ✔	
Pioglitazone	**11.2**							✔ X ✔	7
Vitamin A acetate	**12.2**							✔ ✔ X	
Paroxetine	**10.7**							✔ ✔ X	

sign. decrease/ decreasing trend/ no change/ increasing trend/ sign. increase of signal intensity;
significance = $p < \alpha = 0.0001$ for screen and $p < \alpha = 0.002$ for retesting results; trends = $p < \alpha = 0.05$

Figure 28: Comparison of screen and retest indicates reliable performance of the screen protocol
Color code profiles are shown for controls and library compounds. No difference of the substance effect compared to the negative control (Dex [20 µM]/ Preg [5 µM] + DMSO) is indicated in yellow. A significantly increased or decreased signal intensity compared to the negative control is indicated in green and red, respectively. Non-significant trends ($p < \alpha < 0.05$ for increase or decrease are shown in orange and light green, respectively. Significance is defined as $p < \alpha < 0.0001$ for the screen ($\alpha = 0.05$) and $p < \alpha < 0.002$ for retesting ($\alpha = 0.01$). Validated inhibitors or superactivators from retesting are indicated with their lowest observed effect concentration (LOEC) in the respective GRIZLY assay. Assay reliability is surveyed by direct comparison of a screen result with the respective retesting outcome. Confirmed screen hits or trends are noted with a check. Confirmation was given when at least one of the retesting concentrations significantly reflected the screen result. Diverging results are indicated with a cross.

3.4.2 Combined analysis of compound effects in all three GRIZLY assay modes

For a more detailed analysis of the identified substances and their impacts on GC signaling, compounds showing the same effect patterns in the three GRIZLY assay screens are compared with each other on the following pages. Within this context, also the bioluminescence traces over time, which were measured for each treatment condition, are considered, as they can

possibly provide useful information about compound-specific dynamics. As examples for normal-shaped bioluminescence dynamics, an overview of the signal traces measured over time for this study´s control activators and inhibitors is given in **Figure 29.**

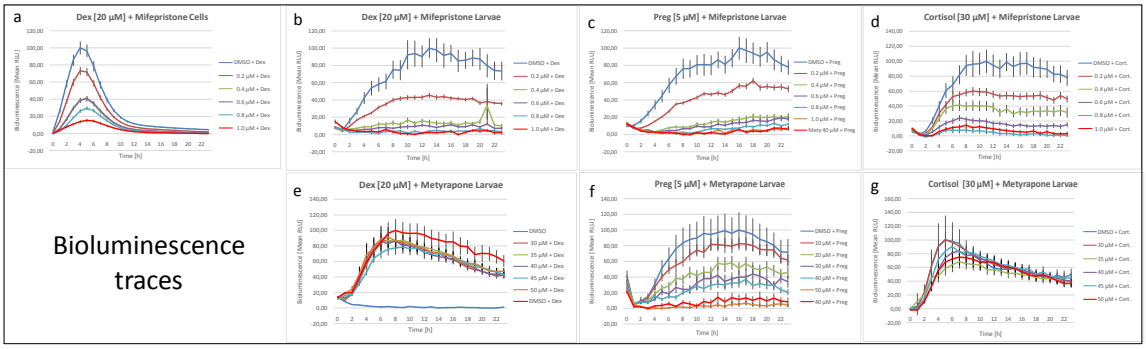


Figure 29: Bioluminescence traces of control activators and inhibitors of glucocorticoid signaling
Bioluminescence traces were detected over 24h of exposure to treatment combinations with control activators (Dex, Preg and Cort) and inhibitors (Mife [0.2 - 1 µM], Mety [10 - 50 µM]). Bioluminescence was measured in relative light units (RLUs). Data are shown as mean relative light units (RLUs) ± SEM. (**a**) Dex [20 µM] + Mife in cells and (**b**) in larvae. (**c**) Peg [5 µM] + Mife, (**d**) Cort [30 µM] + Mife (**e**) Dex [20 µM] + Mety, (**f**) Preg [5 µM] + Mety, (**g**) Cort [30 µM] + Mety, all in larvae. All treatments are presented relative to the respective negative (Dex [20 µM]/ Preg [5 µM]/ DMSO + DMSO; blue) and positive (Dex [20 µM]/ Preg [5 µM] + Mife [1 µM]/ Mety [40 µM]/ Dex [20 µM]; red) control. The amount of DMSO was 0.2% in all treatments.

The activators Dex, Preg, and also directly administered Cort are each co-exposed with either Mife or Mety. The graphs show that the uninhibited bioluminescence traces of all activators (negative controls, blue) form a bell-shaped signal over time. The timepoints of signal peaks vary between the different setups, with cells showing maximal bioluminescence earliest, after 4h. In larvae, the signal peaks later, between 5 h (**g**) and 16 h (**c**), depending on the individual testing conditions. However, it is noticeable in all larvae exposures (**b - g**) that the activator signal starts to decrease between 5h (**g**) and 16h (**c**). This effect may rely on homologous downregulation of the GR (GRα), a mechanisms which is often observable under chronic stimulation of GC signaling (as it will be described in detail below in section **4.4.2.4**). Despite this decrease, the bioluminescence signal measured in larvae does not fall back to the ground level from the exposure start. Instead, it remains elevated until the end of the exposure duration. This is in contrast to the cell behavior, where the signal height falls back to ground intensity after 20 h exposure (**a**).
Regarding the inhibitors (positive controls, red), Mife reduces the height of the signal curve in all exposure scenarios in a concentration-dependant manner (**a - d**). Mety does not affect signal activation by Dex (**e**) or directly administered Cort (**g**), as the bioluminescence signal curves are not altered when compared to the negative controls, but it inhibits concentration-dependently the Preg-induced signal (**f**).

Similar to this example, the bioluminescence traces of the validated inhibitors and superactivators from **Figure 28** will be taken into account for the evaluation of compound effects. Abnormal shapes of signal traces could provide information about the effect dynamics of a compound. Therefore, the bioluminescence signal curves may also constitute a revealing

resource for the pre-catigorization of compound mechanisms of action. In the following figures, compounds with the same effect patterns are grouped together and their bioluminescence traces in all three GRIZLY assay variants are examined for abnormalities.

Pattern group 1

Compounds which inhibited in all three GRIZLY assays were assigned to pattern group 1. In **Figure 30**, their effect patterns with LOECs for each assay and the bioluminescence traces are shown. The five members of this group are Mife, 10-Hydroxycamptothecin (10-HC), Tranilast (Trani), Retinoic acid (Ret. acid) and Acitretin (Aci).

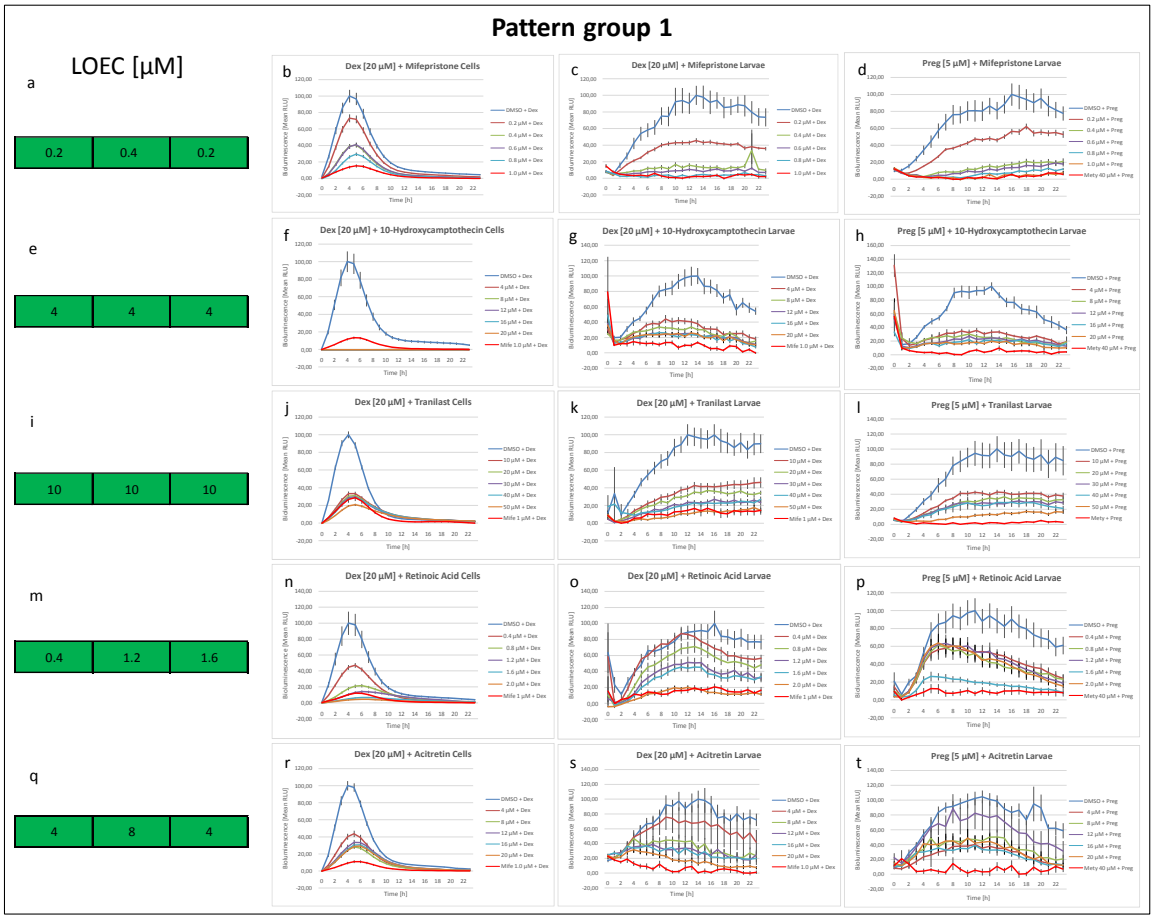


Figure 30: Bioluminescence traces of effect pattern group 1 compounds
Effect patterns with LOECs [µM] and the bioluminescence traces from all three GRIZLY assay variants are shown for Mifepristone (Mife; **a - d**), 10-Hydroxycamptothecin (10-HC; **e - h**), Tranilast (Trani; **i - l**), Retinoic acid (Ret. acid; **m - p**) and Acitretin (Aci; **q - t**) in combination with Dex [20 µM] in cells and larvae and with Preg [5 µM] in larvae. Bioluminescence was measured for 24h and data are shown as mean relative light units (RLUs) ± SEM. All treatments are presented relative to the respective negative (Dex [20 µM]/ Preg [5 µM] + DMSO; blue) and positive (Dex [20 µM]/ Preg [5 µM] + Mife [1 µM]/ Mety [40 µM]; red) control. The amount of DMSO was 0.2% in all treatments.

Mife reduced GC signaling in all three GRIZLY assay variants (**a - d**) because it directly antagonizes the GR, irrespective of whether the agonistic ligand is Dex or Cort. The signal pattern of Mife could thus be expected for every GR antagonist which is active *in vitro* and *in vivo*. As all inhibitors of pattern group 1 showed the same signal kinetics as they are observed Mife, with some minor differences, GR antagonism could be assumed to be their mechanism of

action. However, to my knowledge, none of them has been annotated as a GR antagonist so far in the literature.

Effects on luciferace enzyme activity

Alternative mechanisms have to underlie the observed signal decreases. As these compounds inhibited in all three GRIZLY assays, it may also be possible that they had inhibitory effects on the activity of the luciferase enzyme itself. Therefore, I conducted a control experiment in order to assess the luciferase activity independent from GC signaling. I transiently transfected wildtype AB.9 cells with the pGL3-Control vector, which contains a constitutively active promoter driving luciferase expression. Transfected cells were then co-treated with Dex and the inhibitors from pattern group 1, which were applied in one concentration that showed an equally strong signal inhibition within retesting as the positive control. The relative AUC values of these treatment conditions were normalized to those of transfected cells treated with the negative control condition Dex + DMSO.

The outcomes of the experiment are shown in **Figure 31**. Ret. acid [2 µM] signlificantly reduced the luciferase activity (67 %) compared to the control condition (100 %). All other compounds had no significant effects on the activity of the luciferase enzyme, which excludes the possibility that their inhibitory effects in the GRIZLY assay relay on compound interference with the reporter protein. Only the inhibitory effect of Ret. acid on the luciferase activity may have contributed to the observed GC signaling disruption in all three GRIZLY assays. However, this effect alone cannot have been responsible for the observed inhibition, as the signal decrease was considerably stronger on the GRE-containing promotor sequence than with the pGL3-Control vector. Thus, the reduction of luciferase activity will not be considered in later discussions as the main mechanism by which Ret. acid inhibited the *GRE:Luc* reporter.

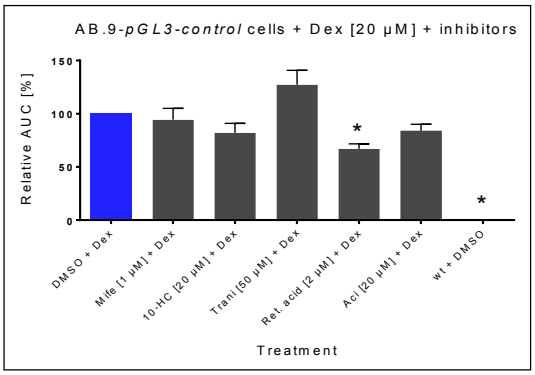


Figure 31: Effects of inhibitors from pattern group 1 on luciferase reporter activity in transiently transfected AB.9-*pGL3-control* cells

Wildtype AB.9 cells were transiently transfected with the pGL3-Control vector that contains a constitutively active promoter driving luciferase expression. AB.9-*pGL3-control* cells were treated either with Dex [20 µM] + DMSO (blue, negative control) or with Dex [20 µM] + the five inhibitors from pattern group 1 (grey) , Mife, 10-HC, Trani, Ret. acid and Aci, respectively. Untransfected AB.9 cells were treated with DMSO as a transfection control (no signal induction). The amount of DMSO was 0.2% in all treatment conditions. AUC values are given relative to the negative control. Data are shown as means + SEM (n = 3). Significanly altered relative AUC values are indicated with an asteriks. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, α=0.05. After multiple testing correction with the Holm-Šídák method, significance is defined as $p < \alpha < 0.005$ = *.

Pattern group 2

All compounds which superactivated the Dex-induced signal in larvae were assigned to pattern group 2. Their effect patterns with LOECs for each assay and the bioluminescent traces are shown in **Figure 32**. The five members of this group are Gestrinone (Gestri), Norethindrone (Nor), Ethisterone (Ethi), Stanozolol (Stano) and Spironolactone (Spiro). Despite the common superactivation in GRIZLY 2, the compound effects in GRIZLY 1 and GRIZLY 3 are diverse. Gestri, Nor, Stano and Spiro inhibited GC signaling in cells but Ethi had no effect in GRIZLY 1 in any retested concentration. Gestri and Nor inhibited also Preg-induced GC signaling in larvae, which is interesting as it is opposite to their *in vivo* effect on Dex-activated GC signaling. Ethi had no effect in GRIZLY 3, which makes the superactivation in GRIZLY 2 the only impact of the compound on GRIZLY activity. Stano and Spiro, similar to their effects in GRIZLY 2, superactivated GC signaling also in GRIZLY 3. Thus, they showed the same effect in larvae with both activators they were co-exposed with.

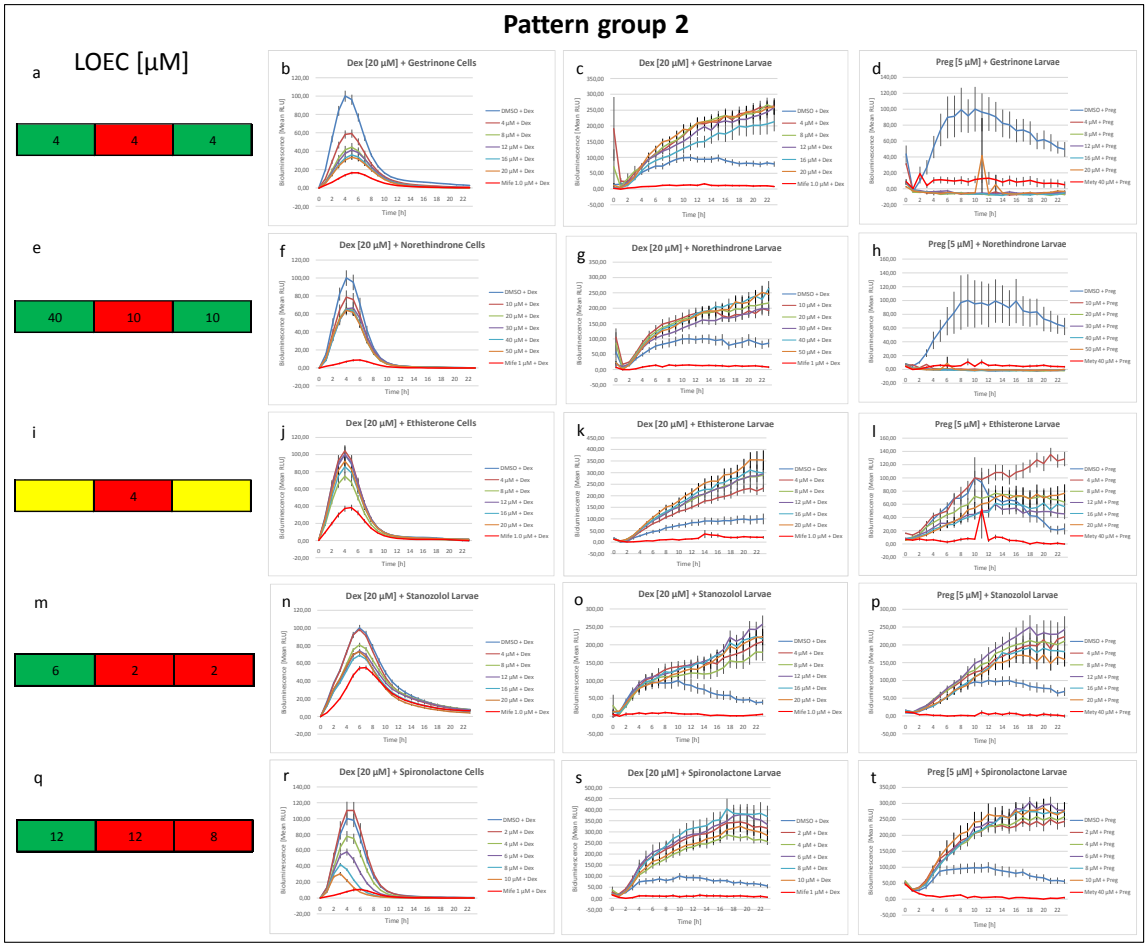


Figure 32: Bioluminescence traces of effect pattern group 2 compounds
Effect patterns with LOECs [µM] and the bioluminescence traces from all three GRIZLY assay variants are shown for Gestrinone (Gestri; **a - d**), Norethindrone (Nor; **e - h**), Ethisterone (Ethi; **i - l**), Stanozolol (Stano; **m - p**) and Spironolactone (Spiro; **q - t**) in combination with Dex [20 µM] in cells and larvae and with Preg [5 µM] in larvae. Bioluminescence was measured for 24h and data are shown as mean relative light units (RLUs) ± SEM. All treatments are presented relative to the respective negative (Dex [20 µM]/ Preg [5 µM] + DMSO; blue) and positive (Dex [20 µM]/ Preg [5 µM] + Mife [1 µM]/ Mety [40 µM]; red) control. The amount of DMSO was 0.2% in all treatments.

The bioluminescence traces reveal that all inhibitors in GRIZLY 1 had concentration-dependent effects (**b**, **f**, **n**, **r**). In GRIZLY 2, where all compounds superactivated the Dex-induced signal, the bioluminescence traces appear to increase constantly with Gestri (**c**), Nor (**g**), Ethi (**k**) and Stano (**o**), without the expectable signal decrease that normally sets in somewhere between 5 - 16 h of GC exposure (see **Figure 29**, e.g. **f** or **c**, blue lines for negative control). For Stano, the increase is not completely constant; a saddle point is visible between 14 - 16 h, before the signal augments a second time until the end of the measurement. With Spiro (**s**), the signal was already relatively high from the beginning. The slope of the bioluminescence signal is much stronger than that of the negative control (blue line). Towards the end of the exposure time, after 18 h, the signal appears to rest on a plateau or to decrease slightly, which is in contrast to the bioluminescence kinetics of all other superactivators in GRIZLY 2.
The two inhibitors in GRIZLY 3, Gestri (**d**) and Nor (**h**), strongly inhibited the Preg-induced signal in all concentrations tested. Retests with lower concentrations will be necessary to assess the concentration-dependency of their effects. For Ethi, which had no significant effect in GRIZLY 3 (**l**), the bioluminescence traces may indicate a slightly altered effect kinetic, as the signal appears to constantly increase over time, similar to the signal curve shape observed with the compound in GRIZLY 2. The bioluminescence signal of Stano increases constantly in GRIZLY 3 (**p**) and shows no saddle point between 14 - 16 h as in GRIZLY 2. Also in contrast to the kinetic observed for this compound in GRIZLY 2, the bioluminescence signal of Stano remains on a constant level after 17 h of larvae exposure until the end of the measurements. Spiro shows a similar effect kinetic in GRIZLY 3 (**t**) as in GRIZLY 2; the bioluminescence signal increases quickly from the beginning before it starts to rest on a plateau after 16 - 18 h of exposure.

Compound interference with GR activity

The superactivation in GRIZLY 2 that was observed with Gestri, Nor, Ethi, Stano and Spiro suggests that the five compounds activated the GR in larvae. They may have agonized the receptor either with higher affinity than Dex, or their ligand binding led to a stronger induction of GC signaling. However, none of the substances is known to agonize the GR. Also, none of these compounds was identified in the pilot chemical screen, where the FDA library was tested with the GRIZLY assay for compounds that activate GC signaling (Weger et al. 2012). As the testing conditions may have been slightly different back then or the current generation of *Tg(GRE:Luc)* zebrafish may have a different sensitivity than that used for the published screen, I retested if the five compounds activated GC signaling under the present testing conditions when they were exposed to the larvae as single substances. In case of GR agonism, I expected that GC signaling induction should also be detectable without the co-treatment with Dex or Preg. However, this was not the case, as shown in **Figure 33**. None of the five compounds induced GC signaling when exposed to the larvae as single substance treatments, although the same concentrations were applied as before.

Controls	Retest LOEC [µM]				
	Cells	Larvae			
DMSO + DMSO					
Preg [5 µM] + DMSO					
Dex [20 µM] + DMSO					
Cort [30 µM] + DMSO					
Dex [20 µM] + Mety [40 µM]	-				
Preg [5 µM] + Mety [40 µM]	-				
Preg [5 µM] + Mife [1 µM]	-				
Dex [20 µM] + Mife [1 µM]					
Compounds	+ Dex	+ Dex	+ Cort	alone	+ Preg
Gestrinone	4	4	4		4
Norethindrone	40	10	10		10
Ethisterone		4	4		
Stanozolol	12	12	4		8
Spironolactone	6	2	2		2

sign. decrease/ decreasing trend/ no change/ increasing trend/ sign. increase of signal intensity; significance = $p < \alpha = 0.002$

Figure 33: GRIZLY assay results for pattern group 2 compounds with exposure alone or in combination with directly administered Cortisol
Effects of the compounds from pattern group 2 in all three GRIZLY assays and during retesting with compound exposure alone and in co-treatment with directly administered Cort are shown. The LOECs for Gestri, Nor, Ethi, Spiro and Stano are indicated for each exposure condition. Color code profiles are shown for controls and compounds. No difference of the substance effect compared to the negative control (Dex [20 µM]/ Preg [5 µM] + DMSO) is indicated in yellow. A significantly increased or decreased signal intensity compared to the negative control is indicated in green and red, respectively. Non-significant trends ($p < \alpha < 0.05$ for increase or decrease are shown in orange and light green, respectively. Significance is defined as $p < \alpha < 0.0001$ for the screen ($\alpha = 0.05$) and $p < \alpha < 0.002$ for retesting ($\alpha = 0.01$). Validated inhibitors or superactivators from retesting are indicated with their lowest observed effect concentration (LOEC) in the respective GRIZLY assay. Assay reliability is surveyed by direct comparison of a screen result with the respective retesting outcome. Confirmed screen hits or trends are noted with a check. Confirmation was given when at least one of the retesting concentrations significantly reflected the screen result. Diverging results are indicated with a cross.

Apparently, the presence of Dex is necessary for the observed effect. Maybe the ligands do not directly activate the GR, but they support GR-mediated signaling by Dex via more indirect means. The effect kinetics observed for the five ligands in co-treatment with Dex, as they were shown in **Figure 32**, may support this hypothesis. The rather continuous signal increase over time that was visible for larvae treated with Dex in combination with Gestri (**c**), Nor (**g**) and Ethi (**k**), the biphasic kinetic with Dex + Stano (**o**) and the extended signal activation with Dex + Spiro (**s**) probably indicate that the compounds intensified, reinforced or stabilized the GR activation by Dex.

As Gestri (**a**), Nor (**e**) and Ethi (**i**) showed different effects on Preg- than on Dex-activated GC signaling, I furthermore wanted to assess if the observed superactivation in GRIZLY 2 might be a Dex-specific effect. Therefore, I co-exposed the compounds with directly administered Cort. In addition to that, I co-treated Spiro and Stano with Cort, as this could provide more information about the activator-dependency of the observed effects.
As also visible in **Figure 33**, all five compounds superactivated Cort-induced GC signaling. This outcome is comparable to the observed overinduction of GC signaling by the compounds in combination with Dex. Apparently, superactivation is not a Dex-specific effect of Gestri, Nor Ethi, Spiro and Stano, but a direct GR agonist is needed. The results imply furthermore that Preg-induced signal inhibition by Gestri and Nor is not a Cort-specific effect, but that the Preg-induced signal probably is inhibited before Cort can be synthesized.

Next, I wanted to know if the superactivation of Gestri and Nor in GRIZLY 2 and their inhibitory effects in GRIZLY 3 would show a concentration-dependency if the compounds were retested in lower concentrations. For each compound, I applied a range of 0.125 - 4 µM to larvae and co-exposed them with either Dex or Preg. The whole range of test concentrations [0.25-20 µM] is shown in in **Figure 34**. Gestri (**a**) and Nor (**c**) still superactivated the Dex-induced signal in all concentration tested. The extend of signal increase was similar for all concentrations, it just varied a little between 1.8 - 3.1-fold of the negative control (Dex/ Preg + DMSO) signal intensity. In co-exposure with Preg, Gestri (**b**) and Nor (**d**) showed a concentration-dependent signal decrease, with a LOEC of 0.5 µM for Gestri and 2 µM for Preg. Interestingly, Nor also showed superactivation of Preg-induced GC signaling in the lowest concentrations [0.25 - 1 µM], with 0.25 µM beign the LOEC for this effect. Gestri did not induce significant superactivation with Preg, but a trend for increased GC signaling might also be indicated for 0.25 µM Gestri + Dex. Apparently, the effect mechanisms of Gestri and Nor on GC signaling are complex and it depends on the applied compound concentration and on the activator substance whether superactivation or inhibition is induced by the treatment.

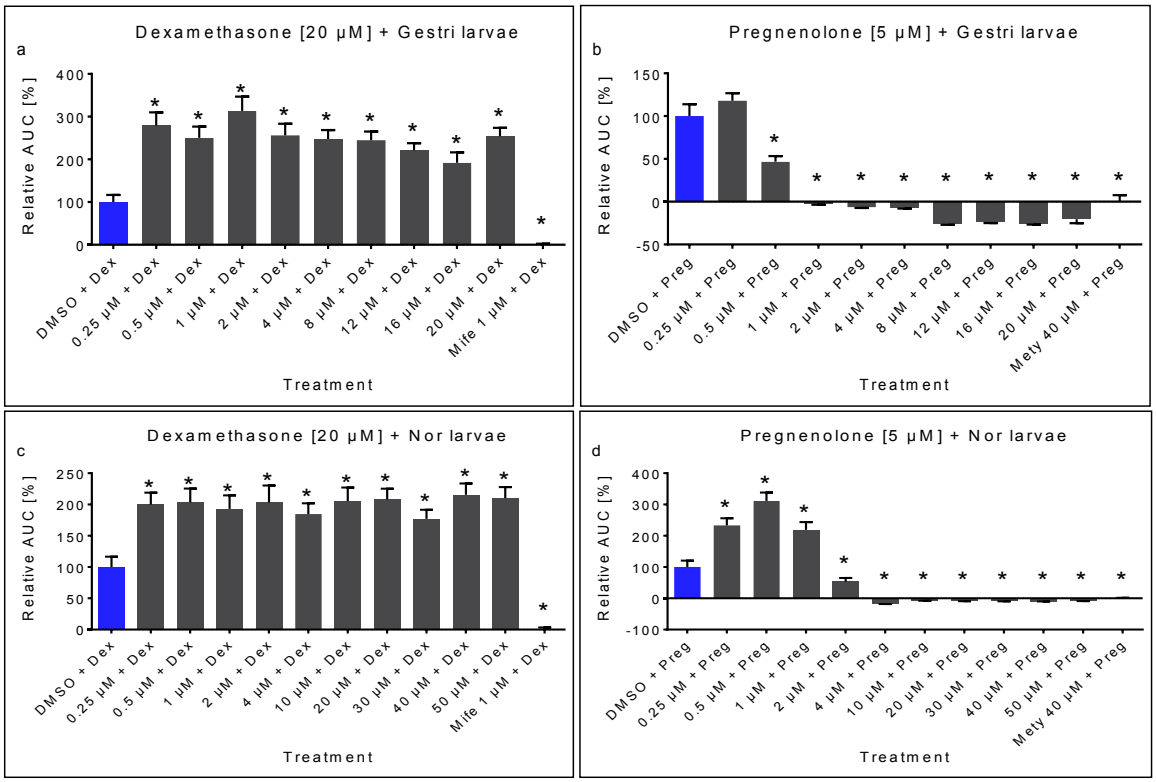


Figure 34: Retest of Gestri and Nor in a range of lower concentrations
Gestri (**a** + **b**) and Nor (**c** + **d**) were retested in lower concentrations [0.25-4 µM] for their effects on Dex-(**a** + **c**) - and Preg- (**b** + **d**) activated GC signaling in larvae. Relative AUC values are shown for the whole range of ten different concentrations of Gestri [0.25-20 µM]/ Nor [0.25-50 µM] + Dex [20 µM] (grey, n = 16). The data are normalized relative to the negative control Dex [20 µM]/ Preg [5 µM] + DMSO (blue, n = 8) and the positive control Dex [20 µM]/ Preg [5 µM] + Mife [1 µM]/ Mety [40 µM] (blue, n = 8). The amount of DMSO was 0.2% in all treatments. Data are shown as means + SEM. Statistical significance is determined by comparison of each treatment condition with the negative control in a multiple t-test, $\alpha = 0.05$. After multiple testing correction with the Holm-Šídák method, significance is defined as $p < \alpha < 0.002$ = *.

Pattern group 3

Compounds that inhibited in both *in vivo* GRIZLY assays but which were ineffective *in vitro* were assigned to pattern group 3. In **Figure 35**, the effect patterns of Nabumetone (Nabu) and Fenbufen (Fen) are shown, with LOECs for each assay and the bioluminescence traces. Nabu strongly inhibited Dex- (**c**) and Preg- (**d**) mediated GC signaling in all concentrations tested. Therefore, I retested the compound in a range of lower concentrations and obtained LOECs of 2 µM for Dex + Nabu (see appendix, **Supplementary Figure I a**) and of 0.25 µM for Preg + Nabu (see appendix, **Supplementary Figure I b**). For Fen, a concentration-dependent inhibition of Dex- (**g**) and Preg (**h**)-mediated GC signaling was indicated by the bioluminescence traces and the compound was therefore not retested in lower concentrations.

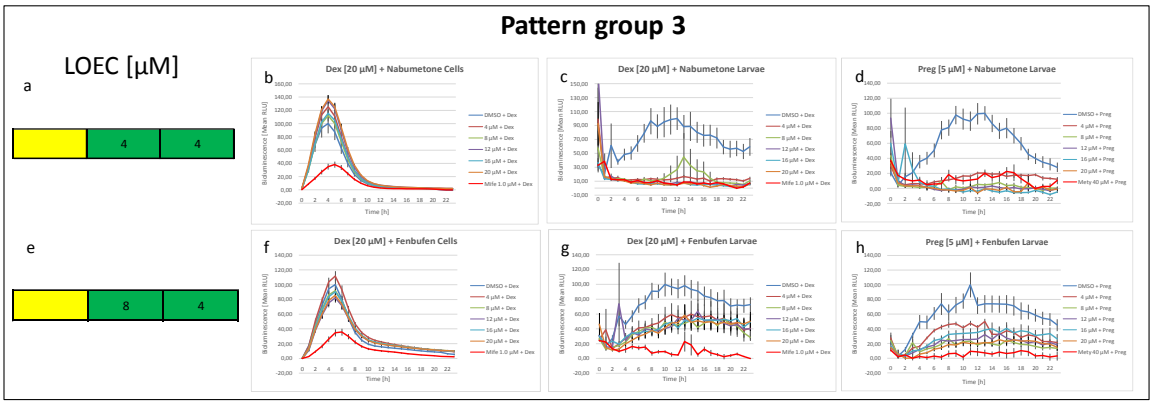


Figure 35: Bioluminescence traces of effect pattern group 1 compounds
Effect patterns with LOECs [µM] and the bioluminescence traces from all three GRIZLY assay variants are shown for Nabumetone (Nabu; **a - d**) and Fenbufen (Fen; **e - h**) in combination with Dex [20 µM] in cells and larvae and with Preg [5 µM] in larvae. Bioluminescence was measured for 24h and data are shown as mean relative light units (RLUs) ± SEM. All treatments are presented relative to the respective negative (Dex [20 µM]/ Preg [5 µM] + DMSO; blue) and positive (Dex [20 µM]/ Preg [5 µM] + Mife [1 µM]/ Mety [40 µM]; red) control. The amount of DMSO was 0.2% in all treatments.

Pattern group 4

All compounds which inhibited in GRIZLY 1 and GRIZLY 3 but not in GRIZLY 2 were assigned to pattern group 4. Their effect patterns with LOECs for each assay and the bioluminescent traces are shown in **Figure 36**.

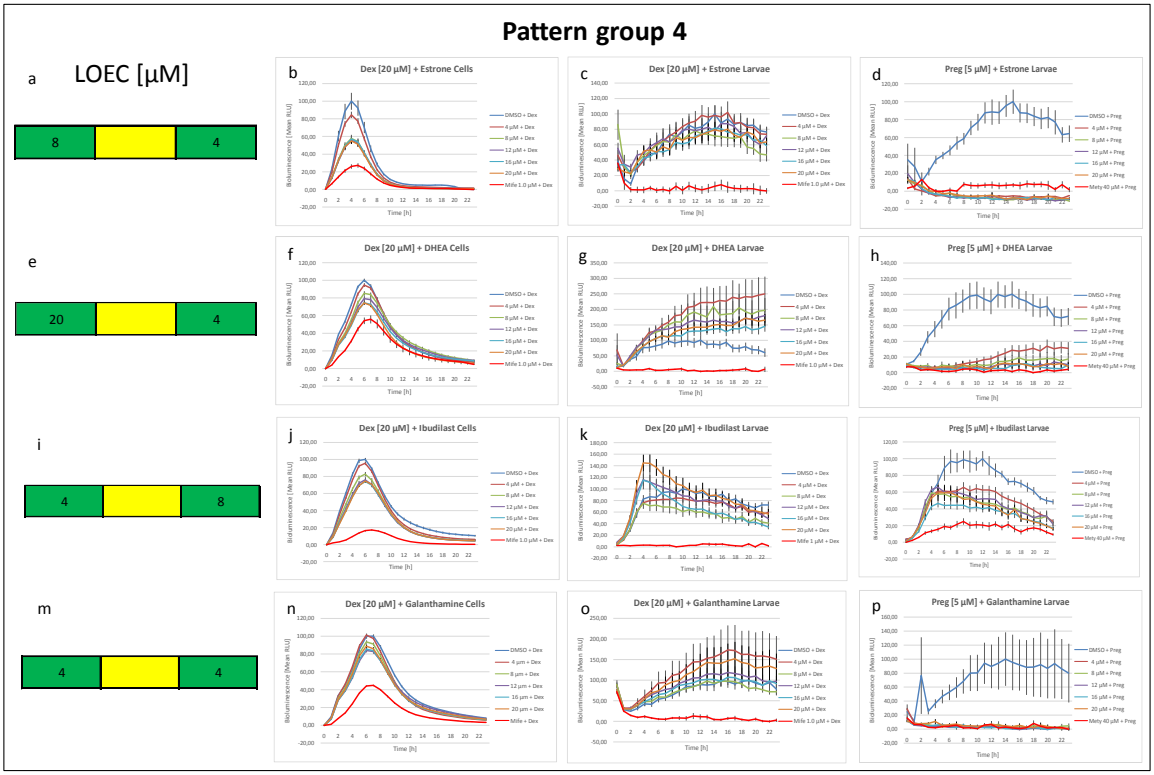


Figure 36: Bioluminescence traces of effect pattern group 5 compounds
Effect patterns with LOECs [µM] and the bioluminescence traces from all three GRIZLY assay variants are shown for Estrone (**a - d**), Dehydroepiandrosterone (DHEA; **e - h**), Ibudilast (Ibudi; **i - l**) and Galanthamine, (Galant; **m - p**) in combination with Dex [20 µM] in cells and larvae and with Preg [5 µM] in larvae. Bioluminescence was measured for 24h and data are shown as mean relative light units (RLUs) ± SEM. All treatments are presented relative to the respective negative (Dex [20 µM]/ Preg [5 µM] + DMSO; blue) and positive (Dex [20 µM]/ Preg [5 µM] + Mife [1 µM]/ Mety [40 µM]; red) control. The amount of DMSO was 0.2% in all treatments.

Estrone, Dehydroepindrosterone (DHEA), Ibudilast (Ibudi) and Galanthamine (Galant) inhibited Dex-induced signaling *in vitro* (**b**, **f**, **j**, **n**) and Preg-induced signaling *in vivo* (**d**, **h**, **l**, **p**). According to this effect pattern, these compounds may be inhibitors of the GC biosynthesis pathway. Estrone (**b**) and DHEA (**f**) showed concentration-dependent inhibition of Dex-induced GC signaling *in vitro*, but of Ibudi and Galant, all concentrations strongly inhibited the signal in GRIZLY 1. In GRIZLY 3, the inhibition of Preg-induced GC signaling in larvae by Ibudi (**l**) was concentration-dependent, but Estrone (**d**), DHEA (**h**) and Ibudi (**p**) strongly suppressed the signal at all concentrations tested. As Estrone and DHEA were selected for follow-up experiments (as it will be described below), I retested the two compounds at lower concentrations [0.25 - 4 µM] for their effects on Preg-induced GC signaling. From this experiment (see appendix, **Supplementary Figure II**), I obtained a LOEC of 0.25 µM for Estrone and a LOEC of 4 µM for DHEA.

Pattern group 5

Compounds which inhibited only in GRIZLY 3 were assigned to pattern group 5. In **Figure 37**, their effect patterns with LOECs for each assay and the bioluminescence traces are shown. As the two compounds Leflunomide (Leflu) and Estriol inhibited Preg-(**d** + **h**) but not Dex-(**c** + **g**) induced GC signaling *in vivo*, they are potential inhibitors of the GC biosynthesis pathway.

Leflu strongly inhibited the Preg-induced signal in all concentrations tested (**d**) and was hence retested in lower concentrations. In this experiment (see appendix, **Supplementary Figure III**), I obtained a LOEC of LOEC 0.5 µM for Leflu. Estriol showed a clear concentration-dependency (**h**) with the higher concentrations and a LOEC of 12 µM.

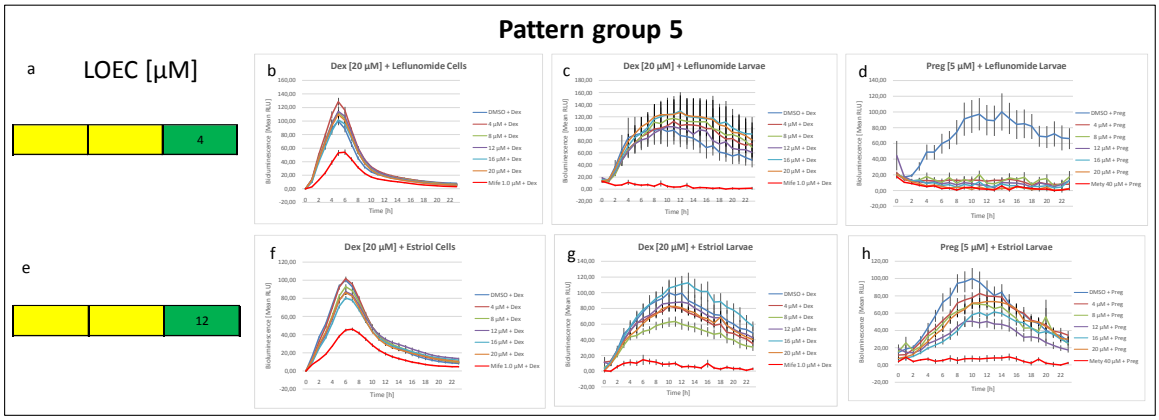


Figure 37: Bioluminescence traces of effect pattern group 5 compounds
Effect patterns with LOECs [µM] and the bioluminescence traces from all three GRIZLY assay variants are shown for Leflunomide (Leflu; **a - d**) and Estriol (**e - h**) in combination with Dex [20 µM] in cells and larvae and with Preg [5 µM] in larvae. Bioluminescence was measured for 24h and data are shown as mean relative light units (RLUs) ± SEM. All treatments are presented relative to the respective negative (Dex [20 µM]/ Preg [5 µM] + DMSO; blue) and positive (Dex [20 µM]/ Preg [5 µM] + Mife [1 µM]/ Mety [40 µM]; red) control. The amount of DMSO was 0.2% in all treatments.

According to their effects, several compounds were assigned to pattern group 6 and 7 (**Figure 28**, highlighted in grey). However, as Amorolfine and Nisoldipine solely inhibited GC signaling *in vitro*, without any *in vivo* effects (pattern group 6) and Erlotinib, Pioglitazone and Paroxetine could not be confirmed to affect GC signaling during retesting (pattern group 7), they are outside the focus of this study and, thus, not discussed further within the combined analysis of compound effects in all three GRIZLY assay screens nor considered for follow-up experiments. Furthermore, two compounds from pattern group 4, Ibudi and Galant (highlighted in grey) were not carried forward, in order to cut down the number of candidates for more detailed investigations within the next steps.

Summary of tier 2

In order to summarize the outcomes of the tier 2 experiments, not only the first aim of the study was achieved, but also the two other objectives, to identify GC biosynthesis disruptors and GC signaling inhibitors with unknown mechanisms of action, were addressed. The combined analysis of compound effects in all three GRIZLY assay screens revealed more detailed insights in the effect dynamics on GC signaling and helped to pre-categorize the substances for their potential mechanisms of action. For instance, Gestri, Nor, Estrone, DHEA, Galant, Leflu, Ibudi and Estriol

potentially act via GC synthesis disruption, while for 10-HC, Trani, Ret. acid and Aci, as for Mife, a more direct effect on the GR appears to underlie signal inhibition.

These observations were followed up on in the next steps of this study. In tier 3 a, several compounds were analyzed for their effects on the endogenous steroid hormone biosynthesis. Especially disruptors of the Cort biosynthesis should be pinpointed by this strategy. In tier 3 b, the compound effects on the expression of selected target genes were examined. This should provide more information about their presumptive effect mechanisms and help to pursue the third aim of this study, to identify GC signaling inhibitors with - so far - unknown mechanisms of action.

3.5 Tier 3 a: Chemical analysis

By means of chemical analysis, I investigated validated GC signaling disruptors for their effects on the endogenous steroid hormone biosynthesis in larvae. As Preg treatment leads to an endogenous overproduction of Cort in larvae, which was measured as GC signaling activation in the GRIZLY assay, I expected to find increased amounts of Cort and of upstream metabolites within the steroid hormone biosynthesis pathway. Therefore, I aimed to determine compound effects on the larvae-internal concentrations of Preg, Progesterone (Prog), Corticosterone (Cortico) and Cort in application of the UPLC-MS/MS technique. As a first step, timecourse experiments with 0 - 24h of exposure to control treatments were carried out in order to identify a suitable timepoint for sample taking.

3.5.1 Timecourse experiments

Timecourse experiment 1: Preg

In **Figure 38**, the outcomes of the timecourse experiment for Preg are shown. Endogenous levels of Cort, Cortico, Prog and Preg were measured after 0, 1, 2, 4, 8 or 24h treatment with either DMSO (**a**), Preg [5 µM] + DMSO (**b**) or Preg [5 µM] + Mety [40 µM] (**c**). Steroid mean concentrations [ng/ 100 larvae] are shown for all exposure times. Concentrations of Cortico were below the limit of detection (LOD) in all exposure conditions, and are, thus, not included in the graphs. The measurements of untreated larvae (0 h) are shown in all graphs in order to visualize treatment-mediated changes of steroid levels. Statistically significant changes between treatment and control conditions are marked with an asteriks.

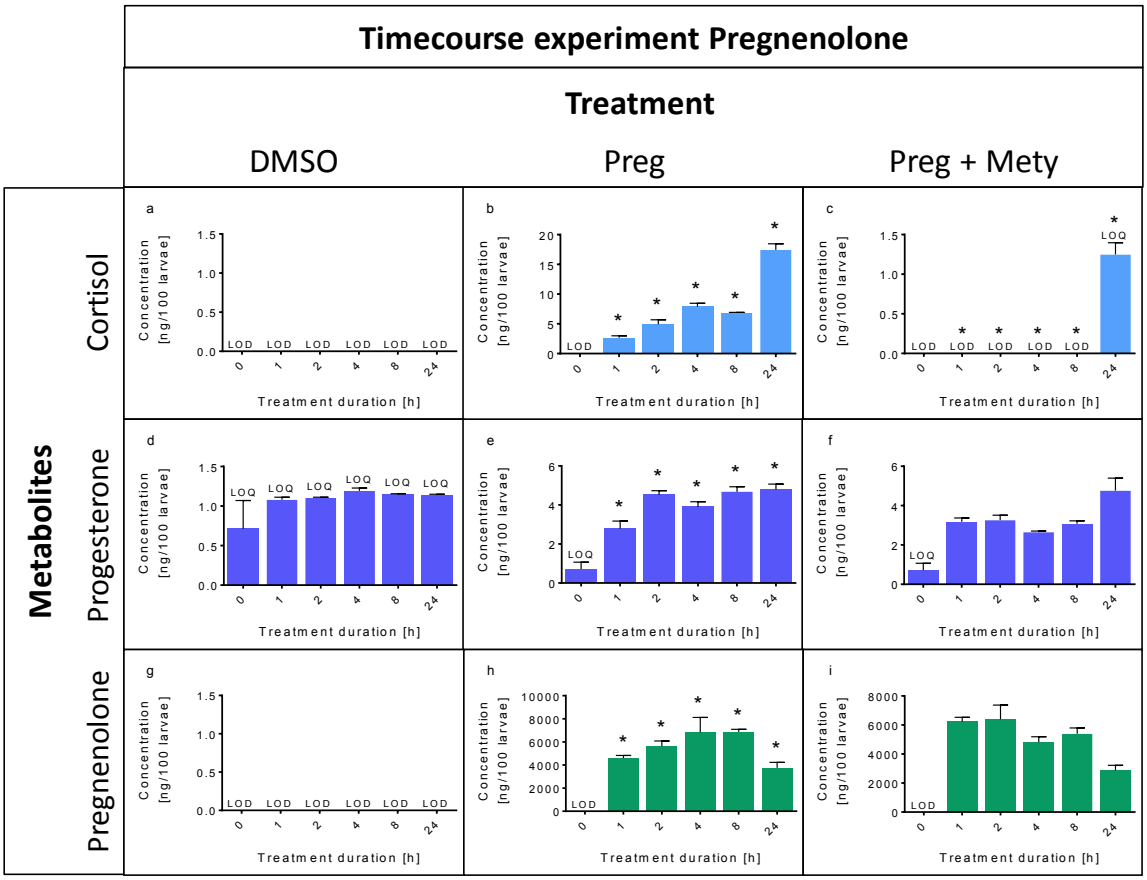


Figure 38: UPLC-MS/MS timecourse experiment Pregnenolone
Steroid hormone concentrations measured in homogenates from larvae exposed to DMSO (**a, d, g**), Preg [5 µM] + DMSO (**b, e, h**) and Preg [5 µM] + Mety [40 µM] (**c, f, i**) for several hours. The amount of DMSO was 0.2 % in all treatments. Concentrations of Cort (blue), Prog (violet) and Preg (green) were measured by UPLC-MS/ MS. Mean concentrations [ng/ 100 larvae] + SEM (n = 3) are shown. Limit of quantification (LOQ) = 1.6 ng/ 100 larvae for Cort and Prog (external calibration standards: 1.6 - 25.0 ng/ 100 larvae) and LOQ = 31.3 ng/ 100 larvae for Preg (external calibration standards: 31.3 - 500.0 ng/ 100 larvae). Concentrations < limit of detection (LOD) were set to 0. Concentrations > LOD < LOQ are shown but have to be considered carefully. Data for Preg are from 1:10 diluted samples, as the undiluted Preg concentrations exceeded the highest internal standard (500 ng/ 100 larvae) for Preg. Data for Cort and Prog are from undiluted samples. Statistical significance was determined by comparison of the mean concentration of a metabolite for each exposure duration with the respective mean concentration of the control treatment. Controls were untreated larvae (0h) for (**a**, **d**, **g**), DMSO 0 - 24h for (**b**, **e**, **h**) and Preg 0 - 24h for (**c**, **f**, **i**). A multiple t-test was applied with $\alpha = 0.05$. After multiple testing correction with the Holm-Šídák method, significance was defined as $p < \alpha < 0.008$ = *****.

In the first column of **Figure 38**, larvae were exposed to DMSO for different durations. Each timepoint was assessed for statistically significant changes of the metabolite concentrations compared to untreated larvae, which were sampled at the timepoint of exposure start (0 h). No significant differences were detected between the steroid concentrations measured in DMSO- or untreated larvae. Levels of Cort (**a**) and Preg (**g**) were below the LOD for all treatments and, thus, set to 0. Prog-(**d**) concentrations were detectable (between 0.7 and 1.2 ng/ 100 larvae), but they were below the limit of quantification (LOQ: 1.6 ng/100 larvae). Nevertheless, with this caveat, the Prog measurements are shown in order to document that the endogenous amount of the metabolite remained relatively constant between 0 and 24h of DMSO exposure.

The second column of **Figure 38** shows the steroid mean concentrations [ng/ 100 larvae] for larvae treated with Preg. For each timepoint, changes in the metabolite concentrations between DMSO- and Preg-treated larvae were assessed statistically. Preg treatment strongly increased the endogenous amounts of Cort (**b**), Prog (**e**) and Preg (**h**). Already after 1h of exposure, the Cort (**b**) concentration significantly increased from LOD to 2.7 ng/100 larvae. With prolonged exposure durations, it further increased. After 24h of exposure, the highest concentration of 17.4 ng/100 larvae was measurable. The effect of Preg treatment on Prog (**e**) concentrations was clearly visible after 1h of exposure, when a significant increase from 0.7 (below LOQ) to 2.8 ng/100 larvae was detectable. An exposure duration of 2h showed further elevated Prog concentrations with 4.5 ng/100 larvae. From 2 to 24h, Prog stayed on a relatively constant level, with concentrations between 3.9 and 4.8 ng/100 larvae. The amount of Preg (**h**) was significantly increased in larvae homogenates at all timepoints measured. Already after 1h of exposure, a concentration 4590 ng/100 larvae was measured. The concentration continuously increased with prolonged exposure durations until 8h, where 6870 ng/ 100 larvae were detected. After 8h, the concentration decreased and only 3760 ng/ 100 larvae were measured after 24h.

The effect of Mety on Preg-treated larvae is visualized in the third column of **Figure 38**. The steroid mean concentrations [ng/ 100 larvae] for larvae treated with Preg + Mety are shown. Changes in the metabolite concentrations between larvae treated either with Preg + DMSO or Preg + Mety were assessed statistically for each timepoint. Mety significantly reduced the endogenous amount of Cort (**c**) in larvae at all timepoints. Between 1h and 8h of exposure, the Cort concentrations were below the LOD. After 24 h, a concentration of 1.3 ng Cort/ 100 larvae was measured, which is slightly below the LOQ. The larvae-internal amount of Prog (**f**) remained relatively constant during the different exposure durations of 1 - 24 h. Prog concentrations between 2.7 and 4.8 ng/ 100 larvae were detected. These concentrations are highly similar to those measured for Prog (2.8 - 4.8 ng/ 100 larvae) when larvae were exposed to Preg alone and were, thus, not significantly altered by Mety. Likewise, the amount of Preg (**i**) in larvae exposed to Preg + Mety was with 2860 - 6400 ng/ 100 larvae similar to that measured for Preg + DMSO treatment (3760 - 6870 ng/ 100 larvae). Interestingly, the highest concentrations of Preg were measured after 1 and 2h of exposure to Preg + Mety, while, when larvae were exposed to Preg + DMSO, the Preg concentrations increased more slowly, and showed the highest values after 4 and 8h. Potentially, when Preg is co-exposed with Mety, it accumulates already after 1-2 h, possibly as Mety blocks the downstream metabolization of Preg by inhibiting 11β-hydroxylase. In contrast, when Preg + Mety are co-exposed for a longer duration, maybe Preg is degraded via other pathways after a while.

Although the measurements of Cortico were removed from the graphs as its concentration was always below the LOD at any treatment condition, the assessment of this metabolite still showed that Preg treatment and increased larvae-internal Preg concentrations had no effect on the amount of Coco. As Cortico is synthesized from Prog via the metabolite 11-deoxyvorticosterone (see the steroid hormone biosynthesis pathway in **1.2.2**, **Figure 3**), elevated concentrations of

Cortico could have been expected from the Preg-induced increase of Prog and Cort, but this was not shown.

Timecourse experiment 2: Dex

The timecourse experiment for Dex is shown in **Figure 39.** Larvae-internal concentrations of Cort, Cortico, Prog, Preg and Dex were measured after 0, 1, 2, 4, 8 or 24h treatment with either DMSO (**a**), Dex [20 µM] + DMSO (**b**) or Dex [20 µM] + Mife [1 µM] (**c**). Steroid mean concentrations [ng/ 100 larvae] are indicated for all exposure times. Here, the endogenous amounts of Preg, Prog and Cortico were below the LOD in all test conditions and were, thus, not included in the graphs. The measurements of untreated larvae (0 h) are shown in all graphs in order to visualize treatment-mediated changes of steroid levels. Statistically significant changes between treatment and control conditions are marked with an asteriks.

The first column of **Figure 39** shows the steroid concentrations measured in larvae that were exposed to DMSO for 0-24 h. Each condition was assessed for statistically significant changes of the steroid concentrations compared to untreated larvae, which were sampled at the timepoint of exposure start (0 h). No significant differences were detected between the steroid concentrations measured in DMSO- or untreated larvae. In case of Cort (**a**), inconsistency of replicate measurements below and above the LOD was the reason why the means were below the LOD in **Figure 38**, but within the Dex timecourse experiment, low concentrations between 0.8 and 1.0 ng/ 100 larvae were detectable in all replicates. Although these values were still below the LOQ (LOQ: 1.6 ng/100 larvae), Cort concentrations are included in the graphs in order to visualize treatment effect trends on endogenous concentrations of Cort. Larvae-internal concentrations of Dex (**d**) were below the LOD and set to 0.

In the second column of **Figure 39**, the steroid mean concentrations [ng/ 100 larvae] for larvae treated with Dex are shown. For each timepoint, changes in the steroid amounts between DMSO- and Dex-treated larvae were assessed statistically. Upon Dex treatment, the Cort concentrations (**b**) appear to be reduced at all timepoints measured. After 4 h and 24 h, the concentration decreases could even be confirmed to be statistically significant. Although these data are not reliable, as they are below the LOQ, they are consistent with the inhibitory effect of Dex on endogenous Cort levels, which is caused by HPA feedback mechanisms as it is reported in various systems, including zebrafish larvae. Regarding the concentrations of Dex (**e**) that were measured in larvae-homogenates, the amount of the synthetic GC increased significantly at all timepoints measured. The highest Dex concentrations were detected after 1h and 24h, each with 15 ng/ 100 larvae. Exposure durations of 2h, 4h and 8h showed slightly lower Dex concentrations, with values between 10.3 and 13.9 ng/ 100 larvae.

The effect of Mife on Dex-treated larvae is visualized in the third column of **Figure 39**. The steroid mean concentrations [ng/ 100 larvae] for larvae treated with Dex + Mife are shown. Changes in the steroid concentrations between larvae treated with Dex + DMSO or with Dex + Mife were assessed statistically for each timepoint. Cort (**c**) concentrations were not altered by

Mife. For the larvae-internal amount of Dex (**f**), a significant concentration increase was detectable after 2h with 38.4 ng/ 100 larvae. At all other timepoints, the measured values lay between 17.2 and 23.8 ng/ 100 larvae, which is considerably higher than when larvae were treated with Dex alone (10.3 and 13.9 ng/ 100 larvae; **e**). Thus, the Dex concentration in larvae is elevated by trend at all timepoints measured when larvae were treated with Dex + Mife; only after 2h and 4h of exposure, the increase of Dex was significant.

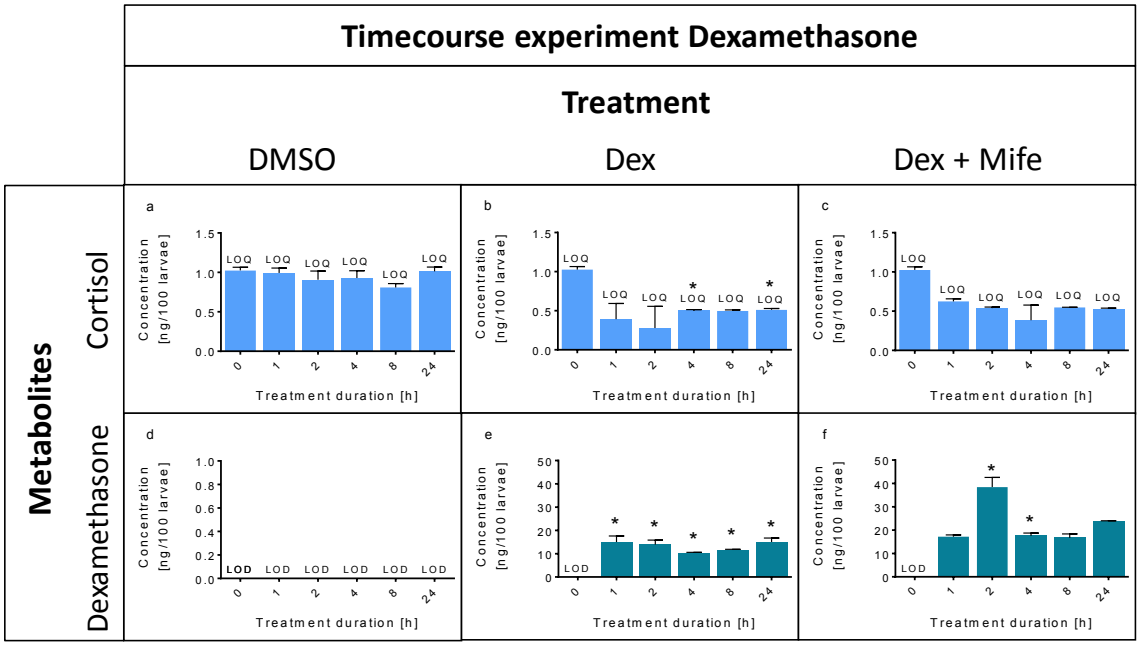


Figure 39: UPLC-MS/MS timecourse experiment Dexamethasone
Steroid hormone concentrations measured in homogenates from larvae exposed to DMSO (**a** and **b**), Dex [20 µM] (**b** and **e**) and Dex [20 µM] + Mife [1 µM] (**c** and **f**) for several hours. The amount of DMSO was 0.2 % in all treatments. Concentrations of Cort (blue) and Dex (turqoise) were measured by UPLC-MS/ MS. Mean concentrations [ng/ 100 larvae] + SEM (n = 3) are shown. Limit of quantification (LOQ) = 1.6 ng/ 100 larvae for Cort and Dex (external calibration standards: 1.6 - 25.0 ng/ 100 larvae). Concentrations < limit of detection (LOD) were set to 0. Concentrations > LOD < LOQ are shown but have to be considered carefully. Data for are from undiluted samples. Statistical significance was determined by comparison of the mean concentration of a steroid for each exposure duration with the respective mean concentration of the control treatment. Controls were untreated larvae (0h) for (**a** and **d**), DMSO 0 - 24h for (**b** and **e**) and Dex 0 - 24h for (**c** and **f**). A multiple t-test was applied with $\alpha = 0.05$. After multiple testing correction with the Holm-Šídák method, significance was defined as $p < \alpha < 0.008$ = *****.

Summary of timecourse experiments

<u>1) Suitability of the applied method to detect steroid concentrations in larvae homogenates</u>

The main purpose of the chemical analysis experiments was to determine uptake and accumulation of Preg and Dex into the larvae and to obtain a first mapping of their effects on endogenous metabolites within the steroid hormone biosynthesis pathway. Furthermore, I aimed to detect disruption of this pathway by compounds, which I was able to see in homogenates from larvae that were co-treated with Preg + Mety.

The applied extraction method and the UPLC-MS/ MS technique were well suitable to measure Preg-induced Cort levels. Also disruption of the Preg-induced Cort production by the reference inhibitor of Cort biosynthesis, Mety, was well detectable with this technique. The UPLC-MS/ MS results furthermore showed that the larvae-internal Cort concentrations continuously increased

within 24h of exposure to Preg, while Prog concentrations remained on a constantly elevated level and Preg concentrations decreased after several hours. Also, it indicated that Prog levels were not affected by Mety treatment. Thus, once induced, compound effects on endogenous steroids and their dynamics were well detectable.

However, the applied technique had some limitations, as non-stimulated endogenous steroid concentrations were below the detection limit of the UPLC-MS/ MS setup (with the exception of Cort in the timecourse experiment with Dex, which however was still below the limit of quantification). Especially in case of the timecourse experiment with Dex, where no induction of endogenous steroid hormones was expected, as Dex treatment rather decreases than induces the Cort biosynthesis pathway due to negative feedback mechanisms via the HPA axis, the possibility to quantify Cort, Cortico, Prog and Preg was very limited. Still, also in these conditions, the method allows to measure the dynamic effects of Dex and co-exposed compounds on Dex concentrations in the larvae extracts.

2) Unequal uptake of Preg and Dex by the larvae

Interestingly, the detected concentrations of Dex were with 10 - 15 ng/ 100 larvae several orders of magnitude lower than the Preg concentrations of 3760 - 6870 ng/ 100 larvae, even though the concentration of Dex treatment was with 20 µM (7.85 mg/l) four-fold higher than the concentration of Preg treatment with 5 µM (1.58 mg/l).

Exemplarily, 10 ng/ 100 larvae were detected when larvae were exposed to 7.85 mg/l Dex. The total treatment volume for 100 larvae was 7.5 ml, wherein 59 µg Dex were available. Accordingly, as calculated below in formula **a)**, 0.017% of the administered amount of Dex were detected in the extract of 100 larvae.

In case of Preg, where 3760 ng/ 100 larvae were measured, a concentration of 1.58 mg/l served 12 µg of Preg in the total exposure volume of 7.5 ml for 100 larvae. As the outcome of calculation **b)**, 31,33% of Preg were recovered from the larvae homogenate, which is 1840-fold more than the quantified amount of Dex. The initial measurement of Preg concentrations in untreated larvae (see e.g. **Figure 38 g**, **h**, **i,** 0 h) showed that endogenous Preg levels are negligible for this calculation.

a) $\frac{10\ ng/\ 100\ larvae}{59000\ ng/\ 100\ larvae}\ x\ 100\% =\ 0.02\%$ b) $\frac{3760\ ng/\ 100\ larvae}{12000\ ng/\ 100\ larvae}\ x\ 100\% = 31.33\%$

As the measurements were conducted with whole larvae extracts, no information is available about the portion of absorbed compound from the recovered percentage. The possibility that parts of the compound were not absorbed, but instead adhered to the larvae skin, cannot be excluded, but several thorough washing steps of the larvae, before they were anesthetized, were aimed to minimize this effect. The varying levels of Preg and Dex over different exposure durations might further indicate dynamic changes in internal larvae concentrations of both compounds. As the endogenous concentration of Cort cumulated statistically significant with increasing Preg levels, potential amounts of externally bound but non-absorbed compounds might be negligible for the aim of the experiment. However, the significantly higher impact of

Preg on endogenous steroid concentrations might be related to the better recovery rate of Preg from larvae extracts. The possibility of externally provided Preg having a higher bioavailability for larvae than Dex should be considered for the Preg effect within all experiments of this study.

3) Selection of exposure duration for the assessment of library compound effects

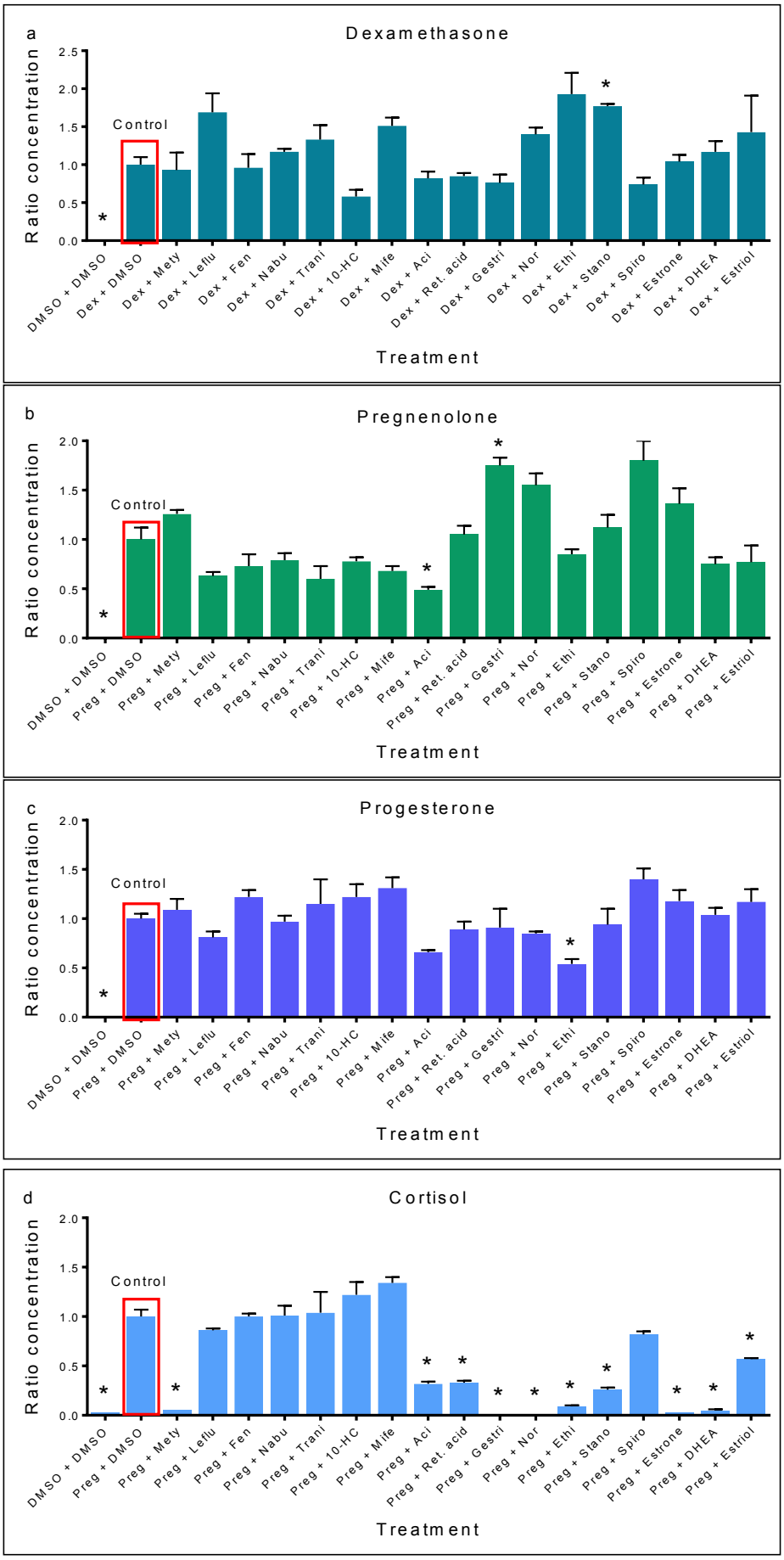
According to the timecourse experiments, the highest concentration of Cort in Preg-treated larvae was measured after 24 h of exosure. For Dex, the highest amount in larvae extracts was also detected after 24 h of Dex treatment, although the amount of Dex was after 1 h of exposure nearly as high. In order to detect the most distinct compound effects on endogenous steroid levels, and to have comparable exposure conditions between Dex and Preg, I selected the exposure duration of 24 h for the assessment of library compound effects on larvae-internal steroid levels.

3.5.2 Library compound effects on larvae-internal steroid levels

For chemical analysis of library compound effects on larvae-internal steroid levels, one effective concentration of each substance was applied to the larvae. This was usually the LOEC from the *in vivo* GRIZLY assays. In cases where the LOEC did not lead to a strong effect on GC signaling in the GRIZLY assay, an appropriate higher concentration was used. After 24 h of exposure, larvae extracts were subjected to UPLC-MS/ MS.

As before, within the timecourse experiments, endogenous concentrations of Cortico could not be detected (< LOD) in larvae treated with Preg or Dex in combination with the library compounds. Likewise, no concentrations of Cort, Preg or Prog were quantifiable (< LOQ) when GC signaling was induced by Dex in larvae. Thus, these metabolite measurements were not visualized.

Changes of Dex levels in larvae treated with Dex + library compounds are shown in **Figure 40 a**. Concentration ratios are indicated for each treatment condition, relative to the negative control Dex + DMSO [20 µM]. No traces of Dex were detectable in the DMSO control. Larvae-internal amounts of Dex significantly increased upon Dex treatment when compared with the DMSO control. Stano significantly increased the measured Dex concentration when compared with the negative control. Further, Mife ($p = 0.0140$), Leflu ($p = 0.0428$), Ethi ($p = 0.0204$) and Nor ($p = 0.0249$) showed trends for higher Dex concentrations. In contrast, 10-HC ($p = 0.0205$) tended rather to reduce the Dex concentration.


a
Dexamethasone
Ratio concentration
Control
Treatment
DMSO + DMSO
Dex + DMSO
Dex + Mety
Dex + Leflu
Dex + Fen
Dex + Nabu
Dex + Trani
Dex + 10-HC
Dex + Mife
Dex + Aci
Dex + Ret. acid
Dex + Gestri
Dex + Nor
Dex + Ethi
Dex + Stano
Dex + Spiro
Dex + Estrone
Dex + DHEA
Dex + Estriol
b
Pregnenolone
Ratio concentration
Control
Treatment
DMSO + DMSO
Preg + DMSO
Preg + Mety
Preg + Leflu
Preg + Fen
Preg + Nabu
Preg + Trani
Preg + 10-HC
Preg + Mife
Preg + Aci
Preg + Ret. acid
Preg + Gestri
Preg + Nor
Preg + Ethi
Preg + Stano
Preg + Spiro
Preg + Estrone
Preg + DHEA
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c
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DMSO + DMSO
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Preg + Mety
Preg + Leflu
Preg + Fen
Preg + Nabu
Preg + Trani
Preg + 10-HC
Preg + Mife
Preg + Aci
Preg + Ret. acid
Preg + Gestri
Preg + Nor
Preg + Ethi
Preg + Stano
Preg + Spiro
Preg + Estrone
Preg + DHEA
Preg + Estriol
d
Cortisol
Ratio concentration
Control
Treatment
DMSO + DMSO
Preg + DMSO
Preg + Mety
Preg + Leflu
Preg + Fen
Preg + Nabu
Preg + Trani
Preg + 10-HC
Preg + Mife
Preg + Aci
Preg + Ret. acid
Preg + Gestri
Preg + Nor
Preg + Ethi
Preg + Stano
Preg + Spiro
Preg + Estrone
Preg + DHEA
Preg + Estriol

Figure 40: UPLC-MS/MS experiment with FDA library compounds
Effects of FDA drug library compounds in combination with Dex [20 µ] on larvae-internal Dex- (**a**) levels and in combination with Preg [5 µM] on endogenous Preg- (**b**), Prog- (**c**) and Cort- (**d**) levels in larvae are shown. Mean ratio concentrations + SEM (n = 4) are indicated for each substance, relative to the respective negative control (Preg [5 µM] + DMSO, n = 4, for Preg + library compound; Dex [20 µM] + DMSO, n = 4, Dex + library compound). The amount of DMSO was 0.2% in all treatment conditions. For Preg, ratio concentrations from measurements of a 1:10 dilution were calculated, for all other steroids, ratio concentrations were calculated from measurements of undiluted samples. Statistical significance was determined by comparison of the mean ratio concentration of each treatment condition with the mean ratio concentration of the negative control in a multiple t-test, $\alpha = 0.05$. After multiple testing correction with the Holm-Šídák method, significance was defined as $p < \alpha < 0.005$ = *****.

The effects of Preg + library compounds on endogenous levels of Preg, Prog and Cort are presented in **Figure 40 b - d**. Concentration ratios are shown for each treatment condition, relative to the negative control Preg + DMSO [5 µM]. Treatment with Preg + DMSO significantly induced larval levels of Preg, Prog and Cort, when compared with the DMSO control (DMSO).
In **Figure 40 b**, library compound effects on larvae-internal Preg levels are shown. Only two compounds significantly changed the amount of Preg in larvae. Aci significantly reduced the Preg level, while Mife ($p = 0.0287$) and Leflu ($p = 0.0139$) show a trend for decreased Preg concentrations. Gestri, signficantly induced Preg accumulation in larvae and Mety ($p = 0.0180$), Spiro ($p = 0.0092$) and Nor ($p = 0.0075$) showed trends for elevated Preg levels.
Library compound effects on endogenous Prog levels are presented in **Figure 40 c**. Ethi significantly reduced the amount of Prog in larvae. For Aci ($p = 0.0110$), also a trend for reduced Prog level is visible. In contrast, Mife ($p = 0.0426$), Fen ($p = 0.0431$) and Spiro ($p = 0.0142$) tend to further increase Prog concentrations.
In **Figure 40 d**, the compound effects on endogenous Cort concentrations are shown. Ten compounds significantly reduced the amount of Cort produced by the larvae. With Mety, DHEA, Estrone, Nor and Gestri, similar Cort levels as for the DMSO control were measured. The other compounds with significant effects, Stano, Estriol, Aci and Ret. acid, reduced the Cort level by 40-70% when of the negative control. Spiro($p = 0.0114$) also showed a trend to reduce endogenous Cort, but the effect was not significant. The only compound, which tended to further increase the endogenous Cort level was Mife ($p = 0.0102$).

Correlation between library compound effects on GC signaling and on endogenous steroid levels

In order to assess the correlation between the compound effects on GC signaling and on the endogenous steroid levels, the chemical analysis outcomes are summarized as steroid level profiles and compared with the GRIZLY retesting results (replotted from **Figure 28**) in **Figure 41**. In case of exposure to library compound + Dex, larvae-internal Dex concentrations from chemical analysis are shown, while in co-treatment with Preg, measurements of Preg, Prog and Cort are indicated. For both experiments, the GRIZLY assay and chemical analysis, the controls are shown with the respective color code profiles. Yellow indicates no difference of the compound effect compared to the negative control. Significant reduction or co-induction of signal intensity/ steroid concentrations are indicated in green and red, respectively; non-significant trends ($p < \alpha < 0.05$) for reduction or co-induction in orange and light green, respectively. Significance is

defined as $p < \alpha < 0.0001$ for the GRIZLY assay and $p < \alpha < 0.005$ for chemical analysis results. The applied concentrations for UPLC-MS/MS experiments are indicated in the right column.

	Retest LOEC [µM]			Chemical analysis				Conc.	
Controls	**Cells**	**Larvae**		**Dex**	**Preg**	**Prog**	**Cort**		
DMSO + DMSO									
Preg [5 µM] + DMSO				-					
Dex [20 µM] + DMSO					-	-	-		
Dex [20 µM] + Mety [40 µM]	-				-	-	-		
Preg [5 µM] + Mety [40 µM]	-			-					
Preg [5 µM] + Mife [1 µM]	-			-					
Dex [20 µM] + Mife [1 µM]					-	-	-		
Compounds	+ Dex	+ Dex	+ Preg	+ Dex	+ Preg			**[µM]**	**Group**
Leflunomide			4					4	
Fenbufen		8	4					4	1
Nabumetone		4	4					4	
Tranilast	10	10	10					10	
10-Hydroxycamptothecin	4	4	4					4	
Mifepristone	0.2	0.4	0.2					1	2
Acitretin	4	8	4					4	
Retinoic Acid	0.4	1.2	1.6					2	
Gestrinone	4	4	4					4	
Norethindrone	40	10	10					10	3
Ethisterone		4						4	
Stanozolol	12	12	8					12	4
Spironolactone	6	2	2					2	
Estrone	8		4					4	
Dehydroepiandrosterone	20		4					4	5
Estriol			12					12	

sign. decrease/ decreasing trend/ no change/ increasing trend/ sign. increase of signal intensity/ steroid concentration in larvae; significance = $p < \alpha = 0.0001$ for GRIZLY and $p < \alpha = 0.005$ for chemical analysis; trends = $p < \alpha = 0.05$

Figure 41: Comparison of GRIZLY retesting results with steroid level profiles
Color code profiles are shown for controls and library compounds. No difference of the substance effect compared to the negative control (Dex [20 µM]/ Preg [5 µM] + DMSO) is indicated in yellow. A significantly increased or decreased signal intensity or steroid level compared to the negative control is indicated in green and red, respectively. Non-significant trends ($p < \alpha < 0.05$) for increase or decrease are shown in orange and light green, respectively. Significance is defined as $p < \alpha < 0.0001$ for the GRIZLY retests ($\alpha = 0.01$) and as $p < \alpha < 0.005$ ($\alpha = 0.05$) for chemical analysis. Validated inhibitors and superactivators from retesting are indicated with their lowest observed effect concentration (LOEC) in the respective GRIZLY assay.

Leflu only inhibited in GRIZLY 3. Although this made it a candidate substance for a disruptor of GC biosynthesis, the level of Cort was unaffected as determined by the chemical analysis. Instead, trends for reduced Preg and induced Dex were observable. This might imply an impact of Leflu on Dex and Preg metabolism in the larvae, but cannot solely explain the inhibition of Preg-induced GC signaling.

Fen and Nabu, the two compounds which had no effect *in vitro* but inhibited Dex- and Preg-activated GC signaling *in vivo*, showed negligible impacts on endogenous steroids. For Nabu, no effect on any of the measured steroids was visible and Fen solely indicated a trend for induced Prog, but effects on Preg and Cort were lacking. Thus, no impact on Cort biosynthesis or Dex metabolism can be hypothesized on these results, the observed inhibition has to result from other mechanisms.

Mife, the positive control for direct inhibition of GC signaling, inhibited in all three GRIZLY screens. By this outcome, the GR antagonistic mechanism of inhibitition was confirmed. In chemical analysis, Mife showed a tendency to increase the larval Dex concentration and, in co-treatment with Preg, trends for reduced Preg, but elevated Prog and Cort levels were observable. These results might suggest that Mife could potentially induce the uptake or reduce the metabolization of Dex. In co-treatment with Preg, the opposite effect might be possible, reduced uptake or induced metabolism of Preg. The elevated Prog and Cort levels resemble a normal expectable *in vivo* response to Mife treatment. GR antagonism by Mife signals an endogenous GC shortage, which is attempted to be compensated via the HPI axis, resulting in a higher Cort production. In this context, the steroid level profile might thus be interpreted by induced Preg metabolism, leading to accumulation of Prog and Cort in larvae. Notably, although induced GC signaling could be expected from the chemical analysis results, due to the elevated Dex and Cort levels, the GR antagonistic effect of Mife dominated GC signaling, as observed in all three GRIZLY screens.

For the other inhibitors in all three GRIZLY screens, Trani, 10-HC, Aci and Ret. acid, an effect mechanism similar to that of Mife was expected. However, not one of the four inhibitors reproduced the steroid profile of Mife. Trani had no effect on any of the tested steroids. As Mife, Trani appears to inhibit GC signaling by a mechanism other than reducing endogenous Dex or Cort.
10-HC solely showed a trend for reduced Dex. With Preg, the steroid levels remained unaffected, which suggests that GC signaling inhibition by 10-HC, at least in GRIZLY 3, was mediated independently from steroid biosynthesis and metabolism.
Interestingly, Aci and Ret. acid significantly decreased endogenous Cort. In addition, Aci showed significant reduction of Preg and a trend for reduced Prog. This response to Aci might suggest a decreased Preg uptake or induced conversion of Preg into metabolites other than Prog and Cort. For Ret. acid, reduced synthesis or induced metabolization of Cort might be a possible explanation. Accordingly, the inhibitory effects of Ret. acid and Aci on GC signaling in GRIZLY 3 could be related to the reduced Cort levels. However, their inhibition in GRIZLY 1 and GRIZLY 2 cannot be explained by this, as changes in endogenous Cort in co-treatment with Dex would not dominate the Dex effect. As chemical analysis did further not reveal any changes in Dex concentrations, the observed inhibition is probably the consequence of mechanisms other than the reduction of endogenous activator concentrations.

In addition to Leflu, the GRIZLY results suggested also a potential for GC biosynthesis disruption for Gestri, Nor, Estrone, DHEA, Estriol. For all five candidates, significantly reduced Cort levels were detected by chemical analysis, which substantiates disruption of GC biosynthesis as one main mechanism of action. However, the compound effects on larval levels of the other steroids Dex, Preg and Prog are diverse, which might imply different points of action among these GC biosynthesis disruptors.

For comparison, the positive control for disruption of GC biosynthesis, Mety, inhibited Preg-induced GC signaling and was ineffective in co-treatment with Dex. Mety significantly reduced Cort, while the Prog concentration remained unaffected and Preg showed a trend to be induced. These results are traceable in **Figure 42**, where Mety inhibits 11β-hydroxylase, the enzyme which enables the last step of the GC biosynthesis pathway. Thus, the conversion of 11-deoxycortisol into Cort is blocked, which consequently leads to an accumulation of the precursor metabolites, including Preg. In literature, strongly elevated levels of 11-deoxycortisol are frequently reported when Mety is applied and serve as a reliable indicator for the inhibition of 11β-hydroxylase (Nicola & Dahl 1971; Spark 1971). As Prog was not altered by Mety treatment and Preg had no inductive effect on Cortico levels in the present study, it appears likely that Preg was predominatly metabolized into Cort via 17α-hydroxyprogesterone and 11-deoxycortisol, whereas conversion of Preg into Cortico via Prog and 11-deoxycorticosterone played a minor role, occurred in a much lesser extend.

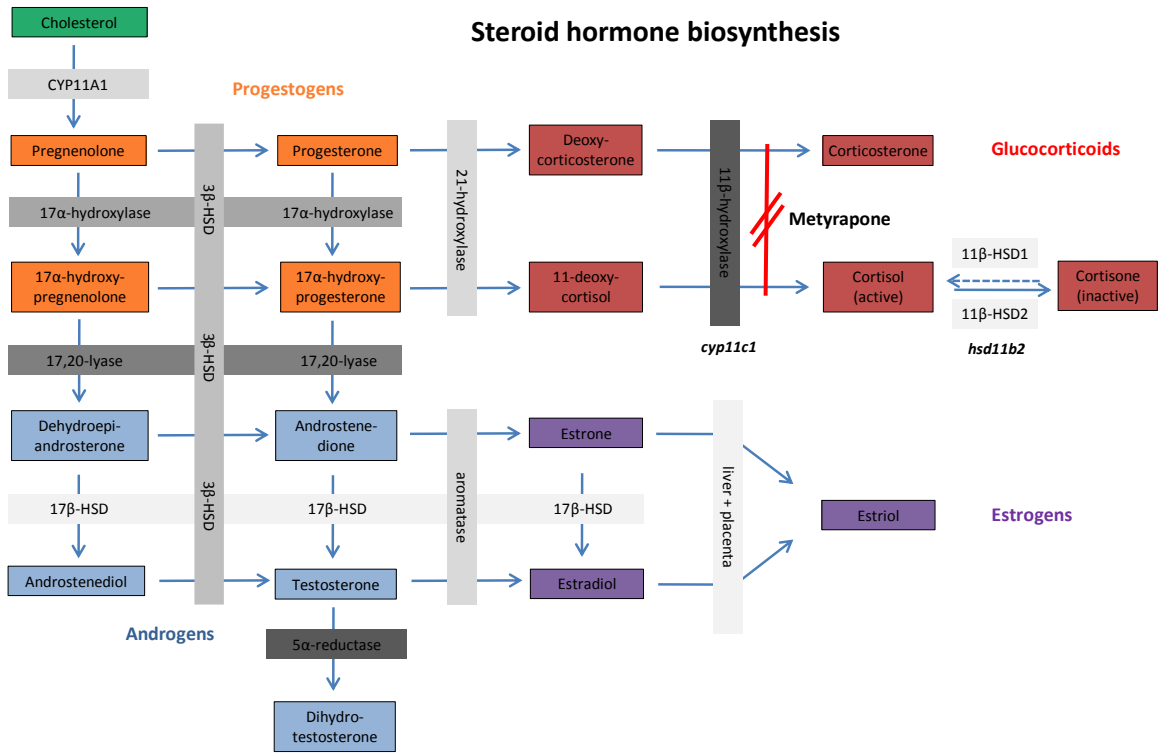

Figure 42: Inhibition of the steroidogenic enzyme 11β-hydroxylase by Metyrapone
The scheme of the steroid hormone biosynthesis pathway is replotted from **Figure 3** and slightly modified in order to show the point of Cort biosynthesis disruption by Mety. Also the enzyme 5α-reductase and an additional androgen, Dihydrotestosterone are included.More detailed information about all other metabolites and enzymes was provided in the original scheme in **Figure 3** (chapter **1.2.2**).

Interestingly, among the putative other GC biosynthesis inhibitors, Nor showed the same steroid level profile as Mety in co-exposure with Preg, which suggests that this compound also inhibits 11β-hydroxylase activity. The only difference in the profile of Mety and Nor is that Nor treatment led to higher Dex levels in the larvae, while they were not affected by Mety. However, this is in accordance with the GRIZLY results, as Mety did not interfere with Dex-induced GC signaling, but Nor superactivated it. Correspondingly, this effect of Nor might potentially be explained by interference of the compound with induced uptake or reduced metabolism of Dex.

Very similar to that of Nor is the steroid level profile of Gestri, with significant reduction of Cort, unaffected Prog and even significantly elevated Preg. Here, also inhibition of 11β-hydroxylase might be the point of disruption, but the more distinct accumulation of Preg could further imply a stronger effect than that of Mety, potentially involving the inhibition of other enzymes, for instance 3β-HSD or 17α-hydroxylase. The effect of Gestri in the GRIZLY assay was the same as for Nor, inhibition with Preg but co-induction with Dex. However, in contrast to Nor, no tendency for an elevated Dex concentration was detectable for Gesti, which makes interference with Dex uptake or metabolism a rather unlikely explanation for the observed GRIZLY effect. Potentially, as hypothesized within the comparative compound analysis for all candidates from **Figure 32**, a more direct interference of Gestri (and Nor, Ethi, Spiro, Stano) with Dex-mediated ativation of the GR could rather explain the co-induction of Dex signaling.

For Stano, Spiro and Ethi, the comparative compound analysis of the GRIZLY results gave no direct indications for inhibition of GC biosynthesis, as Preg-induced signaling was not reduced. However, chemical analysis revealed reduced Cort levels for all of them.

Stano superactivated GC signaling in co-treatment with both activators. Chemical analysis showed a significantly elevated Dex concentration in the larvae, which might explain the co-inductive effect with Dex. However, this does not apply for the observed superactivation with Preg, as Stano significantly reduced Cort without affecting Preg and Prog. Therefore, the Stano effect on GC signaling is independent of the endogenous Cort levels in co-treatment with Preg, which might also be true for Dex. The observed reduction of Cort probably underlies disrupted GC biosynthesis, but Stano has apparently additional mechanisms of action that are capable to disrupt/ induce GC signaling.

Spiro also co-induced GC signaling with Dex, as well as with Preg. As the UPLC-MS/MS data showed unaffected Dex levels, this superactivation with Spiro apparently did not involve changes in Dex metabolism. The same applies for co-treatment with Preg, as the measured steroid levels do not indicate increased Cort. Thus, like for Stano, the co-inductive effect on GC signaling appears to be independent of the endogenous steroid levels. Instead, chemical analysis of Spiro + Preg showed trends for elevated Preg and Prog and reduced Cort. Irrespective of the GRIZLY results, this steroid level profile indicates disruption of GC biosynthesis, possibly by inhibition of 17α-hydroxylase and potentially in combination with 11β-hydroxylase or 21-hydroxylase. As blocking of 11β-hydroxylase led to reduced Cort and a trend for elevated

Preg in case of Mety, but as Prog levels were not changed by this, another enzyme might be inhibited by Spiro, explaining the Prog accumulation. As visible in the schematic steroid hormone biosynthesis pathway above in **Figure 42**, blocking of 17α-hydroxylase would most likely lead to accumulation of Preg and Prog while Cort would be reduced. Inhibition of 11β-hydroxylase and/ or 21-hydroxylase could also play a role in this context, but, compared to the Mety effect, accumulation of Prog would not be expectable, as long as 17α-hydroxylase is blocked.

Ethi solely superactivated GC signaling with Dex in larvae. The steroid level profile shows a trend for increased Dex in the larvae, which suggests an effect of Ethi on Dex metabolization that might explain the co-induction in the GRIZLY assay. Moreover, Ethi significantly reduced Prog and Cort, but the Preg concentration remained stable. According to the UPLC-MS/MS measurements in **Figure 40 c + d**, the amount of Prog is reduced by 50%, while the inhibition of Cort is more pronounced, with only 10% of the steroid remaining. This implies that Cort production was nearly completely prevented, while the disruption of Prog was incomplete or partly compensated via other metabolites. Regarding the enzymes involved in the steroid hormomone biosynthesis pathway (**Figure 42**), only disruption of 3β-HSD has the potential to reduce Prog levels. Accumulation of Preg could be expected in case of 3β-HSD inhibition, as Preg levels were even elevated when the downstream enzyme 11β-hydroxylase was blocked by Mety. However, maybe the inhibition of 3β-HSD involves a different effect cascade, which could imply accumulation of 17α-hydroxypregnenolone or induction of other enzymes, enabeling the metabolization of Preg and 17α hydroxypregnenolone into androgens.
Regarding the GRIZLY response to Ethi + Preg, no significant change in GC signaling was detectable. However, as it was described in the combined analysis of compound effects in all three GRIZLY assay approaches and as it is visible in **Figure 32 l**, Ethi tended to further increase the bioluminescent signal with Preg, similar to the compound effect on Dex-induced signaling (**Figure 32 k**) or the response of Stano + Preg (**Figure 32 t**), but weaker, and thus, not significant.

Estrone, DHEA and Estriol inhibited Preg-induced GC signaling in the GRIZLY assay, but had no effect on Dex. Within chemical analysis, all three significantly reduced Cort, but Preg, Prog and Dex were not affected. As none of the three compounds showed accumulation of any of the metabolites, prevention of cortisol production is indicated, but probably via inhibition of steroidogenic enzymes other than those inhibited by Mety, Gestri and Nor.

In summary, the UPLS-MS/MS experiments largely supported the hypotheses from the combined analysis of compound effects in all three GRIZLY assays (section **3.2.2.2**) and provided important information about the compound effects on endogenous steroid levels. For Mife, Trani, 10-HC, Ret. acid, Aci, Fen, Nabu, Spiro, Stano and Ethi, GC signaling inhibition independent from the endogenous Cort levels was assumed, which was substantiated by chemical analysis. Although Mife, Ret. acid, Aci, Spiro, Stano and Ethi interestingly had effects on the Cort levels, these observations do not (fully) reflect the compound effects within the GRIZLY assay.

Like for Mety, disruption of GC biosynthesis was assumed to underly the effects of Leflu, Gestri, Nor, Estrone, DHEA and Estriol on Preg-induced GC signaling. This was further supported by chemical analysis, as reduced Cort levels were detected for all candidates, except Leflu.

Accordingly, the second aim of this study was achieved, a number of GC biosynthesis disruptors was identified from the drug library. Gestri, Nor, Estrone, DHEA, Estriol, Ret. acid, Spiro, Stano and Ethi led to reduced Cort levels when larvae were co-treated with Preg. For several, the putative point of disruption could even be narrowed down to certain enzymes. Aci may or may not be considered a presumptive disruptor of GC biosynthesis, as also Preg and Prog levels were strongly reduced, which might possibly indicate inhibition of cholesterol biosynthesis or also a disrupted Preg-uptake or induced -metabolization.

Regarding the observed superactivation of GC signaling with Spiro, Stano, Gestri, Nor and Ethi, a potential direct interaction at the level of the GR was hypothesized. Additionally or alternatively, elevated Dex levels with Stano, Nor and Ethi were found, which could potentially explain their effect on Dex-induced GC signaling.
In order to obtain more information about the compounds, whose effect mechanisms could not be further clarified by chemical analysis, gene expression analysis was conducted in the next step. Assessment of substance effects on a set of GC target genes and selected indicator genes for xenobiotic metabolism should help to specify the origin of GC signaling disruption and to further examine aspects of the GC signaling disruption

3.6 Tier 3b: Gene expression analysis

In tier 3b, the compound effects on selected target genes were examined in order to gain more insight into the mechanisms of GC signaling disruption. Prior to that, validation studies were carried out for the selection of suitable target genes and an assessment of the exposure time optimum.

3.6.1 Pretests for the selection of target genes and exposure duration

In order to identify the optimal exposure time for compound effects on target gene expression, larvae were exposed to Preg [5 µM, 0.2%] and DMSO [0.2%] for durations between 1h and 24h. Subsequently, qPCR analysis of a set of GC-signaling related genes was conducted. In **Figure 43**, the expression ratios of the six selected genes are shown upon exposed for 1, 2, 4, 12 or 24h to Preg or for 24h to E3. Values are given relative to those of the DMSO control for the respective durations.

For the GC target genes *hsd11b2* (**b**), *gilz* (**d**) and *fkbp5* (**e**), mRNA expression significantly increased already after 1h of exposure to Preg. The strongest up-regulation at this timepoint, with a ratio of 15, was measured for *fkbp5*. The mRNA levels for all three genes increased further when larvae were exposed for 2 and 4h. The highest expression ratios for *hsd11b2* and *gilz* were detected after 4h, with 9 and 3.5, respectively. For *fkbp5*, the strongest up-regulation was

measurable after 2h, with a ratio of 28, but after 4h the ratio was with 27.5 only slightly lower. After 12h of exposure, the mRNA levels of all three target genes were moderately less induced; in case of *hsd11b2* and *gilz*, the expression was not significantly elevated anymore.

The other selected targets, *cyp11c1* (**a**), *pomca* (**c**) and *gr* (**f**)were not affected by Preg treatment. Potentially, their mRNA transcription is not mediated by Cort binding to the GR, or they are regulated by mechanisms other than receptor binding. Especially in case of *cyp11c1*, encoding 11β-hydroxylase, GC effects on the enzyme activity and availability probably underlie more dynamic effects than transcription regulation. In the case of *pomca*, downregulation of expression in the anterior pituitary (as seen with Dex treatment by Liu et al. 2003) may be masked by unchanged expression levels in the posterior pituitary or the arcuate nucleus, rendering this effect not detectable by qPCR from larval extracts.

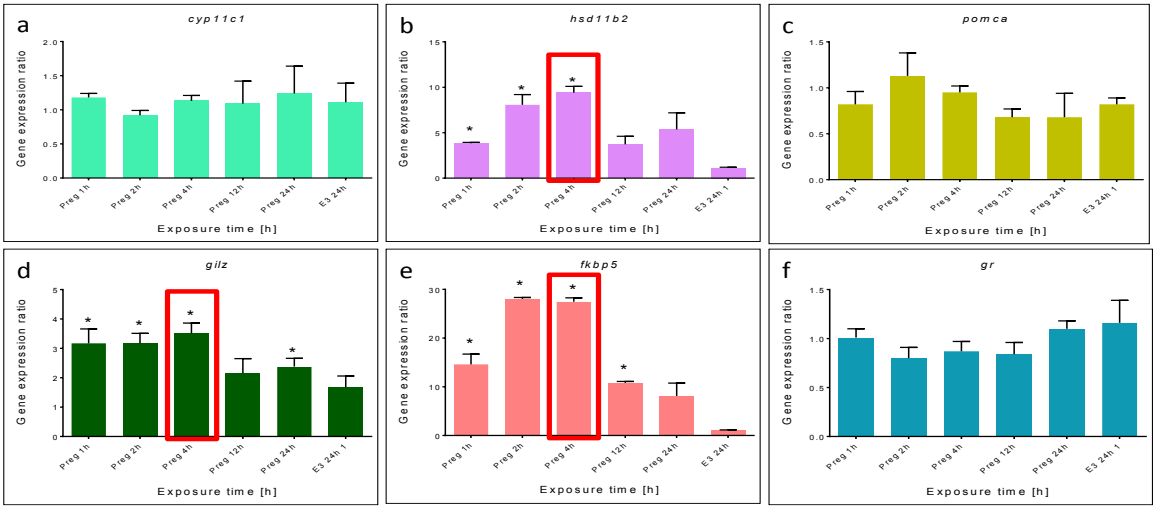

Figure 43: Gene expression timecourse experiments Pregnenolone
Expression ratios of GC-signaling related target genes in exposure to Preg. Expression of mRNA is measured for *cyp11c1* (**a**), *hsd11b2* (**b**), *pomca* (**c**), *gilz* (**d**), *fkbp5* (**e**) and *gr* (**f**) after larvae were exposed for 1,2,4,12 or 24 h to Preg ([5 µM], 0.1%) or DMSO (0.1%, data not shown] or to E3 for 24 h.The ratios of treatment with DMSO to treatment with Preg/ E3 are calculated for the respective exposure times. Mean gene expression ratios of three biological replicates + SEM (n=3) are shown. Statistic evaluation of gene expression data was conducted with the 2△CT method, measured with two technical replicates per plate and two housekeeping genes, *rpl13a* and *ef1α*. Statistical significance is determined by comparison of the mean ratio of each treatment condition with the mean ratio of the DMSO control for each exposure time in a multiple t-test, α=0.05. After multiple testing correction with the Holm-Šídák method, significance is defined as $p < \alpha < 0.02$ =*.

According to the pretesting results, *hsd11b2*, *fkbp5* and *gilz* were selected as GC target genes for gene expression analysis of the library compounds. As for these three genes the exposure time of 4h showed the highest expression ratios, this duration was selected as the optimal exposure time for the assessment of compound effects on target gene expression.
Furthermore, although *cyp11c1* was not regulated by Preg treatment, the library compound effects on this target gene were still assessed because of its role in GC metabolism. In addition, without pretesting, two genes related to xenobiotic metabolism, *cyp1a1* and *cyp3a65,* were selected based on their implication in GC drug metabolism. Regulation of *cyp1a1* or *cyp3a65* might indicate compound effects on xenobiotic metabolism, which could affect endogenous concentrations of Dex and Preg and would in turn disrupt GC signaling.

Next, the compound gene expression patterns are examined upon co-exposure with Dex and Preg *in vivo*. This will provide more information about their presumptive effect mechanisms and should help to pursue the third aim of this study, to identify GC signaling inhibitors with - so far - unknown mechanisms of action.

3.6.2 Library compound effects on target gene expression

For library compound effect analysis on the selelcted genes, larvae were exposed for 4 h to the same compound concentrations as applied for chemical analysis, each substance in co-treatment with Dex [20 µM] as well as with Preg [5 µM]. Afterwards, qPCR experiments were conducted. Gene expression ratios were calculated by comparing the treatment with the negative control results. The results are shown in **Figure 44**. As Preg had an overall stronger impact on target gene expression, the results for Preg will be described first.

The expression ratios for all genes after co-exposure to Preg + library compounds are shown in **Figure 44.II**. Preg (Preg [5 µM] + DMSO, negative control) significantly induced the expression of *fkbp5* (**a**), *hsd11b2* (**b**), *gilz* (**c**), *cyp3a65* (**e**) and *cyp1a1* (**f**) when compared to the DMSO control (DMSO + DMSO, 0.2%). The strongest up-regulation was observable for *fkbp5* with a 25-fold and *hsd11b2* with a 17-fold induced gene expression by Preg, consistent with the obsevations from the time course experiments, while the increase of *gilz*, c*yp3a65* and *cyp1a1* was between four- and five-fold. As the negative control effect served as reference for all other treatments, lower or higher induction of gene expression by drug library compounds was defined as reduced or induced gene expression. Also the DMSO control leads to “reduced” expression in this view, as no induction can occur here.

The data reveal that most library compounds reduced the expression of all tested genes, except that of *cyp11c1*. Only Trani ($p=0.0354$) and Estrone ($p=0.0329$) showed a trend to decrease *cyp11c1* mRNA levels (**d**). This low effect on the gene encoding 11β-hydroxylase is consistent with the results from pretesting experiments and strengthens the assumption that GC effects on the enzymatic action of 11β-hydroxylase may not predominatly be mediated via changes in mRNA transcription, but rather by more dynamic mechanisms.

For *fkbp5* (**a**), *hsd11b*2 (**b**) and *gilz* (**c**), distinct patterns of regulation are observed, showing that several library compounds had common effects on all three genes. Especially Estrone, Gestri, Nor, Mety, Mife and DHEA strongly decreased the Preg-induced gene expression, with mRNA levels for Estrone, Gestri and Nor even comparable to those of the DMSO control. Only three compounds did not affect *gilz* expression, namely Ret. acid, Leflu and Ethi, while *fkbp5* expression was unaffected by six compounds, Nabu, Spiro, Stano, Ethi, Fen, and Leflu. All other substances showed at least a trend ($p < \alpha < 0.05$) to reduce the expression of *fkbp5*, *hsd11b*2 and *gilz*, most of them had a significant ($p < \alpha < 0.0.0027$) effect.

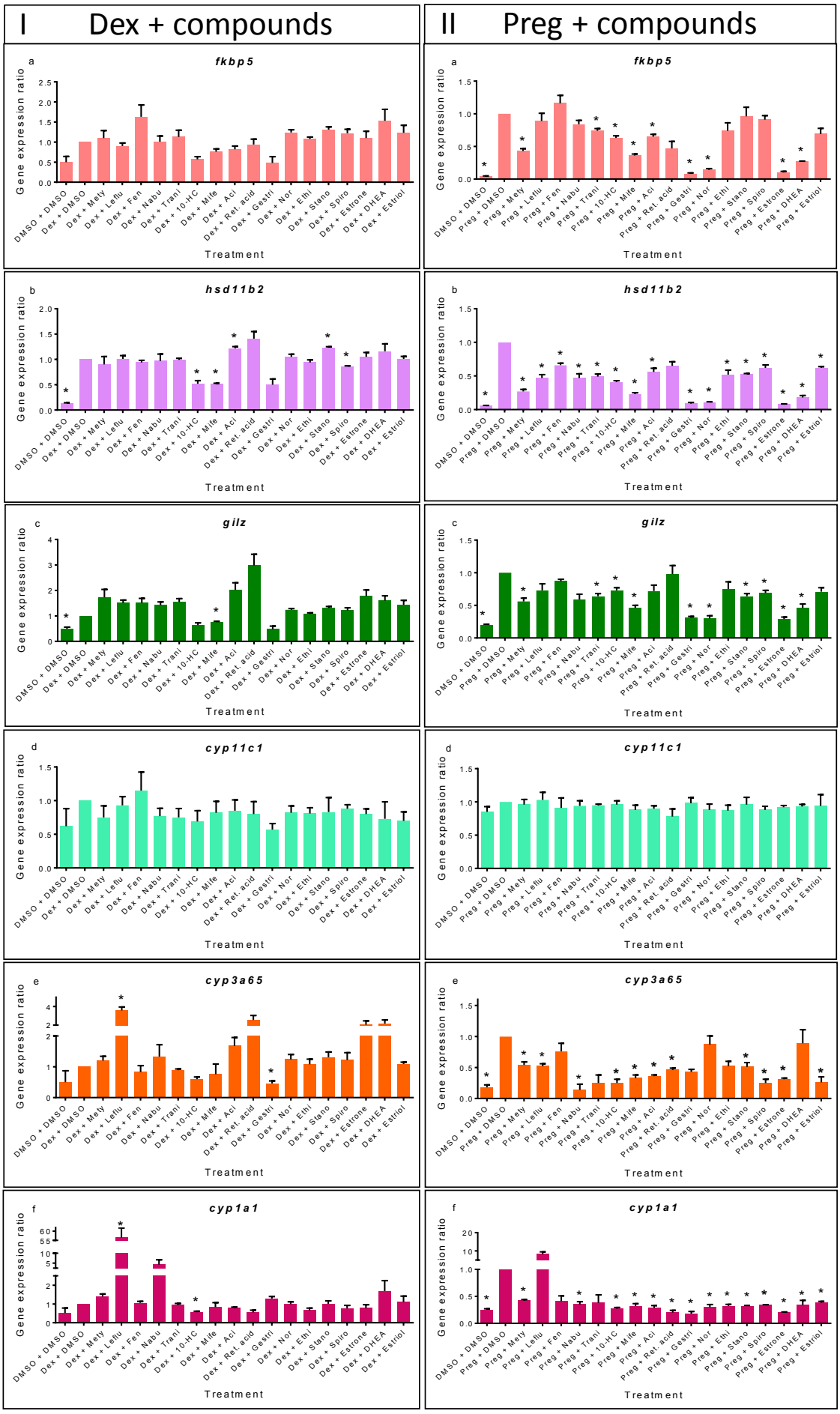
I Dex + compounds
II Preg + compounds
a
fkbp5
b
hsd11b2
c
gilz
d
cyp11c1
e
cyp3a65
f
cyp1a1
Gene expression ratio
Treatment
DMSO + DMSO
Dex + DMSO
Dex + Mety
Dex + Leflu
Dex + Fen
Dex + Nabu
Dex + Trani
Dex + 10-HC
Dex + Mife
Dex + Aci
Dex + Ret. acid
Dex + Gestri
Dex + Nor
Dex + Ethi
Dex + Stano
Dex + Spiro
Dex + Estrone
Dex + DHEA
Dex + Estriol
Preg + DMSO
Preg + Mety
Preg + Leflu
Preg + Fen
Preg + Nabu
Preg + Trani
Preg + 10-HC
Preg + Mife
Preg + Aci
Preg + Ret. acid
Preg + Gestri
Preg + Nor
Preg + Ethi
Preg + Stano
Preg + Spiro
Preg + Estrone
Preg + DHEA
Preg + Estriol

Figure 44: Gene expression experiments with FDA library compounds

Expression ratios of GC-signaling- (**a**, **b**, **c**, **d**) and xenobiotic metabolism- (**e**, **f**) related genes in co-exposure to Dex (**I**) or Preg (**II**)+ library compounds. Expression of mRNA is measured for *fkbp5* (**a**), *cyp11c1* (**b**), *hsd11b2* (**c**), *gilz* (**d**) *cyp3a65* (**e**) and *cyp1a1* (**f**) after larvae exposure for 4 h to Preg [5 µM]/ Dex [20 µM] + library compound (different concentrations) or to the negative control Preg + DMSO [5 µM]/ Dex + DMSO [20 µM]. The amount of DMSO was 0.2% in all treatment conditions. The ratios of treatment and negative control are calculated. Mean gene expression ratios of three biological replicates + SEM (n=3) are shown. Statistic evaluation of gene expression data was conducted with the 2△CT method, with two technical replicates per plate and two housekeeping genes, *rpl13α* and *ef1α*. Statistical significance is determined by comparison of the mean ratio of each treatment condition with the mean ratio of the negative control for each exposure time in a multiple t-test, α=0.05. After multiple testing correction with the Holm-Šídák method, significance is defined as $p < \alpha < 0.0027$=*****

Regarding the genes related to xenobiotic metabolism, Preg-induced mRNA expression of *cyp3a65* (**d**) was attenuated by all compounds except Fen, Nor and DHEA, which had no impact at all. The expression of *cyp1a1* (**f**) was significantly downregulated by all candidates except Leflu, which interestingly even intensified the induction by Preg.

In **Figure 44.I**, the gene expression ratios for larvae exposure to Dex + library compounds are shown. Dex (Dex + DMSO, negative control) generally had a lower impact on mRNA expression than Preg, as only two genes were significantly up-regulated, *hsd11b2* (**b**) and *gilz* (**c**), and an induced trend was visible for *fkbp5* (p=0.0239) (**a**). The most sensitive gene was *hsd11b2*, with a seven-fold induction, while mRNA expression of all other targets was two-fold higher with Dex. However, compared to the 17-fold *hsd11b2* increase by Preg, the Dex effect was considerably weaker.

Beside the overall lower effect of Dex on target gene expression, the most obvious difference betwen Dex and Preg treatment is the lacking induction of xenobiotic target genes by Dex, while *cyp1a1* and c*yp3a65* were highly induced by Preg. This absence of a xenobiotic marker gene response to Dex treatment suggests that at least the drug metabolism pahway involving these two CYP enzymes was not activated by larvae treatment with the synthetic GC. Keeping in mind the low Dex recovery rate found with UPLC-MS/MS, compared to the retrieved amount of Preg, it may be possible that the uptaken amount of Dex was high enough to trigger a therapeutic response (induce GC signaling and gene expression of several target genes), but not to activate the phase I drug metabolism enzymes *cyp1a1* and c*yp3a65 .*

Corresponding to the weak Dex impact on target gene expression, only few library compounds significantly altered the mRNA levels in response to Dex induction. However, most substances showed a trend to further induce mRNA expression of *gilz* (**c**), and, in few cases, also *fkbp5* (**a**) and *hsd11b2* (**b**) were up-regulated with Dex, although the same compounds decreased the levels of these genes in co-treatment with Preg.

Trends for downregulation of *fkbp5* (**a**) are visible with 10-HC (p=0.0030), Mife (p=0.0266) and Gestri (p=0.0302), whereas tendencies for induced expression can be observed with Nor (p=0.0254) and Stano (p=0.0109)

Five compounds significantly alterd the expression of *hsd11b2* (**b**), 10-HC, Mife and Spiro reduced gene expression, while Aci and Stano further induced it. In addition, Gestri (p=0.0111) shows a trend to increase and Ret. acid (p=0.0420) a tendency to decrease the mRNA level.
The expresion of *gilz* (**c**) was altered by all compounds except Spiro, Ethi and Estriol. Mife reduced mRNA expression significantly while Leflu, Fen, Nabu, Trani, Aci, Ret. acid, Nor, Stano, Estrone and DHEA showed trends to upregulate mRNA expression (p=0.0472-0.0067) and 10-HC (p=0.0161), as well as Gestri (p=0.0123) tended to decrease *gilz* levels.
The mRNA levels of *cyp11c1* (**d**) were not altered by any of the tested compounds, similar to the observations with Preg treatment.
Two compounds had significant effects on *cyp3a65* (**e**), Gestri reduced mRNA expression while Leflu induced it. Furthermore showed Ret. acid (p=0.0341) and DHEA (p=0.0399) trends to further induce gene expression, while a tendency for down-regulation was visible for 10-HC (p=0.0046).
The other marker gene for xenobiotic metabolism, *cyp1a1* (**f**), was significantly regulated by two compounds. Gene expression was remarkably high induced by Leflu with a ratio of 56 while 10-HC down-regulated the mRNA level. Also Aci (p=0.0046), Ret. acid (p=0.0194) and Ethi (p=0.0483) showed trends to reduce *cyp1a1* expression.

Evaluation of the gene expression results in the context of GRIZLY assay and chemical analysis outcomes

Figure 45 summarizes the data of the whole tiered testing approach, i.e. the substance effects on GC signaling, their steroid level profiles and the gene expression patterns. Again, color code profiles are shown for controls and library compounds. No difference of the substance effect compared to the negative control is indicated in yellow. Significant reduction or co-induction of signal intensity/ steroid concentration/ gene expression compared to the stimulated untreated (negative) control is indicated in green and red, non-significant trends ($p < \alpha < 0.05$) for reduction or co-/induction in orange and light green, respectively. Significance is defined as $p < \alpha < 0.0001$=***** for the GRIZLY assay, $p < \alpha < 0.005$=***** for chemical analysis and for gene expression results $p < \alpha < 0.0027$=*****. The applied concentrations for UPLC-MS/MS and qPCR experiments are indicated for each substance. In order to facilitate comparisons, the compounds were divided into four groups according to their GRIZLY behaviour and their impact on endogenous Cort levels.

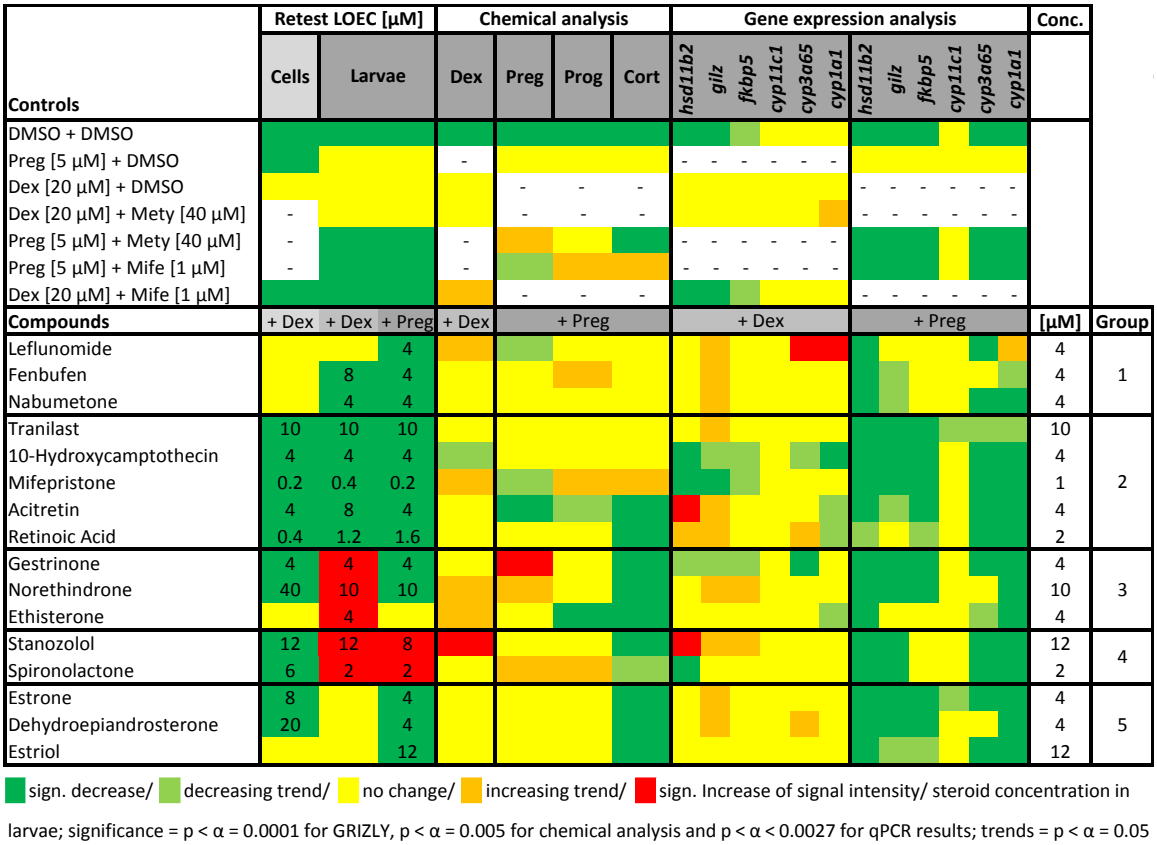

Figure 45: Comparison of compound effects in GRIZLY, UPLC-MS/MS and qPCR experiments
Color code profiles are shown for controls and library compounds. No difference of the substance effect compared to the negative control (Dex [20 µM]/ Preg [5 µM] + DMSO) is indicated in yellow. A significantly increased or decreased GRIZLY signal intensity, steroid level or gene expression compared to the negative control is indicated in green and red, respectively. Non-significant trends ($p < \alpha < 0.05$) for increase or decrease are shown in orange and light green, respectively. Significance is defined as $p < \alpha < 0.0001$ for the GRIZLY retests, as $p < \alpha < 0.005$ for chemical analysis and as $p < \alpha < 0.0027$ for qPCR results. Validated inhibitors and superactivators from retesting are indicated with their lowest observed effect concentration (LOEC) in the respective GRIZLY assay.

Controls

Regarding the gene expression analysis controls, the positive control for Dex-induced GC signaling (Dex + Mife) apparently counteracted the negative control (Dex + DMSO) effect on mRNA levels. Thus, the gene expression pattern of Dex + Mife is identical with that of the DMSO control. This, in turn, reflects the Mife effect on GC signaling within the GRIZLY assay, where Mife also produced the same signal patterns as the DMSO control.

Likewise, this applies for the positive control of Preg-induced GC signaling (Preg + Mety). Mety completely reversed the effect of the negative control (Preg + DMSO) on mRNA expression levels, leading to identical gene expression patterns as the DMSO control. This, again, reproduced the effect of Mety on Preg-induced GC signaling within the GRIZLY assay.

The same pattern of target gene expression as for Preg + Mety was observable for Preg + Mife, which also resembles the respective GRIZLY result. This further indicates that both control inhibitors, although acting via different mechanisms, have the same impact on Preg-induced GC signaling, even on the level of mRNA expression of the selected gene set. Within this set, also the Preg-induced expression of the xenobiotic clearance-related targets *cyp1a1* and *cyp3a65*

was counteracted by Mety and Mife, which strongly suggests a GR-mediated trancriptionl regulation of these genes in co-treatment with Preg, instead of, or in addition to, an AhR/ PXR-initiated mechanism. Furthermore, within the Preg timecourse experiment from **Figure 38**, the endogenous amount of Preg was with 6833 ng/ 100 larvae 30% higher under treatment with Preg + DMSO (**h**) than with Preg + Mety (**i**), where 4810 ng/ 100 larvae were detected at the timepoint of qPCR analysis (4 h). The lower Preg level in the larvae could thus, also result in a lower xenobiotic activity and may contribute to the decrease of *cyp3a65* und *cyp1a1* mRNA expression upon Mety treatment. As Dex had no measurable effect on *cyp1a1* and *cyp3a65*, expression of these targets was accordingly not altered by Mife.

In view of the positive control effects on endogenous steroid levels, reduced Cort by Preg + Mety correlates with the GRIZLY and the gene expression outcomes. Also as expected, steroid level profiles of Dex + Mife and Preg + Mife are not following the Mife effects within GRIZLY and qPCR experiments, as Mife inhibits GC signaling by GR antagonism, a mechanism which does not necessarily involve reduction of the endogenous Cort concentration.
Furthermore , it was shown that Mety had no impact on Dex-induced GC signaling, neither in the GRIZLY assay, nor on GC target gene expression or endogenous steroid levels. The only exception is a trend to induce the expression of *cyp1a1* with Dex, potentially suggesting disrupted xenobiotic clearance, but the endogenous Dex concentration was not altered by Mety.

Group 1

The library compounds Leflu, Fen and Nabu were assigned to group 1. Fen and Nabu reduced GC signaling in GRIZLY 2 and GRIZLY 3, while Leflu inhibited the signal only in GRIZLY 3 and was, thus, suspected to disrupt the Cort biosynthesis. However, the steroid level profiles gave no indications for disruption of GC biosynthesis for any of the three compounds.

Regarding the gene expression patterns of **Leflu**, the significant up-regulation of *cyp3a65* and *cyp1a1* in co-treatment with Dex is eye-catching. Also for Leflu + Preg was a strong induction of *cyp1a1* visible, but the gene expression ratio was seven-fold higher with Dex (**Figure 44 I+II f**). This strong induction of the xenobiotic clearance marker gene by Leflu in co-treatment with both activators cannot be explained by GRIZLY or UPLC-MS/MS results, as the outcomes are different with Preg and Dex. Possibly, the effect of Leflu on *cyp1a1* expression is reasoned by GC-signaling independent mechanisms. Although the GR is known to have a regulatory function on the expression of *cyp1a1*, this gene is also the major target of the Ahr transcriptional activity. Accordingly, Leflu may have triggered Ahr-medited *cyp1a1* expression, independent from GR signaling.
Regarding the other gene for xenometabolic activity, *cyp3a65*, mRNA induction was obserrvable with Dex while expression was down-regulated with Preg. Potentially, Leflu affected the uptake of Dex and Preg into the larvae differently, as it is supported by chemical analysis data, showing elevated Dex and reduced Preg in the larvae. Thus, the *cyp3a65* expression in larvae may be induced as a response to the increased Dex concentration, while the decreased Preg level resulted in downregulation of *cyp3a65.* Although the elevated Dex in the larvae did not lead to a

significant increase in GRIZLY signal, the bioluminescent traces for Dex + Leflu in **Figure 37 c** are in accordance with the chemical analysis findings, indicating a tendency for increased GC signaling. The increased expression of the Dex target gene *gilz* further supports this assumption.
In contrast, the observed inhibition of GC signaling by Preg + Leflu is supported by qPCR results. Although endogenous Preg levels were reduced, additional mechanisms must have led to the observed, strong, signal inhibition within the GRIZLY assay. A Preg-induced *hsd11b2* expression was decreased by Leflu, but mRNA levels of *gilz* and *fkbp5* were not affected, the compound disrupted the transcriptional regulation of several, but not all GR target genes. Due to these differences in the gene expression patterns of Leflu and Mife, Leflu certainly had no full antagonistic effect on all Preg-induced genes, but may still have directly antagonized the expression of some GR targets. Potentially, GR binding of Leflu may have induced conformational changes of the LBD, which led to differential recruitment of coactivators and repressors and resulted in gene-specific transrepression.

Fen and **Nabu** had similar effects in all experiments. They inhibited Dex- and Preg induced GC signaling in the GRIZLY assay, thus, disruption of the Cort biosynthesis was not assumed. This was confirmed by chemical analysis, as the measured steroid levels neither gave indications for inhibition of Cort production, nor suggested they changes in drug uptake due to treatment with Fen and Nabu. On the level of gene expression, both compounds showed a trend to induce *gilz* in co-treatment with Dex, while a tendency to down-regulate this gene was observable in co-exposure with Preg. Accordingly, with Dex, the gene expression patterns of Fen and Nabu show no resemblance with that of Mife, where Dex-induced expression of *hsd11b2*, *gilz* and *fkbp5* was counteracted. With Preg, Fen and Nabu down-regulated several target genes of the GR antagonist Mife, but *fkbp5* expression was not reduced. As for Leflu, the differences between the respective gene expression patterns suggest that Fen and Nabu inhibit GC signaling differently than Mife and Mety, but they may still compete with Dex and Cort for GR binding and mediate differential expression of several GR target genes.

<u>Group 2</u>
Group 2 contains the five compounds which inhibited in all three GRIZLY approaches, Trani, 10-HC, Mife, Aci and Ret. acid.
Trani showed no effect on any steroid measured by UPLC-MS/MS. On target gene expression, a trend for induced *gilz* was observable with Dex, while the mRNA levels of all targets were decreased with Preg. In co-treatment with Dex, Trani revealed the same gene expression pattern as Fen and Nabu, which reflects not the GRIZLY response and unfortunately provides no further information about the origin of Dex-induced signal disruption. Co-exposed with Preg, the Trani effect on target gene expression was similar to that of Mife and Mety, suggesting here parallels between the underlying mechanisms of GC signaling inhibition. However, in contrast to the positive controls, the substance showed a trend to decrease also *cyp11c1*, which might imply compound impacts on GC signaling different from or additional to the control inhibitor response.

For **Mife**, chemical analysis did not reveal reduced Cort levels, excluding this as an explanation for the observed inhibition in GRIZLY 3. Although Cort and Dex levels were even elevated, Mife dominated GC signaling via GR antagonism, which led to the observed inhibition in all three GRIZLY screens and which was also reflected on the level of gene expression, as Mife antagonized all targets of Dex and Preg.
Interestingly, **10-HC** had the same effect as Mife when co-treated with Preg, not only within the GRIZLY assay, but also in qPCR experiments, as it produced almost exactly the same gene expression pattern as Mife. The endogenous steroid levels were not found to be altered by 10-HC. However, a minor trend for elevated Prog and Cort and reduced Preg might be perceived from Preg + 10-HC treatment in **Figure 40 b-d**, very similar to Preg + Mife, but with higher replicate variations and thus, less considerable. Accordingly, given the gene expression patterns and GRIZLY responses, a distinct comparability between the impact of 10-HC and Mife on Preg-induced GC signaling is observable, which is also rather supported by chemical analysis.
In combination with Dex, the gene expression pattern of 10-HC showed differences to that of Mife. Although both compounds inhibited *hsd11b2*, *gilz* and *fkbp5*, the effect of 10-HC on *gilz* was not significant, but instead, *cyp3a65* and *cyp1a1* were reduced. As these two xenobiotic genes were no signifcant targets of Dex, down-regulation by 10-HC appears to be a compound-specific effect, potentially independent from Dex-mediated GC signaling. Furthermore anduniquely with 10-HC, a lower Dex concentration was measured in the larvae, which might explain the reduced expression of all Dex targets. The lower Dex level may be a consequence of reduced Dex uptake, an idea which appears to be supported by the down-regulation of *cyp3a65* and *cyp1a1*, indicating less xenometabolic activity due to the lower amount of Dex in the larvae.
In summary, these results may suggest that the GRIZLY response to 10-HC underlies different mechanisms when GC signaling is stimulated either with Dex or with Preg. While GR antagonism of Cort by 10-HC might possibly have lead to reduced GC signaling in GRIZLY 3, reduced uptake of Dex might potentially explain the effect of 10-HC in GRIZLY 2. However, in GRIZLY 1, reduced uptake of the synthetic GC due to some effect of 10-HC cannot be completely excluded as an explanation for the observed GC signaling inhibition, but appears rather unlikely.

The two other inhibitors in all three GRIZLY approaches, **Aci** and **Ret. acid**, both showed reduced Cort levels in co-treatment with Preg. Aci further also reduced Preg and Prog, which is why disrupted uptake or metabolism of Preg was hypothetized to underly the reduced Cort and thus, decreased GC signaling by Aci in GRIZLY 3. For the effect of Ret. acid, a disrupted Cort biosynthesis was considered a possible explanation. Regarding their impact on target mRNA levels, both compounds broadly counteracted the effect of Preg and showed high similarity with the gene expression patterns of Mife and Mety. For Aci, the only difference to the positive controls was a trend for reduced *gilz*, while the target was significantly down-regulated by Mife and Mety. Ret. acid had no effect on *gilz* and the decrease of *fkbp5* was not significant. Thus, Ret acid may have a different target gene response profile than the positive controls but effect similarity on Preg-induced GC signaling is still suggested.

Regarding the impacts of Aci and Ret. acid on Dex-induced GC signaling, the Dex levels in larvae were not altered. Interestingly, both compounds had a similar effect on target gene expression. Aci and Ret. acid showed trends to induce *gilz* and to reduce *cyp1a1*. Also, *hsd11b2* was induced by both substances, significantly by Aci. Ret. acid furthermore showed a tendency to induce *cyp3a65*. Especially the up-regulation of *hsd11b2* is a specific effect of Aci and Ret. acid among the inhibitors in GRIZLY 2. As the induced levels of *hsd11b2* and *gilz* are contrary to the Mife effect on target gene expression, rather increased than reduced GC signaling might be suspected from this. However, the GRIZLY response did not reflect GC signaling induction. This suggests that the two compounds apparently triggered gene-specific responses on *hsd11b2*, *fkbp5* and *gilz*, probably via interaction within the promotor region of these target genes. Additional, unknown mechanisms of Aci and Ret. acid must have led in the end to the observed inhibition of GC signaling.
The disrupted regulation of *cyp3a65* and *cyp1a1* indicates that impaired xenobiotic clearance may play a role within the observed effects, although the Dex levels in larvae were not altered. Alternatively, *cyp3a65* and *cyp1a1*, which are no targets of Dex or Mife, might also have been regulated by a mechanism other than GC activation of the GR or receptor antagonism.

Group 3

The substances which were assigned to group 3 superactivated in GRIZLY 2 and inhibited in GRIZLY 3. Furthermore, they decreased endogenous Cort in co-treatment with Preg.
For **Gestri** + Preg, the qPCR results correlate with the inhibition in GRIZLY 3 and the reduced Cort levels. Gestri decreased the expression of all Preg targets, identical with the effects of Mety and Mife. This further confirms Cort biosynthesis disruption as the underlying mechanism of action, without any indications for additional impacts of Gestri on Preg-induced GC signaling.
Together with Dex, Gestri superactivated GC signaling. Chemical analysis did nor indicate significant changes of endogenous Dex, neither can the qPCR data explain this effect. Gestri reduced the expression of all genes but *cyp1a1* and addressed, thus, the same targets as Mife. Moreover, uniquely among all compounds, Gestri decreased the expression of *cyp11c1* in co-treatment with Dex down to a lower level than for the DMSO control. Although a potential interaction of Gestri with Dex uptake or metabolism might be suggested by the disrupted *cyp3a65* expression, this was not confirmed by UPLC-MS/MS experiments, and might thus be less of an indicator for the effect mechanism of Gestri than the decrease of *cyp11c1*.

With **Nor**, a high effect similarity to Mety on Preg-induced GC signaling was described according to the steroid level profile. The gene expression pattern supports this assumption, as Nor reduced the same targets as Mety (and Mife), except *cyp3a65*, which was not altered by Nor.
Together with Dex, Nor had the same effect on GC signaling as Gestri, it also showed superactivation. Interestingly, the Nor effect on target gene expression had no similarity with the response to Gestri, as Nor showed a tendency to induce *gilz* and *fkbp5*, while the other genes remained unaffected. The upregulation of these two GC targets correlates with the elevated Dex level in larvae, observed by chemical analysis, and the superactivation in the GRIZLY assay.

Group 4

All compounds from group 4 superactivated GC signaling in GRIZLY 2 and had the same or no effect in GRIZLY 3. Furthermore, for all of them, disrupted GC biosynthesis due to reduced endogenous Cort levels was hypothesized as a mechanism of action, but which cannot explain the superactivation of GC signaling in GRIZLY 2.

When **Stano** was co-treated with Dex, it significantly elevated the Dex level in larvae. In accordance with this, the expression of the GC targets *hsd11b2, gilz* and *fkbp5* was also induced. Accordingly, Stano up-regulated all targets which were reduced by Mife, and had thus, the opposite effect of the GR antagonist, on mRNA expression, as well as on GC signaling within the GRIZLY assay. While several compounds showed induction of *gilz* without inducing GC signaling in the GRIZLY assay, Stano was beside Nor the only compound which up-regulated *hsd11b2* and superactivated in GRIZLY 2. Further, Stano and Nor were the only compounds which induced *fkbp5* when co-treated with Dex. As Stano also revealed an elevated Dex level, without disruption of *cyp3a65* and *cyp1a1*, like Nor, it is equally suggested for Stano that the effects in GRIZLY 2 and on GC target genes might be caused by the higher Dex concentration. Also comparable for both compounds, no alterations in the expression of *cyp3a65* and *cyp1a1* were observed in co-treatment with Dex, although it may have been exprectable from the elevated Dex level. Maybe, some other pathway counteracted the effect on *cyp3a65* and *cyp1a1* or xenometabolism was primarily regulated via other mechanisms.

Regarding the qPCR results of Preg + Stano, *hsd11b2*, *gilz*, *cyp3a65* and *cyp1a1* were reduced. Stano decreased the same targets as Mife and Mety, except *fkbp5*. Accordingly, the gene expression pattern correlates well with reduced endogenous Cort, but the GRIZLY assay showed the opposite effect on GC signaling. The qPCR data strengthen the hypothesis that Stano disrupted GC biosynthesis, but also acted by another mechanism which led to co-induced GC signaling with Preg, at least on isolated GRE elements as used in the GRIZLY assay. Potentially, this mechanism involved induction of *fkbp5*, which could possibly have compensated its reduced expression due to lacking Cort, leading in the end to an unchanged mRNA expression level.

Spiro showed the same gene expression pattern as Stano in co-treatment with Preg. This also correlates with the assumed disruption of Cort biosynthesis by Spiro, but as for Stano, another mechanism must have led to induced GC signaling in GRIZLY 3 and unchanged levels of *fkbp5*.

For Dex + Spiro, no indication for induced Dex levels were given by chemical analysis. On target gene expression, Spiro had a rather low impact as it only reduced *hsd11b2*. Accordingly, no direct hint is given by UPLC-MS/MS or qPCR experiments why Spiro co-induced GC signaling in GRIZLY 2.

In contrast to Stano, where superactivated GC signaling in GRIZLY 2 probably is the consequence of increased endogenous Dex concentrations, but the effect in GRIZLY 3 must have had a different reason, for Spiro, the effect in GRIZLY 2 and GRIZLY 3 can possibly be caused the same mechanism, regardless of the Cort biosynthesis disruption with Preg.

Ethi had a co-inductive effect on GC signaling in GRIZLY 2 but not in GRIZLY 3. Although a minor trend for increased bioluminescent traces was also seen with Preg in **Figure 32 l**, suggesting

effect similarity of Ethi on both Preg- and Dex-induced GC signaling, the signal increase was not significant in GRIZLY 3.

Chemical analysis showed elevated endogenous Dex with Ethi, which might explain the effect in GRIZLY 2. Dex target genes were not increased by Ethi, but, as visible in **Figure 43. I**, *fkbp5* (**a**), *hsd11b2* (**b**) and *gilz* (**c**) showed rather a slightly higher expression with Ethi than without. Thus, even though elevated Dex did not significantly increase the respective target mRNA levels, qPCR data rather support an Ethi-mediated accumulation of endogenous Dex to be the reason for superactivation in GRIZLY 2. Disrupted Dex metabolism might further be suggested by the decreased level of *cyp1a1* by Ethi.

With Preg + Ethi, disrupted Cort biosynthesis was suggested by chemical analysis. As Ethi had no significant effect in GRIZLY 3, the reduced Cort level did not result in a decrease of GC signaling. Within qPCR experiments, Ethi counteracted the Preg effect on *hsd11b2*, *cyp3a65* and *cyp1a1*, similar to Mety and Mife, which partly correlates with the disrupted Cort biosynthesis. However, in contrast to the positive controls, Ethi did not decrease *gilz* and *fkbp5*, which may relate to the lacking inhibition of GC signaling in the GRIZLY assay. Apparently, similar to Stano and Spiro, Ethi acted beside the disruption of Cort synthesis via an additional mechanism, which compensated the effect of reduced Cort on GC signaling, and led to the insignificant effect on GC target genes, visible in **Figure 44 II**, as mentioned above.

To highlight the effect similarities between Stano, Spiro and Ethi, the missing decrease of *fkbp5*, noticeably correlates with their lacking GC signaling inhibition in GRIZLY 3. As, in contrast to all other disruptors of Cort biosynthesis (groups 2-5), Stano, Spiro and Ethi commonly did neither inhibit in GRIZLY 3, nor decreased *fkbp5* expression, their effect mechanism might be suggested to involve regulation of this target.

Group 5

Estrone, **DHEA** and **Estriol** had no effect in GRIZLY 2, but showed reduced Cort as possible explanations for their inhibition in GRIZLY 3. Unlike Mety and several other GC biosynthesis disruptors, Estrone, DHEA and Estriol did not affect endogenous Preg or Prog levels. Accordingly, disruption of Cort biosynthesis might be their mechanism of action, but also reduction of endogenous Cort via different pathways could underlie these findings.

In co-treatment with Preg, the gene expression patterns of all three compounds show high similarity with those of Mety and Mife, although none of them is identical to that of the positive controls. This observation might imply certain mechanistic differences, but they appear minor, regarding the high effect similarity of Estrone, DHEA and Estriol within all experiments.

Estrone significantly down-regulated all target genes except *cyp11c1*, identical to Mety and Mife, but showed moreover a tendency to decrease also the expression of *cyp11c1*. DHEA significantly reduced the same targets as the positive controls, except *cyp3a65*, which remained unchanged. Therewith, the gene expressio pattern of DHEA is identical to that of Nor, suggesting high effect similarity between two putative disruptors of Cort biosynthesis, but in contrast to Nor, DHEA did not reveal elevated Preg levels during chemical analysis. Thus, down-regulation of *cyp3a65* appears to be independent from the compound mechanism on Cort reduction. Estriol also

decreased the expression of all positive control targets, but its effect on *gilz* and *fkbp5* was not significant.

When co-exposed to Dex, trends for induced *gilz* were observed for Estrone and DHEA and a tendency for elevated *cyp3a65* was also visible with DHEA. However, as neither the Dex level was altered, according to UPLC-MS/MS experiments, nor GC signaling was affected, this observation seems to be rather unimportant in the context of the study.

Chapter 4

4. Discussion

In this thesis, I applied the transgenic *Tg(GRE:Luc)*zebrafish line, as well as the AB.9-*GRE:Luc* cell reporter, and modified the GRIZLY assay for the identification of GC signaling inhibitors. I selected and validated control conditions for activation and inhibition of GC signaling (section **3.1.1**) and performed three different variants of the GRIZLY assay in order to detect compound interference with the GC system at different points of action. In GRIZLY 1, I tested for inhibition of directly activated GC signaling *in vitro* in the cell culture system. In GRIZLY 2, inhibition of directly activated GC signaling was examined *in vivo* with *Tg*(*GRE:Luc)* zebrafish larvae, and in GRIZLY 3, inhibition of indirectly activated GC signaling was targeted.

With these assays, I pursued a tiered testing approach (section **3.2**) and screened an FDA approved drug library of 640 established compounds, with the aims 1) to assess the suitability of the GRIZLY assay for the detection of GC signaling inhibitors, 2) to identify disruptors of the GC biosynthesis pathway and3) to identify compounds with - so far - unknown potential to interfere with GC signaling or with unknown mechanisms of action. In tier 1 (section **3.2.1**), I screened the drug library for toxic effects in cells and in larvae (section **3.2.1.1**) and tested for inhibition of GC signaling in the three GRIZLY approaches (section **3.2.1.2**). Interestingly, I identified not only inhibitors, but also superactivators of the *GRE:Luc* reporter. All compounds that significantly modulated (inhibited or superactivated) GC signaling *in vivo* and showed no toxic potential *in vivo* or *in vitro* were carried over to tier 2 (section **3.2.2**), where I retested these candidates concentration-dependently under the same treatment conditions. From the confirmed hits, promising *in vivo* modulators of GC signaling were subjected to tier 3, where I elucidated the drug mechanisms of action in more detail. In tier 3a (section **3.2.3**), I assessed by means of chemical analysis if the substances indirectly inhibited GC signaling, for instance by disruption of Cort biosynthesis. Additionally, in tier 3b (section **3.2.4**), I performed qPCR analysis in order to examine effects of the compounds on a set of target genes.

The tiered approach was a reasonable testing strategy, as it enabled me to identify and validate GC signaling disruptors from a library of substances by the combined testing in three different GRIZLY approaches. Furthermore, the follow-up studies in tier 3 helped me to narrow down the mechanisms of action possibly underlying the compound effects and gave me ideas which future experiments will be necessary to characterize their mechanisms in detail.

In the following sections, I will first discuss the toxicity assessment results from tier 1 (section **4.1**) before I summarize the suitability of the GRIZLY assay for my intention to identify inhibitors of GC signaling (section **4.2**). Then I will focus on the compound effects in the GRIZLY assay and the follow-up studies. I will survey first several general (section **4.3**) and more specific (section

4.4) mechanisms of GC signaling disruption, which may have played a role within the effect outcomes of the identified substances. Based on the results of the tiered testing strategy (section **4.5**), and according to the available literature, I will then discuss the identified GC signaling disruptors and their mechanisms of action in detail (section **4.6 - 4.11**).

4.1 Differences in compound toxicity between the three testing approaches

Prior to the evaluation of the screening results for the detection of GC signaling inhibitors, I assessed the toxicity of the library compounds for each of the three GRIZLY testing approaches in order to distinguish GRIZLY signal inhibition between general compound toxicity and specific effects. As an outcome, several compounds showed toxicity in two or three testing setups, but also a number of candidates were considered toxic in only one single assay (as it was shown in **Figure 24**). I will discuss possible reasons for these discrepancies in the next sections.

4.1.1 Stringency of toxicity assessment

The disparity that I found a considerably lower number of toxic compounds *in vitro* than *in vivo* possibly relies on the different rigor by which toxicity was defined. *In vitro*, only statistically significant data from the cytotoxicity screen were considered, with a stringency of $p < \alpha < 0.0001$. The *in vivo* screens, in contrast, were assessed more conservatively, as a compound was considered as potentially toxic when 10% of all larvae replicates were immotile or dead. Although I intended a high stringency in order to not include potential false-positive inhibitors in the following data analysis and testing procedures, some compounds thereby might have been false-positively determined as toxic.

4.1.2 Increased *in vitro* toxicity

In addition to the different stringency of toxicity assessment, which was probably the major reason for the different outcomes of the toxicity assessment in larvae and cells, also other mechanisms may have contributed to the observed discrepancies of toxicity between the three assays. For instance, disparities between *in vitro* and *in vivo* drug uptake could have played a role. While zebrafish fibroblast-like AB.9-*GRE:Luc* cells were directly exposed to the chemicals in their culture medium, for larvae, the bioaccessibility of compounds may be lower and substance-specific, depending strongly on the particular chemical properties which control the potency to pass the skin barrier. Moreover, 5 dpf old larvae not only absorb chemicals via the skin and perhaps via the developing gills, they also start already to ingest substrates orally, which may allow for a certain amount of drugs to pass the intestinal tract. This furthermore increases the role of compound-specific bioavailability for the extent of pharmacological effects in larvae upon uptake. *In vivo*, the portion of a drug which reaches the systematic circulation is usually considerably reduced during the first-pass effect, where the liver metabolizes a big part of the incorporated amount (e.g. by the CYP enzymes), which might even lead to complete inactivation of an active compound. Accordingly, a decreased drug bioaccessability and bioavailability, possibly involving reduced compound activity and toxicity, might be suspected for the larvae

when compared to the *in vitro* exposure, where these whole-organism abilities for drug biotransformation and translocalization are lacking. This context could explain why seven identified compounds were only toxic *in vitro* but not *in vivo*.

4.1.3 Increased *in vivo* toxicity

On the other hand, a chemical´s bioavailability can also be increased by the organism. Numerous pharmaceuticals are orally inactive until they are transformed by liver enzymes into active metabolites. Also, drugs are often designed to be transported to and to function in a specific target organ or tissue. In the zebrafish larvae, several compounds could therefore have been activated metabolically and translocated to their pharmacological targets, which might then have led to toxic effects *in vivo*. For some of the 128 compounds, which were only toxic in the *in vivo* screens, this might have been the case.

4.1.4 Xenometabolism

Also the compound co-exposure with different activators, either Dex or Preg, can influence the pharmacological effect of the substance. As already described in **1.3.1.3**, Dex and Preg can stimulate the xenobiotic metabolism and differentially induce the expression of several CYP enzymes (Gentile et al. 1996; Martignoni et al. 2004; Petkam, Renaud, and Leatherland 2003; Zanger and Schwab 2013). Especially the results from chemical analysis (section **3.5.1**), which showed that the retrieved amount of Preg (31.33%) from larvae extracts was over three orders of magnitude higher than that of Dex (0.02%), suggest that an unequal amount of activator was taken up by the larvae. As a consequence, the larvae showed probably a different xenometabolic activity with the library compound, depending on the co-exposed activator. This consideration is also supported by the qPCR results, as Dex induced the expression of the two xenometabolic marker genes *cyp3a65* and *cyp1a1* about two-fold, while Preg triggered a five-fold induction of *cyp3a65* and a four-fold increase of *cyp1a1* expression (DMSO control compared to the negative control: Dex/ Preg + DMSO; **Figure 44 e** and **f**). Accordingly, this different xenometabolic activity may have contributed to compound toxicity in one, but not in the other *in vivo* GRIZLY assay. An increased activity of xenometabolic enzymes, for instance of members of the CYP1, CYP2 and CYP3 families, can lead to higher toxicant removal rates and reduced blood concentrations, not only of Dex or Preg, but also of the co-treated substance, or could also stimulate the production of toxic metabolites, depending on the pharmacological properties of the library compound.

4.1.5 Future steps to investigate compound toxicity

In order to determine the origin of compound toxicity, a systematic effect evaluation of the parent and its metabolites measured in all three GRIZLY approaches would be insightful. Also, a more detailed investigation of the expression and activity of the involved (xeno-) metabolic enzymes could help to follow up on the toxic potential of compounds. However, as it was the aim of this study to identify specific disruptors of GC signaling and to elucidate their mechanisms

of action, the indication of potential toxicity by a compound was sufficient reason to exclude it from subsequent experiments and to focus on the remaining substances.

4.2 GRIZLY assay suitability for the detection of glucocorticoid signaling inhibitors

The first main objective of this study was to assess the suitability of the GRIZLY assay for the detection of GC signaling inhibitors. Therefore, the GRIZLY assay was modified and applied in three different testing approaches to screen a library of 640 FDA approved compounds. After 140 putatively toxic compounds were removed from the screen outcomes, 29 inhibitors and 5 superactivators remained as significant screen hits in tier 1. This demonstrates the assay suitability for the detection of GC signaling inhibitors, even in large compound libraries and in higher throughput applications. Moreover, the GRIZLY assay enables not only to detect inhibitors, but also superactivators of GC signaling, compounds which can apparently not activate the reporter gene by themselves, but increase or elongate the GC signal when co-exposed in combination with a GR agonist. These findings were validated in tier 2. Out of the screen hits, 24 compounds were selected for concentration-dependent retesting in all three GRIZLY assay approaches. With a retrieval rate of 78%, the GRIZLY assay was proven to be a reliable tool to identify disruptors of GC signaling.

4.3 General mechanisms underlying treatment-specific effects

In accordance with the relatively low mutual toxicity found for the compounds among the different testing setups, also the identified disruptors of GC signaling revealed only a moderate overlap between the three GRIZLY assays. After retesting, the correlation increased, due to concentration-dependency of the compound effects, but still, several substances disrupted GC signaling in one, but not in the other GRIZLY assay. Of the mechanisms that I discussed in section **4.1.4**, several may also apply for compound interference with pharmacological targets such as GC signaling.

4.3.1 Drug uptake and metabolism

Drug uptake and -metabolism plays a major role not only for compound toxicity (see section **4.1.4**) but also for specific pharmacological effects. The bioavailability and bioaccessability of a substance can be divergent *in vitro* and *in vivo*, which may affect the activation of metabolic enzymes (such as CYPs) differently (Gentile et al. 1996; Martignoni et al. 2004; Petkam et al. 2003). Also, numerous pharmaceuticals are specifically designed to decrease the transcription and activity of the most abundant members of the CYP1, CYP2 and CYP3 families, which are involved in vertebrate drug and steroid metabolism (Zanger and Schwab 2013). Depending on their chemical properties, drugs can affect the production and the activity of xenobiotic enzymes, which can result in a fast elimination of the substance, but also in disruption of the endogenous hormone homeostasis (Creusot et al. 2015; Hotchkiss et al. 2008).

Ligand activated transcription factors such as PXR, AHR, CAR, VDR and GR can regulate the transcription of CYP genes directly or indirectly interfere with xenometabolic gene expression via receptor cross-talk and are, thus, possible targets for such pharmaceutical actions in humans (Bainy et al. 2013; Creusot et al. 2015; Goodwin et al. 2001; Kliewer et al. 1998; Moore and Kliewer 2000; Pascussi et al. 2000). For instance, the involvement of PXR and AhR in the transcriptional regulation of *cyp3a65* has also been describedin zebrafish (Tseng et al. 2005). Thus, within the present study, changes in *cyp3a65* and *cyp1a1* expression by the compounds may indicate disruption of xenometabolic metabolism, which potentially also affected the endogenous hormone homeostasis. Also an indirect involvement of GR-mediated signaling may be indicated by such an effect.

As a consequence of xenometabolic enzyme expression changes, the first-pass effect, biotransformation (metabolic activation or inactivation) and translocalization, not only of the substance tested, but also of the compound it is co-exposed with, can be altered. Within the present study, the unequal amount of absorbed activator (specified in section **4.1.4**) certainly affected the activity of xenometabolic enzymes differently, which in turn may have altered the metabolism of the drug library compound. *Vice versa*, also the library compound could have altered the xenometabolism activity in larvae, which may have affected co-exposed Dex or Preg differently.

As will be described in more detail in the following sections, drug uptake and -metabolism could have been altered with 10-HC (section **4.7.2.3**) and the progestins (section **4.9.2**). Spiro possibly was metabolically activated *in vivo* (section **4.10.2.3**) and also Estrone, Estriol and DHEA may have been converted *in vivo* into the more potent endogenous estrogen Estriol (section **4.11.2**).

4.3.2 Context specificity

GC effects are highly specific given the physiological and cellular context in which they occur. This is also true for the three GRIZLY approaches. For instance, the different exposure scenarios constitute diverging organismal and cellular environments. Thus, the *in vitro* assay (GRIZLY 1) is carried out in fibroblast-like cells in culture medium, while the *in vivo* assays (GRIZYL 2 and GRIZLY 3) comprise a large number of different tissue contexts with two different types of GC signaling activation (Preg-derived endogenous Cort and external Dex). This, in turn, will affect the biological activities, binding affinities and ant-/agonistic potencies of ligands for a specific receptor. Furthermore, different ligands can induce different conformational changes of the receptors and thereby create ligand-specific interaction surfaces for cofactors or specific DNA binding sequences and their genomic environments (Weikum et al. 2017). This results in ligand-receptor complexes specific for certain target gene regulatory regions. Transcriptional regulation is also dependent on the availability of certain coactivators, corepressors and other transcription factors in the respective contexts (Africander et al. 2011; Hapgood et al. 2004; Heitzer et al. 2007; Inhoffen and Hohlweg 1938; Louw-du Toit et al. 2017; Weikum et al. 2017).

In application of the three GRIZLY assays and the follow-up studies on steroid metabolite and gene expression levels, I scanned with distinct compound treatment combinations a relatively broad range of cellular and physiological contexts. This allows me to detect context-specific

effects, that may have occurred for instance with the progestins (section **4.9.2**), Stano (section **4.10.1.3**), Spiro (section **4.10.2.3**), Estrone, Estriol and DHEA (section **4.11.3**), as it will discussed in more detail in the respective sections.

4.4 Specific mechanisms of glucocorticoid signaling disruption

In addition to these general effects, the compounds may have interfered with one or several specific mechanistic pathways related to the GC system. I will describe a selection of important mechanisms in the following sections. Note that all of these mechanisms are also subject to the general considerations described in the previous chapter; they may therefore have diverse impacts in the different assays.

4.4.1 Pre-receptor mechanisms of glucocorticoid signaling disruption

Certain chemicals can disrupt the GC system without directly interfering with the GR. For instance by targeting specific enzymes that are involved in the production and availability of Cort, a compound can limit or increase the amount of GR ligand and thereby affect GC signaling.

4.4.1.1 Disruption of glucocorticoid biosynthesis

Several endocrine disruptive compounds (EDCs) can prevent the production and release of steroid hormones via the HPA axis (Gore 2010). Also, some chemicals are capable to disrupt the steroid hormone biosynthesis by inhibiting steroidogenic enzymes, for instance 3β-hydroxysteroid dehydrogenase (3β-HSD) or 11β-hydroxylase (Louw-du Toit et al. 2017) (for an overview of the steroid hormone biosynthesis pathway see **Figure 3**).

Beside direct compound interference with these enzymes, also indirect mechanisms of steroidogenic enzyme activity disruption are possible. For example, ligands of the PXR (such as Rifampicin) can alter the adrenal steroid homeostasis via drug - hormone interactions (Ihunnah et al. 2011; Zhai et al. 2007). Activation of the PXR may lead to increased plasma levels of certain steroid hormones (e.g. Coco), which can be accompanied by activation of several steroidogenic enzymes (e.g. CYP11a1, CYP11b2, 11β-hydroxylase, 3β-HSD). This could lead to disruption of the GC system, as it was for instance observed by (Zhai et al. 2007) in mice, where the PXR was genetically and pharmacologically activated.

Within the present study, several compounds may have disrupted the steroid hormone biosynthesis pathway. For retinoids (with Preg; section **4.8.3**), progestins (maybe with Dex; section **4.9.2**), Stano (section **4.10.1.3**), Spiro (section **4.10.2.3**), Estrone, Estriol and DHEA (section **4.11.3**), some indications for their potential to disrupt the biosynthesis of Cort are given, as will be discussed in the respective sections.

4.4.1.2 Cortisol activation and inactivation

Also other enzymes regulating the availability of active and inactive forms of steroid hormones in the blood can be affected by compound treatment, such as 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2). This enzyme oxidizes active Cort (and Corticoin rodents) into the inactive metabolite Cortisone (and 11-dehydrocorticosterone in rodents)(**Figure 3**) and prevents

it from binding to the MR in sodium-transporting epithelia in mineralocorticoid target tissues such as kidney and colon (Leckie et al. 1998; Mune et al. 1995; Ferrari 2010; White et al. 1997; Louw-du Toit et al. 2017; Sanderson 2006; Alderman & Vijayan 2012). *In vitro*, the MR can be activated by both Cort and the natural mineralocorticoid Aldosterone, with equal affinity. In humans and vertebrates, whose major active GC is Cort (and not Cortico, as in rodents), Cort circulates at 100-1000-fold higher concentrations than Aldosterone (MILLER 1988). Thus, Cort would out-compete Aldosterone and activate the MR, if it were not for 11β-HSD2 (Leckie et al. 1998; Mune et al. 1995; Ferrari 2010; White et al. 1997).
Inactivation of 11β-HSD2 via enzyme inhibition (e.g. by licorice) or by mutation of the *hsd11b2* gene (in patients suffering from apparent mineralocorticoid excess (AME)) results in Cort-induced mineralocorticoid effects such as sodium retention, hypokalaemia or salt-dependent hypertension (Ferrari 2010; Omar et al. 2012; Stewart et al. 1996). Human patients with deficient 11β-HSD2 activity usually exhibit increased ratios of Cort to Cortisone and a prolonged Cort half-life (Ferrari 2010).
In this thesis work, UPLC-MS/MS data did not indicate an increase in endogenous Cort levels for any compound. However, according to literature, Spiro (section **4.10.2.3**) and the estrogens (section **4.11.1**) are likely to interfere 11β HSD2, which may to some extend have contributed to their effects on diverse levels, as discussed in detail in the specific paragraphs.

4.4.2 Direct interference with GR signaling

Endocrine disruptive chemicals (EDCs)can also bind to steroid hormone receptors and alter their transcriptional function on target gene expression. The receptor activity can be agonized or antagonized by EDCs via transactivation, transrepression, tethering (section **1.2.4**) or other mechanisms and, thus, all types of mechanisms involved in GR-mediated regulation of transcription may possibly be targeted.

4.4.2.1 Ligand-receptor binding affinity and nuclear localization

Pharmaceuticals can potentially alter the binding affinity of the GR for the ligand (e.g. via conformational changes within the LBD of the GR) and reduce the localization of the receptor into the nucleus. For instance, as described in section **1.3.1.1**, the immunosuppressive-drug-binding proteins FKBP51 and FKBP52 are known regulators of the GR ligand-receptor binding affinity and nuclear localization. Immunosuppressive ligands (e.g. synthetic GCs) can substitute the negative regulator FKBP51 (*fkbp5*) for the positive regulator FKBP52 within the receptor-protein heterocomplex, which induces the nuclear localization of the complex and/or elevates the intranuclear ligand concentration by inhibiting the steroid export proteins and, thus, increases GR activity (Davies et al. 2002, 2005; Mazaira et al. 2015; Pereira et al. 2014). Interestingly, in adipose tissue, increased expression of *fkbp5* has been observed in response to Dex treatment, which probably serves to prevent excessive GC signaling by inhibiting GC-GR binding (Pereira et al. 2014). Such inhibitory mechanisms may constitute first indications for GC resistance. Similarly, in New World squirrel monkeys, which have high levels of circulating Cort

but show no indication of hypercorticism, it has been found that FKBP51 levels are increased, which resulted in a low ligand binding affinity of the GR (Denny et al. 2000; Storer et al. 2011). Likewise, within the present study, Preg strongly induced the expression of *fkbp5* (**Figure 44.II a**). Most inhibitors of Preg-activated signaling (e.g. Trani, 10-HC, Gestri) showed reduced *fkbp5* mRNA levels, indicating a decreased GR signaling activity, while superactivators of Preg-induced GC signaling such as Stano (section **4.10.1.3**) and Spiro (section **4.10.2.2**), showed no reduced *fkbp5* expression, although the mRNA levels of all other Preg target genes were decreased. This may suggest an increased GR activity with these compounds.

4.4.2.2 GR activity modulation via posttranslational modifications

The activity of the GR can also be modulated via posttranslational modifications (PTMs) in response to external stimuli. PTMs can enhance or decrease protein functionality by regulating stability, structure, activity and intracellular localization of the receptor and its interaction with other proteins (Liberman, Antunica-Noguerol, and Arzt 2014; Scheschowitsch et al. 2017). Examples for PTMs include phosphorylation, acetylation, ubiquitination, methylation, nitration, nitrosylation and SUMOylation (Anbalagan et al. 2012; Duma, Jewell, and Cidlowski 2006; Liberman et al. 2014).
For compounds where direct interactions with GR activity are suggested in the present study, PTMs probably played important roles in numerous effects. Although it is hard to tell which of this study´s inhibitors interfered with GR signaling via PTMs, there is evidence in the literature for some of them (e.g. estrogens, see below, section **4.11.1**).

Receptor phosphorylation

One important mechanism of PMT is receptor phosphorylation. As the GR is a phosphoprotein, phosphorylation modulates its functionality (Liberman et al. 2014; Wallace and Cidlowski 2001). The phosphorylation occurs generally within the DBD, where kinases such as mitogen-activated protein kinases (MAPKs) or cyclin-dependent kinases (CDKs) can modify the phosphorylation status ligand-dependently (Galliher-Beckley and Cidlowski 2009; Miller et al. 2005). For instance, cytokines (e.g. IL-2 and IL-4) can induce GR phosphorylation via the p38 MAPK pathway, which reduces the nuclear import of the GR and leads to GC resistance during chronic inflammation (Irusen et al. 2002). Furthermore, several MAPK proteins involved in nucleocytoplasmic GR shuttling can enhance the nuclear export of the GR and, thus, reduce the transcriptional efficiency and induce the hormone-dependent downregulation of the GR (Liu &DeFranco 2000).
Generally, agonistic effects of GCs are accompanied by hyper-phosphorylation of the GR upon ligand binding, which induces a conformational change of the receptor that enables coactivator binding and which also plays an important role in the receptor protein turnover (Wallace and Cidlowski 2001). Antagonists, in contrast, do not induce hyper-phosphorylation but trigger conformational changes which enable corepressor recruitment (Africander et al. 2011; Nettles and Greene 2005; Schaaf and Cidlowski 2002). The phosphorylation state of the GR influences also the receptor stability, as well as its selectivity and sensitivity to GCs for downstream activity (Galliher-Beckley and Cidlowski 2009; Webster et al. 2001).

Within the present study, the GR agonists Dex and Cort certainly induced hyper-phosphorylation of the GR, but also other compounds could have had (at least partial) agonistic properties for the receptor (e.g. the progestins (section **4.9**), Stano (section **4.10.1**) or Spiro (section **4.10.2**) and accordingly may have interfered with its phosphorylation state.

4.4.2.3 GR beta as a dominant negative regulator of GR alpha

The GR exists in various isoforms, of which GRα and GRβ have been most studied. Of these two, only GRα, the major isoform, possesses transcriptional activity, while it is commonly accepted that GRβ is not ligand-inducible and functions as a dominant negative regulator of GRα (Oakley, Sar, and Cidlowski 1996). GRβ can suppress the transcriptional activity of GRα, for instance by altering the receptor dephosphorylation status (Webster& Cidlowski 1994; Oakley & Cidlowski 1993).
Within this study, for instance the anti-inflammatories (section **4.6.3**) may have altered the expression of GRα and GRβ and thereby decreased the activity of GRα, as it will be described below in detail.

4.4.2.4 Ligand-dependent homologous downregulation

The activity of the GRα isoform can be altered by hormone-dependent homologous downregulation via autoregulatory transrepression of the GR gene (Oakley and Cidlowski 1993; Shimojo et al. 1995). As described above (in section **4.4.2. 3**), GRβ cannot bind ligands by itself, it is thus not susceptible to ligand-dependent homologous downregulation and accumulates instead (Oakley and Cidlowski 1993), which may in turn increase the impact of this dominant-negative regulator on GRα. This mechanism often underlies GC resistance in response to long-term medication of chronic inflammations with synthetic GCs (Corrigan et al. 1991; Okret et al. 1986; Ramamoorthy and Cidlowski 2013; Rosewicz et al. 1988; van Rossum and Lamberts 2006; Schaaf and Cidlowski 2002).

Within the present study, ligand-dependent homologous downregulation probably underlies the normally observable signal decrease of the *GRE:Luc* reporter after several hours of constant GC treatment. Upon activation, the *GRE:Luc* reporter lines respond with a strong signal increase, before a decline in bioluminescence activity is observed, which normally sets in after 4h of Dex treatment in cells (e.g. **Figure 29 a**, blue line) and after 10 - 14 h of exposure to Dex (e.g. **Figure 29 e**, blue line) or Preg (e.g. **Figure 29 f**, blue line) in larvae. This decrease may arise from GRα inactivation or decreases in GR protein levels.
Certain GC signaling disruptors may have interfered with the homologous downregulation of GRα which is considered "normal" in GRIZLY experiments after continuous exposure to GCs for several hours. For instance, Stano (section **4.10.1.3**) may have prevented the downregulation, while the anti-inflammatories (section **4.6.3**), Estrone, Estriol and DHEA (section **4.11.1**) potentially induced this process.

4.4.2.5 Receptor interaction with other signaling pathways

Also other transcription factors can interfere with GR signaling. For instance, steroid hormone receptor crosstalk between the GR and the ER has been shown in human breast-cancer cell lines, where estrogen agonists provoked GR degradation and inhibited GR target gene expression (Kinyamu & Archer 2003). Crosstalk between the GR and diverse other transcription factors has been reviewed by Kassel & Herrlich (2007).

Especially interactions between the GR and proinflammatory transcription factors such as AP-1 and NF-κB have been the focus of numerous studies. For example, NF-κB has been shown to induce the expression of GRα and GRβ (Webster et al. 2001). Also mutual interactions between NF-κB and GR signaling have been reported (Auphan et al. 1995; Scheinman et al. 1995) and it was observed that GC treatment repressed the transcriptional activity of NF-κB (Bamberger et al. 1995; Webster and Cidlowski 1999). Also interactions between GC and retinoic acid signaling have been extensively described (Aubry and Odermatt 2009; Bonhomme et al. 2014; Brossaud et al. 2013; Lefebvre et al. 1999; Tóth et al. 2011; Yamaguchi et al. 1999).

Several compounds from the FDA library may have interfered with GR signaling via receptor crosstalk or via interactions with other transcription factors. For instance, for the anti-inflammatories (section **4.6.3**), retinoids (section **4.8.3**) and progestins (section **4.9.2**), such interactions have been described.

4.4.2.6 Coactivator and corepressor recruitment

Upon binding, ligand-specific recruitment of coactivators, such as the steroid receptor coactivator-1 (SRC-1), and corepressors, like the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), affects the transcriptional efficiency of the GR and the GC signaling response. The ratio of coactivators and corepressors within the promotor region regulates target gene transcription or transrepression (Heitzer et al. 2007). For instance, overexpression of the corepressor SMRT can counteract coactivator effects on GR-mediated target gene transcription (Szapary et al. 1999).

In this study, two retinoids (section **4.8.3**) were identified to inhibit GC signaling. Possibly, as they have been described to repress AhR-mediated induction of *cyp1a1* through the SMRT corepressor, they may also have increased the amount of SMRT within the promotor environment of the 4 x GRE construct, which potentially contributed to the observed GRIZLY signal inhibition.

4.5 Assessment of glucocorticoid signaling disruption by means of the tiered testing approach

As a first step, the combined compound analysis of the GRIZLY assay allowed me to identify compounds with either similar or diverse *in vitro* and *in vivo* effects. Furthermore, I was able to pre-categorize potential disruptors of the Cort biosynthesis pathway, as several substances inhibited Preg- (GRIZLY 3), but not Dex- (GRIZLY 2) induced GC signaling, such as Leflu, Gestri, Nor, Estrone, DHEA and Estriol).

Chemical analysis helped me to confirm that these compounds (all except Leflu) indeed significantly reduced the amount of Preg-induced Cort production in larvae. Interestingly, some additional substances (Aci, Ret. acid, Ethi, Stano and Spiro), where the GRIZLY results gave no clear indication for a disrupted Cort biosynthesis, were shown via UPLC-MS/MS to decrease Cort levels in larvae. Also effects on drug uptake and metabolism could be detected with this technique for several compounds.

Via qPCR, I analyzed the expression of a set of target genes in larvae co-treated with Dex/ Preg + the compounds. The two markers *cyp1a1* and *cyp3a65* should give further indications for xenometabolic disruption by the compounds. The mRNA levels of *cyp11c1*, encoding the Cort-producing enzyme steroid 11β-hydroxylase and *hsd11b2*, the transcript of the Cort-metabolizing enzyme hydroxysteroid 11-β dehydrogenase 2, were measured in order to monitor changes of the Cort availability. Two GC target genes, *gilz* and *fkbp5*, were selected to detect changes in Preg- and Dex-induced GC signaling. The transcriptional induction of *gilz* is closely related to anti-inflammatory mechanisms of action while upregulation of *fkbp5* (as described in section **4.4.2.1**) indicates an increased GR activity. With the combined assessment of these markers, I aimed to assess compound effects on three major mechanisms of GC signaling disruption, direct interference with GR signaling, inhibition of GC biosynthesis and disruption of GC homeostasis via drug metabolism.

In the following sections I will discuss the identified compounds in groups according to their functionalities. Consulting the available literature, the biological functions and the major mechanisms of action of the substances will be described, with regard to potential interfaces with the GC system. Then I will summarize the compound effect profiles from the present study and interpret them within the context of evidences and hints provided by literature.I will survey their potential to interfere with the GC system via diverse mechanisms and outline perspectives for future investigations in order to validate putative mechanisms of GC signaling disruption for each functional group.

4.6 Anti-inflammatories

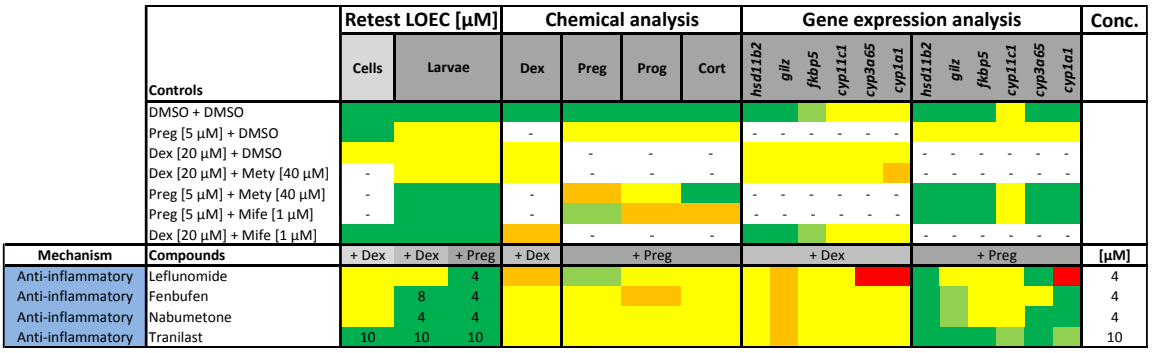

Figure 46: Anti-inflammatories (extract from Figure 45)

Leflu, Fen, Nabu and Trani are substances with anti-inflammatory properties. Although they are partly associated to different compound classes with diverse pharmacological targets, they have in common certain major anti-inflammatory mechanisms of action. In the following paragraphs, I

will first characterize the compound- and class-specific properties before I specify their respective anti-inflammatory pathways for the discussion of putative mechanisms of GC signaling disruption.

4.6.1 The biological functions of anti-inflammatories and their mechanisms of action

Leflunomide

Leflu is an immunosuppressive, disease-modifying, antirheumatic drug (DMARD) (Singh et al. 2012). Upon uptake, Leflu is converted via first-pass metabolism within colon and liver into its active metabolite teriflunomid (A771726) (O'Connor et al. 2006). Mechanistically, this DMARD is a pyrimidine biosynthesis inhibitor that has been approved for the treatment of immune-diseases such as rheumatoid arthritis and multiple sclerosis (Cherwinski et al. 1995; O'Connor et al. 2006).

1) Pyrimidine biosynthesis inhibition leads to immunosuppression

Leflu prevents the biosynthesis of pyrimidine via inhibition of the enzyme dihydroorotate dehydrogenase (Davis et al. 1996). Although the immunosuppressive effects of the compound are not yet completely understood, they are believed to result from the depletion of pyrimidine supply, which inhibits the reproduction of fast-dividing cells, such as lymphocytes (Breedveld and Dayer 2000; Rückemann et al. 1998). However, also other mechanisms have been described for Leflu, including disruption of interleukin (IL)-mediated pathways (Miceli-Richard and Dougados 2003; Munier-Lehmann et al. 2013; O'Donnell et al. 2010) and inhibition of prostaglandin production (Burger et al. 2003).

2) Pyrimidine biosynthesis disruption disables TNF-mediated activation of NF-κB and AP-1

Manna et al. (2000) demonstrated that Leflu also inhibits TNF-induced activation of NF-κB and AP-1. For instance, the anti-inflammatory inhibited the activation of I-κBα kinase (IKK) and suppressed the degradation of I-κBα, the inhibitory subunit of NF-κB. Interestingly, the researchers found out that the repressing effects of Leflu on TNF signaling could be rescued by uridine, which led them to conclude that the biosynthesis of pyrimidine plays a crucial role in TNF-mediated signaling.

3) AhR agonism

Moreover, Leflu is a known agonist of the AhR (Miceli-Richard and Dougados 2003; O'Donnell et al. 2010). In one case study where O'Donnell and collegues (2010) investigated the mechanisms of action of this drug, Leflu, but not the active metabolite teriflunomid, increased the expression of endogenous AhR target- and reporter genes via the AhR. For instance, they observed in zebrafish that Leflu (but not teriflunomid) was able to inhibit *in vivo* tissue regeneration AhR-dependently.

Fenbufen and Nabumetone

Fen and Nabu are non-steroidal, anti-inflammatory drugs (NSAIDs) (Huerta et al. 2005). Fen is a member of the class of propionic acid derivatives, which includes also Ibuprofen and Ketoprofen (Chiba et al. 1985). Fen and one of its major metabolites, 4-biphenylacetic acid (BPAA), have been shown to be potent *in vitro* and *in vivo* inhibitors of prostaglandin synthesis in a variety of tissues (Palmer et al. 1990; Sloboda et al. 1980).

Nabu belongs to the class of acetic acid derivatives, with Diclofenac being another prominent representative of this pharmacological group (Boyle et al. 1982). Nabu itself is non-acidic, but it is rapidly metabolized in the liver into its principal active metabolite 6-methoxy-2-naphthyl acetic acid. This metabolitewith its carboxylic acid group is primarily responsible for the therapeutic effect of Nabu, which is the reason why the anti-inflammatory is classified as a derivative of acetic acid (Haddock et al. 1984).

Inhibition of cyclooxygenase enzymes leads to disruption of prostaglandin synthesis

NSAIDs belong to the most commonly used drugs in the world (Green 2001). Most NSAIDs exert their anti-inflammatory properties by inhibiting cyclooxygenases (COXs), enzymes that are involved in the synthesis of prostaglandins (Smith et al. 2017). The COX enzymes are conserved among all vertebrate groups and two isoenzymes have been characterized in numerous species, including human and fish (Corcoran et al. 2010; Havird et al. 2008; Ishikawa and Herschman 2007; Zou et al. 1999). COX-1 regulates prostaglandin baseline levels, while COX-2 needs to be stimulated by growth factors and pro-inflammatory cytokines in order to produce prostaglandins at inflammation sites. The majority of NSAIDs act on both COX isoforms and, thus, broadly inhibit prostaglandin synthesis (Fent et al. 2006; Lister and Van Der Kraak 2008; Mustafa and Srivastava 1989; Vane 1994).

Tranilast

Trani is a derivative of the amino acid tryptophan which has been used predominantly in Japan and South Korea to treat inflammatory diseases due to its anti-allergic properties (Xia et al. 2017). The compound is also used as a medication for several other diseases including autoimmune disorders, renal fibrosis, proliferative disorders, cardiovascular problems and cancer (Darakhshan and Pour 2015).

Inhibition of multiple pro-inflammatory mediators

Trani inhibits the production and release of many inflammatory mediators including cytokines and chemokines and thereby also suppresses the activation of NF-κB (Darakhshan and Pour 2015). For instance, the compound reduces production and activity of pro-inflammatory cytokines such as TNF-α, IL-1 β and transforming growth factor (TGF)-β 1 in different cell types (Yamada et al. 1994; Xia et al. 2017; Martin et al. 2005; Pae et al. 2008). Furthermore, Trani was described to inhibit the release of prostaglandins and histamines from mast cells (Yamada et al. 1994). Also, in a study by Pae et al. (2008), the drug has been shown to downregulate the expression of COX-2 to decrease thereby levels of COX-2-derived PGE2 in macrophages.

Common anti-inflammatory mechanisms of the four compounds

In summary, although Leflu, Fen, Nabu and Trani originate from different chemical groups, they exert their anti-inflammatory properties via partly overlapping mechanisms. Together with natural and synthetic GCs, which also possess anti-inflammatory capacities (see section **1.2.5**), the four compounds inhibit inflammatory processes via common biological and mechanistic targets.

1) Inhibition of prostaglandin production

All four anti-inflammatories are potential inhibitors of prostaglandin production. Also GCs can suppress the transcription of COX-2 via GR-mediated transrepression (e.g. via tethering to NF-κB) and thereby prevent the synthesis of prostaglandins (Anacker et al. 2011; De Bosscher &Haegeman 2009). Prostaglandins can be found in almost every vertebrate tissue and they contribute to numerous physiological processes such as regulation of inflammation, reproduction, calcium transport, cell growth and hormone production. Accordingly, inhibition of prostaglandins by drugs with anti-inflammatory properties may potentially affect any of these functions.

2) Suppression of the NF-κB pathway

Prostaglandins can furthermore induce the transcriptional activation of NF-κB (Camandola et al. 1996). Accordingly, inhibition of prostaglandin production might lead to reduced NF-κB signaling. Beside this hypothesis of an indirect inhibition of NF-κB via disruption of the prostaglandin synthesis, all four anti-inflammatory compounds, as well as GCs, can directly suppress the activation of the NF-κB pathway. Exemplarily, for the NSAIDs Aspirin and Sodium salicylate, it was described that inhibition of NF-κB activity may be involved in their anti-inflammatory and growth inhibitory properties. Also for GCs, the direct interaction of the GR with NF-κB is one of their multiple anti-inflammatory mechanisms of action (as described in section **1.2.4**). In addition, GCs are able to upregulate I-κB, the inhibitory subunit of NF-κB, which suppresses the activation of NF-κB (Cuzzocrea et al. 2007; de Lima Souza et al. 2012).

4.6.2 The effects of anti-inflammatories within the present study

Leflunomide

In the GRIZLY assay, Leflu only inhibited Preg-induced GC signaling in larvae. A clear concentration-dependency was shown by retesting, where a LOEC of 0.5 µM was found for the compound (see appendix, **Supplementary Figure III**). Due to the effect of Leflu to inhibit Preg- but not Dex-induced GC signaling *in vivo*, the compound was considered a potential disruptor of Cort biosynthesis. However, chemical analysis gave no indications for changes in endogenous Cort levels, although the larvae-internal amount of Preg tended to be decreased compared to the control. This may indicate that the uptake of Preg from the medium was hampered in the presence of Leflu, but the strong signal reduction observed in GRIZLY 3 is unlikely to rely on this, as otherwise the Cort level would have to be reduced. Some other mechanisms must have played a role for the inhibition in GRIZLY 3.

For Leflu + Dex treatment, the internal amount of Dex in the larvae showed a trend to be increased, which is the opposite of the compound effect on Preg. Therefore, it was suspected that Leflu may have affected the uptake of Dex and Preg into the larvae differently, for instance by interfering with xenometabolic pathways. As the elevated Dex level did not significantly alter GC signaling in GRIZLY 2, although a tendency for a signal increase was visible (as shown in **Figure 37 c**), it may be suspected that the accumulation of Dex did not suffice to increase the effect on the reporter construct. Alternatively, Leflu may have had also an inhibitory effect on Dex-induced GC signaling, similar to the impact on Preg activation, but it could have been counteracted by the increased amount of Dex in the larvae.

In qPCR experiments, Leflu showed heterogenous effects. Under Dex stimulation, the two xenometabolic marker genes were induced significantly and *gilz* was upregulated by trend. These results may be related to the elevated Dex level in the larvae. With Preg, *hsd11b2* and *cyp3a65* were significantly decreased, which could as well have to do with the lower amount of Preg measured in larvae homogenates. Interestingly, *cyp1a1* was also strongly increased with Preg (ratio 8.3), although not statistically significant. Certainly, several general (section **4.3**) or specific (section **4.4**) mechanisms of GC signaling disruption contributed to these gene-specific effects. Only the strong increase of *cyp1a1* in co-treatment with both activators is striking, as Leflu was the only compound leading to an induction of this marker.

This effect most likely relies on the AhR agonistic property of Leflu that was described above (Miceli-Richard and Dougados 2003; O'Donnell et al. 2010). Although *cyp1a1* can be regulated by the GR, it is predominantly a major target of the AhR. Thus, the expression of *cyp1a1* was probably induced by Leflu via Ahr agonism and was maybe largely independent from the transcriptional activity of the GR.

Fenbufen and Nabumetone

The two NSAIDs showed very similar effects in all experiments. They both inhibited Dex- and Preg induced GC signaling in the GRIZLY assay *in vivo* but did not alter the bioluminescent signal *in vitro*. The lacking *in vitro* effect may reflect a need for compound metabolization and that the signal inhibition *in vivo* was triggered by metabolites of the two substances. For instance, BPAA and 6-methoxy-2-naphthyl acetic acid are metabolites of Fen and Nabu, respectively, for which therapeutic activities are known (Haddock et al. 1984; Palmer et al. 1990; Sloboda et al. 1980).While a clear concentration-dependency was visible for Fen in GRIZLY 2 and GRIZLY 3 (**Figure 35 g + h**) within the tested range of 4 - 20 µM, Nabu showed strong signal inhibition at all tested concentrations (**Figure 35 c + d**). Retesting with lower concentrations of Nabu [0.25 - 4 µM] confirmed a concentration-dependency for the inhibitory effect of the substance and LOECs of 2 µM and 0.25 µM could be determined for Nabu + Dex and Nabu + Preg, respectively. The data from these experiments are shown in the appendix as **Supplementary Figure I**.

As the two anti-inflammatories inhibited in both *in vivo* assays, a disruption of Cort biosynthesis was deemed unlikely to underlie this effect. This was confirmed by chemical analysis, as the

measured steroid levels neither gave indications for inhibition of Cort production, nor did they suggest alterations in drug uptake upon treatment with Fen and Nabu.
On the level of gene expression, both compounds showed a tendency to further increase *gilz* expression in co-exposure with Dex, while trends for reduced mRNA levels of this target were observed co-treatment with Preg. In addition to that, both NSAIDs decreased the Preg-induced expression of *hsd11b2* and *cyp1a1*, and Nabu also down-regulated *cyp3a65*. As the two anti-inflammatories disrupted the transcriptional regulation of several, but not all GR target genes, certain general (section **4.3**) or specific (section **4.4**) mechanisms of GC signaling disruption gene may be at work gene-selectively, similar to what was observed for Leflu.

Tranilast
Trani inhibited GC signaling in all three GRIZLY assays but showed no effect on any steroid measured by UPLC-MS/MS. For target gene expression, a trend for increased *gilz* induction was observed with Dex, while the Preg-induced expression of all targets was decreased with Trani. Thus, the anti-inflammatory showed the same gene expression pattern as Fen and Nabu when co-treated with Dex, indicating similarities in the gene-selective regulation by the three compounds.
In co-exposure with Preg, Trani had a similar effect on target gene expression as Mife and Mety, but in contrast to the positive controls, the substance showed also a trend to decrease *cyp11c1* expression, which might imply an additional specific effects of this compound. The striking discrepancy between the rather low effect of Trani on Dex-induced, but the strong impact on Preg-induced target genes strongly suggests treatment-dependent, general (section **4.3**) or specific (section **4.4**) mechanisms of GC signaling disruption.

4.6.3 Potential mechanisms of glucocorticoid signaling disruption by antiinflammatories

Leflu, Fen, Nabu, Trani, Dex and Cort inhibit pro-inflammatory processes via common biological and mechanistic targets. Therefore, from a combined treatment with two immunosuppressive substances, additive effects, addressing common immunomodulatory targets such as inducing the same anti-inflammatory gene or suppressing a pro-inflammatory pathway, could be anticipated. However, the effects of the four anti-inflammatories on GC signaling were not additive, as all four compounds showed inhibition in the GRIZLY assay. Based on common effect, it may still be suspected that similar mechanisms underlie the results. Accordingly, it appeared reasonable to me to discuss the major common mechanisms of the compounds, suppression of the prostaglandin production and inhibition of NF-κB signaling, for the potency to interfere with GC signaling.

Interference with GR signaling
1) Suppression of the prostaglandin production may decrease GR levels
Prostaglandins can increase the expression of GRs. For instance, one research group found significantly reduced GR levels when they incubated synovial fibroblast cells with NSAIDs.

Interestingly, this effect could be reversed by prostaglandin treatment, as prostaglandin E2 (PGE2), and also a synthetic analog of prostaglandin E1, Misoprostol, increased the GR levels in human chondrocytes (DiBattista et al. 1991; Pelletier et al. 1994). Thereby, a reciprocal regulation of prostaglandin synthesis and GR signaling is strongly suggested. Potentially, in the present study, the anti-inflammatories decreased the GR levels by inhibiting the prostaglandin production and interfered via this mechanism with the GC system.

2) Suppression of the NF-κB pathway may affect GR signaling due to mutual antagonism

Although the suppression of the NF-κB pathway is a common anti-inflammatory target of Leflu, Fen, Nabu, Trani, Cort and Dex, which not necessarily affects GC signaling, it may indirectly disrupt the GC system. Several proinflammatory transcription factors, such as NF-κB (as mentioned in section **4.4.2.5**) or AP-1, can interact with the GR and may, thus, disrupt GC signaling (Dejager et al. 2014; Webster et al. 2001). For instance, NF-κB and the GR are known to have antagonistic effects on target gene transcription (De Bosscher, Vanden Berghe, and Haegeman 2006; McKay and Cidlowski 1998, 2000). Related to this, Webster et al. (2001) investigated whether the capability of NF-κB to repress GRα activity via tethering may rely on NF-κB-mediated alterations in GRα and GRβ expression. They showed that two different activators of NF-κB (the proinflammatory cytokines TNF-α and IL-1) increased the mRNA and protein levels of both GRα and GRβ in human cells of epithelial (HeLaS3) and lymphoid (CEMC7) origin. Interestingly, under all treatment conditions, GRβ expression increased to a greater extent than GRα. In this context, they furthermore showed that upon GC (Dex) treatment, GRα, but not GRβ, underwent ligand-dependent homologous downregulation (section **4.4.2.4**), which aggravated the proinflammatory effect.

Disruption of cortisol biosynthesis

Inhibition of prostaglandin production can disrupt the biosynthesis of cortisol

Prostaglandins are also involved in the production of Cort. For instance, it has been shown for NSAIDs that they can disrupt Cort biosynthesis in fish (Gravel and Vijayan 2006; Mommsen, Vijayan, and Moon 1999; Wendelaar Bonga 1997). Accordingly, inhibition of Cort synthesis is a mechanism of GC signaling disruption which may accompany prostaglandin-inhibiting, anti-inflammatory drugs. However, in the present study, the Preg-induced endogenous Cort levels appeared unchanged according to chemical analysis, making it unlikely that this mechanism has contributed to the observed GC signaling disruption by the compounds. However, I cannot exclude that the compounds could still affect basal Cort levels in untreated larvae, which were not measured within this thesis work.

Suppression of pro-inflammatory mediators may reduce the production of cortisol

Also, upon inflammation, pro-inflammatory mediators (cytokines TNF-α, IL-1 β and transforming growth factor (TGF)-β 1) normally induce the production of Cort via the HPA axis (Adcock et al. 2004; Heitzer et al. 2007; Schaaf and Cidlowski 2002; Weikum et al. 2017). Thus, the suppression of these mediators by the anti-inflammatories could also have led to reduction of endogenous Cort, but I observed no such effect.

Induced expression of *gilz*

Interestingly, all four compounds overinduced the expression of *gilz* when they were co-treated with Dex. This observation might suggest that *gilz* is a common target of Dex, Cort, Leflu, Fen, Nabu and Trani, and that the four library compounds had a synergistic effect on its expression.

GILZ as an indicator for suppression of the NF-κB pathway

The glucocorticoid-induced leucine zipper (GILZ) protein is a known mediator for the anti-inflammatory effects of GCs and plays, thus, an important role in GC immunomodulation (Ayroldi and Riccardi 2009; Ronchetti et al. 2015; Scheschowitsch et al. 2017). This inhibitory protein suppresses several pro-inflammatory mechanisms, including pathways of NF-κB, AP-1, rapidly accelerated fibrosarcoma 1 (Raf-1) protein and rat sarcoma (Ras) protein, which are all involved in mediating GC effects, and GILZ is also necessary for GC inhibition of cytokine-induced COX-2 expression (Ayroldi and Riccardi 2009; Coutinho and Chapman 2011; Mittelstadt and Ashwell 2001). For instance, GILZ inhibits the activity of NF-κB in T-cells and macrophages by binding to the p65 subunit of this transcription factor. Thereby, it inactivates NF-κB, which leads to impairment of target gene transcription (e.g. of cytokines, chemokines, growth factors) and suppression of inflammation (Ayroldi and Riccardi 2009; Scheschowitsch et al. 2017).

Accordingly, gene expression induction of this target by GCs indicates activity of anti-inflammatory mechanisms, possibly including suppression of the NF-κB pathway. Its increased induction by Dex treatment upon co-exposure with the anti-inflammatories may act synergistically with the other anti-inflammatoryproperties of the compounds.

4.6.4 Next steps

In summary, Leflu, Fen, Nabu and Trani may have affected the production of prostaglandines in larvae and possibly suppressed certain pro-inflammatory pathways, such as NF-κB and AP-1. This hypothesis is supported by the common effect of all compounds on the expression of *gilz*. However, more experiments will be necessary for a detailed investigation of these anti-inflammatory mechanisms. Measurements of COX enzymes, prostaglandin levels and NF-κB activity will certainly help to evaluate such hypotheses.

In order to follow-up on the compound mechanisms to disrupt GC signaling, a direct interference with the GR can be assessed by measuring the GRα/β expression in larvae under the same treatment conditions.

Disruption of the GC biosynthesis by the four compounds was deemed unlikely in the present study as no changes in Preg-induced Cort levels were observed in chemical analysis experiments. The anti-inflammatories may still have decreased basal Cort levels, but this will have to be quantified with a more sensitive analysis equipment.

Beyond this, it will be interesting to assess whether the production of selected pro-inflammatory mediators in zebrafish can be stimulated in larvae via an external stressor and if this will increase the endogenous Cort synthesis. Larval treatment with the anti-inflammatories should then suppress the activation of these mediators and prevent an increased Cort production.

4.7 Glucocorticoid receptor antagonist and anti-neoplastic

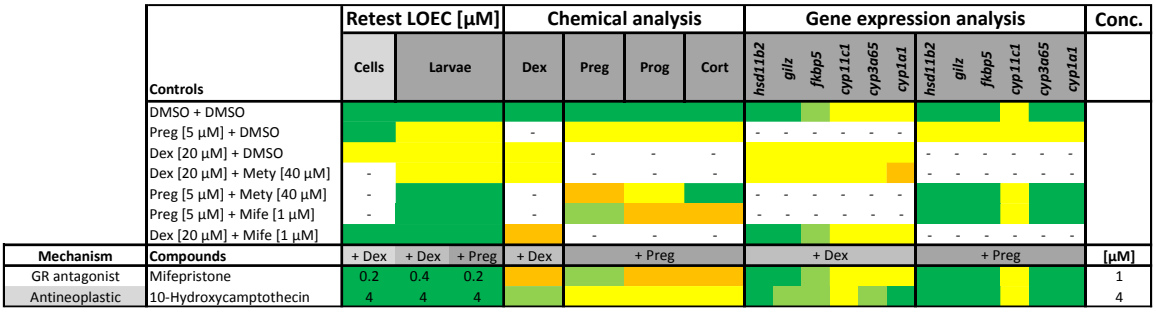

Figure 47: Glucocorticoid receptor antagonist and anti-neoplastic (extract from Figure 45)

This chemical group consists of two compounds with different pharmacological properties. Mife, a GR antagonist, was the reference inhibitor of directly activated GC signaling within the present study and also a compound in the drug library. 10-HC is an antineoplastic drug. However, the two compounds showed very similar effects in GRIZLY and qPCR experiments, which is why I grouped them together in order to discuss potential mechanistic similarities.

4.7.1 Mifepristone

4.7.1.1 The biological function of Mifepristone and its mechanisms of action

Mife (RU 486) is a synthetic 19-norsteroid and a derivative of the progestin norethindrone that was developed in the early 1980s (Healy and Fraser 1985). The compound antagonizes PR, GR and AR via competitive binding and has, thus, strong antiglucocorticoid and antiprogestogenic as well as antiandrogenic properties (Pecci et al. 2009).

Anti-progestin

Due to its antiprogestogenic activity, Mife is majorly used as an abortifacient as it antagonizes the pregnancy-maintaining effects of progesterone, but also finds application for the treatment of endometriosis (von Hertzen et al. 2002; Michael Kettel et al. 1996; Pecci et al. 2009).

Anti-glucocorticoid

In an early study by Gagne et al. (1985), who assessed the potency of Mife to antagonize the GR *in vitro* and *in vivo*, the compound was observed to have a threefold higher affinity for the GR in the cytosol of rat hepatoma tissue culture (HTC) cells than Dex. This high affinity was described by the authors to rely on a very low dissociation rate of the receptor complexes. Also, Mife was a potent full antagonist of dexamethasone-induced enzyme activity of tyrosine aminotransferase (TAT) in whole rat cells. *In vivo*, the compound completely inhibited the Dex-induced activity of hepatic tryptophan oxygenase (TO) in adrenalectomized rats.

Due to its strong GR antagonistic potential, Mife is frequently applied as an established reference substance for the assessment of anti-GC effects, although the interactions of the compound with PR and AR are undesirable for this purpose. Nevertheless, up to now, Mife is the only GR antagonistic drug on the market (Pecci et al. 2009).

In addition to being a reference GR antagonist, Mife also finds medical application. The anti-GC properties make it a useful medication to treat Cushing´s syndrome, as the drug counteracts hypercortisolism (Chu et al. 2001; Fleseriu et al. 2012). Also, Mife has been shown to be an effective treatment for major depression, where GR antagonism induces an HPA feedback loop mechanism which increases ACTH levels and induces endogenous Cort production (DeBattista et al. 2006; Flores et al. 2006).

4.7.1.2 The effects of Mifepristone within the present study

Within the present study, Mife served as a reference GR antagonist for *in vitro* and *in vivo* experiments. Accordingly, the compound inhibited GC signaling in all three GRIZLY assays. The anti-GC effect of Mife was also validated in qPCR experiments, as it suppressed the expression of all genes that were induced by Dex (*hsd11b2*, *gilz* and *fkbp5)* and Preg (*hsd11b2*, *gilz*, *fkbp5*, *cyp3a65* and *cyp1a1)* within the set of selected targets.

4.7.2 10-Hydroxycamptothecin (10-HC)

4.7.2.1 The biological function of 10-HC and its mechanisms of action

10-HC (also known as SN-38) is the active metabolite of irinotecan, an analogue of camptothecin. Irinotecan is an antineoplastic agent and one of the major drugs used in metastatic colorectal cancer treatment (Basseville et al. 2011; Kehrer et al. 2002; Raynal et al. 2010). It serves as a prodrug which is *in vivo* converted by carboxylesterases into the pharmacologically active compound 10-HC (Humerickhouse et al. 2000). 10-HCexerts its antineoplastic properties via inhibition of the enzyme DNA topoisomerase I (Ebrahimnejad et al. 2010; Gupta et al. 1997). The compound interacts with DNA topoisomerase I, whereby cleavage complexes are formed which prevent DNA replication and transcription (Basseville et al. 2011). This mechanism induces cytotoxicity in cancer-diseased tissues (Ebrahimnejad et al. 2010).

The potential of 10-HC to interfere with glucocorticoid signaling

I grouped 10-HC together with Mife as the two compounds had very similar effects on GC signaling within the GRIZLY assay and in qPCR experiments. Therefore, I suspected that they might share mutual mechanisms of action. However, I was unable to retrieve direct indications for 10-HC to antagonize the GR from the searched literature. Only two publications that I found suggest links between 10-HC and the GC system:

Akagi and collegues (2009) described an enhanced chemotherapeutic effect of 10-HC in co-treatment with Dex in rat-1 cells, indicating potential common mechanisms of the two compounds.

In a study by Ahowesso et al. (2011), 10-HC induced circadian disruption in mice. Treatment with the antineoplastic affected body temperature, rest-activity, plasma corticosterone (the major GC in rodents) and the expression of several clock genes (e.g. *Rev-erbα*, *Per2*, and *Bmal1*) in the livers of the animals.

4.7.2.2 The effects of 10-HC within the present study

Within the present study, 10-HC inhibited GC signaling in all three GRIZLY assays, like the GR antagonist Mife. Also, the gene expression patterns of 10-HC in co-treatment with Dex and Preg showed high similarity with those of Mife. The only remarkable difference between the responses of Dex + 10-HC and Dex + Mife on target gene transcription was that the antineoplastic decreased the mRNA levels of the two xenometabolic marker genes *cyp3a65* and *cyp1a1*, which were no targets of Dex and Mife. This might correlate with the results from chemical analysis, where a lower Dex level was measured in the larvae. Although upregulated xenometabolic marker genes could be expected in correlation with a lower amount of Dex, effects on gene expression (after 4 h of exposure) may underlie different temporal dynamics than changes on larvae internal amounts of xenobiotics (24 h of exposure) and thus, the mRNA levels of *cyp3a65* and *cyp1a1* could indeed have been increased at another timepoint.
The endogenous concentrations of Preg, Prog and Cort were not altered by 10-HC, thus, disruption of the Cort biosynthesis by 10-HC was neither suggested by UPLC-MS/MS experiments, nor by the GRIZLY results. Also, in contrast to the effect of 10-HC in co-exposure with Dex, no metabolic changes were indicated in larvae co-treated with Preg + 10-HC.

4.7.2.3 Potential mechanisms of glucocorticoid signaling disruption by 10-HC

1) Interference with GR signaling

Although the effect similarities with Mife may suggest common mechanisms of action between the GR antagonist and 10-HC, the searched literature gave no direct indications for the antineoplastic to antagonize the GR. Neither have alterations in GR expression or -activity been directly described for this compound. According to the two studies mentioned before (section **4.7.2.1**; Akagi et al. 2009; Ahowesso et al. 2011), 10-HC may interfere with GC signaling at diverse levels, but the underlying mechanisms are unclear. However, also the function of the circadian clock and other GC-unspecific pathways (section **4.3**) that were triggered by 10-HC treatment may have indirectly affected the GRIZLY response and Dex/ Preg target gene expression within the present study.

2) Extensive metabolization of 10-HC in detoxification organs via the pregnane X receptor

Interestingly, after irinotecan is converted into 10-HC, this active metabolite is extensively metabolized in detoxification organs such as liver and the intestine, where it can be inactivated by glucuronidation (via UDP-glucuronosyl transferases (UGTs) such as UGT1A1, UGT1A6, UGT1A7, UGT1A9) (Ihunnah et al. 2011; Mathijssen et al. 2004; Raynal et al. 2010), by CYP enzymes (such as CYP3A4 and CYP3A5) (Basseville et al. 2011) and by detoxification processes via different xenobiotic transporters (e.g. multidrug resistance protein 1 (MDR1) or breast cancer resistance protein (BCRP) (Basseville et al. 2011).
In chemosensitivity studiesseveral research groups examined if these cellular defense mechanisms may underlie the frequently observed resistance of colon cancer to irinotecan (Mathijssen et al. 2004; Raynal et al. 2010). As the PXR is predominantly expressed in the liver and the gastrointestinal tract, the xenoreceptor is suspected to play a major role in the response of colon cancer cells to 10-HC. The transcription factor is activated by multiple drugs and

environmental pollutants and regulates the expression of genes involved in xenometabolism and xenobiotic transport. Thus, considering the metabolic pathways of 10-HC and the tissue distribution of the PXR, the receptor is believed to mediate the cellular defense against drugs by upregulating the expression of detoxification genes (Basseville et al. 2011; Raynal et al. 2010).
Within this context, for instance Basseville et al. (2011) demonstrated via Chromatin immunoprecipitation (ChIP) in a colon cancer cell line that upon treatment with 10-HC, the PXR heterodimerized with the RXR in the nucleus and induced the expression of the CYP3A4 gene. They also confirmed via RNA interference experiments that the RXR was indeed involved in the overexpression of CYP3A4 and furthermore identified CYP3A5 as an additional target gene of the xenoreceptor. As upregulation of CYP3A4 is a major mechanism of drug resistance, they concluded that the PXR plays a pivotal role in tumor resistance to 10-HC.

These findings may relate to the present study where with 10-HC + Dex, less Dex was measured in larvae extracts and the expression of *cyp3a65* and *cyp1a1* was altered. Possibly, 10-HC was strongly metabolized in the larvae, which also led to a decrease of the internal Dex concentration. An involvement of the PXR within this xenometabolic process may be suggested by the regulation of *cyp3a65*, a supposed PXR target in zebrafish(Bainy et al. 2013; Kubota et al. 2015; Tseng et al. 2005). These correlations may indicate thatchanges in drug uptake and -metabolism (section **4.3.1**) possibly contributed to the observed effects of 10-HC + Dex in GRIZLY 2 and on target gene expression, but they can neither explain the inhibition of GC signaling *in vitro*, nor the impact of 10-HC on Preg-induced GC signaling in GRIZLY and qPCR experiments. Thus, additional mechanisms have to be involved in these effects.

3) Suppression of gene transcription via DNA topoisomerase I inhibition
Considering the pharmacological activity of 10-HC as an antineoplastic which prevents DNA replication via inhibition of topoisomerase I, the obvious conjecture is that the compound had a cytotoxic impact on cells and larvae that might have reduced their responsiveness to GC signal activation. However, no toxicity was detected in both cells (via the LDH and the alamarBlue® assay; [11 µM]) and larvae (reaction to a tactile stimulus and lethality; [4-20 µM]) (section **3.3.1**). In contrast, the parent compound of irinotecan, camptothecin, was itself present in the FDA library and showed toxicity in cells and larvae; hence, it was excluded from further experiments. Accordingly, some subliminal toxic effects might still have occurred with 10-HC, which may not have led to a significant result in the two cytotoxicity assays and neither decreased the motility nor increased the lethality in larvae at the timepoint assessed, but potentially it prevented DNA transcription via DNA topoisomerase I inhibition. This could have led to the observed inhibition within the GRIZLY assay and the qPCR experiments, as the transcription of the luciferase reporter gene and the selected GC target genes may have been hampered by DNA topoisomerase I inhibition. Although toxicity would be expected to result from this sooner or later, maybe it occurred not immediately and the exposure duration was too short for transcription inhibition to lead to cell death.

4.7.2.4 Next steps

In order to pursue whether the effects of 10-HC might involve effects on the expression of the GR or its isoform ratio, the mRNA and protein levels of the two GR isoforms and their ratio will be assessed in future experiments. Also *in vitro* GRα binding studies may provide more detailed information about direct or indirect interactions of the compound with receptor activity. Should these findings indicate a potency of 10-HC to interact with GR signaling, this would be a so far unknown effect mechanism of the drug.

Regarding the considered increased larval xenometabolism of Dex + 10-HC and a putative involvement of the PXR in these processes, it will be insightful to assess the expression levels of the xenoreceptor and its target genes (e.g. *cyp3a65*) in time series with short intervals. An increased expression of CYP genes would be expected at a certain time point in larvae treated with Dex + 10-HC in case that the PXR played a role in the decreased Dex level found in larvae homogenates.

Considering suppression of gene transcription via DNA topoisomerase I inhibition as a possible effect mechanism for 10-HC within the present study, toxicity should occur in cells and larvae under prolonged exposure. It might thus be surveyed if toxic effects can be observed if cells and larvae are treated with the same conditions for 36 or 48 h.

4.8 Retinoids

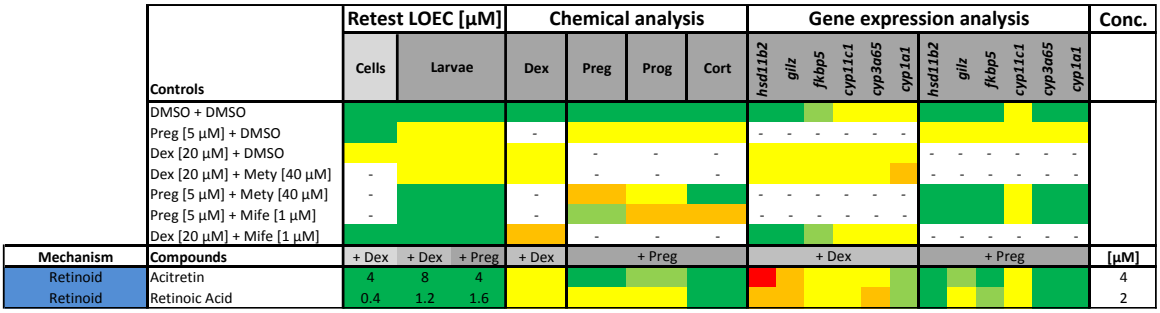

Figure 48: Retinoids (extract from Figure 45)

4.8.1 The biological functions of retinoids and their mechanisms of action

Ret. acid and Aci belong to the group of retinoids, which are derivatives of Vitamin A (retinol). Ret. acid is the carboxylic form and the main active metabolite of retinol (Aubry and Odermatt 2009; Park et al. 1999). Synthetic retinoids such as Aci, a second generation retinoid, are produced to mimic retinoid signaling and -function for specific medical applications (Sue Lee and Koo 2005).

During vertebrate growth and development, Ret. acid plays an important role, as it is involved in the embryonic anterior/ posterior patterning. After embryogenesis, Ret. acid is essential for cognitive functions, adult brain plasticity, anxiety-like behavior and epithelial differentiation (Aubry and Odermatt 2009; Bonhomme et al. 2014; Brossaud et al. 2013; Chen et al. 2014). In medicine, retinoids are applied in chemoprevention and for the therapy of some types of cancer, as they inhibit several biological functions including tumor growth, angiogenesis and metastasis

(Bonhomme et al. 2014; Chen et al. 2014; Marill et al. 2003; Soprano, Qin, and Soprano 2004). Aci usually finds application for the treatment of psoriasis (Lee and Li 2009).

Classical pathway

Ret. acid acts by binding and activating the retinoic acid receptor (RARα, β, or γ), which forms a heterodimer with the retinoid X receptor (RXRα, β, or γ) (Huang, Chandra, and Rastinejad 2014; Ihunnah et al. 2011; Park et al. 1999). RXRs can also be liganded by Ret. acid, but only by the 9 cis (not the all-trans) isomer of the compound (Chen et al. 2014; Huang et al. 2014). The heterodimeric RAR/ RXR complexes bind to specific retinoic acid response elements (RAREs) on the DNA upstream of Ret. acid target genes, where they act as transcription factors and regulate the expression of genes which are involved for instance in cellular differentiation, arrest and apoptosis (Chen et al. 2014; Ihunnah et al. 2011; Lee and Li 2009).

Non-classical pathway

Beyond this classical pathway, Ret. acid function involves interplays with numerous other important signaling molecules. It can modulate NF-κB and diverse other transcription factors, which act for instance downstream of IFN-γ, VEGF, TGF-β and MAPK, and through these interactions, Ret. acid can affect their transcriptional activity (Chen et al. 2014; Connolly, Nguyen, and Sukumar 2013; Cras et al. 2012). Also, RAR and RXR can heterodimerize with other receptors and regulate the signaling pathway of the partner receptor. These interactions are referred to as non-classical pathways, which may have opposite functions to those of the classical, RAR/RXR heterodimer-mediated pathway. For instance, interference with the signaling of estrogen receptor alpha (ERα), peroxisome proliferator-activated receptor (PPAR), liver X receptors (LXRs) and vitamin D receptor (VDR) have been described (Chen et al. 2014; Connolly et al. 2013; Páez-Pereda et al. 2001).

The potential of Retinoic acid to interfere with GR activity

Also, retinoid signaling can interfere with GR pathways and either enhance or decrease the effects of GCs (Aubry and Odermatt 2009; Bonhomme et al. 2014; Brossaud et al. 2013; Lefebvre et al. 1999; Tóth et al. 2011; Yamaguchi et al. 1999). For instance, Tóth et al. (2011) observed that Ret. acid had a synergistic effect on GR activity in thymocytes, as the Ret. acid-liganded RARα/ RXR heterocomplex interacted with the GC-liganded GR and enhanced its transcriptional activity. In contrast, Aubry & Odermatt (2009) reported an inhibitory effect of the retinoid on GR activity, where Ret. acid prevented GR transactivation in skeletal muscle cells.

Apparently, Ret. acid and GCs can also influence the expression of their receptors reciprocally. Brossaud et al. (2013) found in a hippocampal cell line that Ret. decreased the expression of GR via a negative GR feedback loop when the cells were co-treated with Dex + Ret. acid. They also observed that Dex and Ret. acid both interact through regulations of RARβ and GR and that the two compounds can regulate the mRNA and protein expression of their own and of the other receptors. In accordance with an inductive effect of Dex treatment on RAR and RXR expression, Yamaguchi et al. (1999) observed in cultured rat hepatocytes that Dex increased mRNA and protein levels of RXRα. Furthermore, the researchers showed that this Dex-induced increase in

RXRα expression could also influence the ligand-dependent transcriptional activation of other receptors that heterodimerize with RXRs, as Dex treatment intensified the Ret. acid-dependent upregulation of RARβ mRNA expression. In contrast to this, also counteracting effects of Dex on retinoid-mediated mechanisms have been reported. Lefebvre et al. (1999) showed in a human myeloma cell line that several Ret. acid-induced apoptotic features were inhibited by Dex treatment, such as the expression of the enzyme transglutaminase. Thus, the regulatory interactions between the two systems appear to be context dependent.

The potential of Retinoic acid to disrupt the Cortisol biosynthesis pathway

Ret. acid can also interfere with the Cort biosynthesis pathway. For instance, Páez-Pereda et al. (2001) observed that Ret. acid suppressed the production of ACTH *in vitro*, in human pituitary ACTH-secreting tumor cells and in a lung cancer cell line, as well as *in vivo*, in tumors of mice secreting ACTH. Within this context, the researchers furthermore found that Ret. acid inhibited the proliferation of cells within the adrenal cortex and through this also the production of corticosterone.

4.8.2 The effects of retinoids within the present study

Within the present study, the two retinoids inhibited GC signaling in all three GRIZLY assays. Chemical analysis revealed reduced Cort levels for both compounds, and in larvae treated with Aci also lower concentrations of Preg and Prog were measured. Accordingly, a disrupted Cort biosynthesis pathway is suggested for Ret. acid and Aci when co-treated with Preg. This is further supported by the gene expression profiles, as the two compounds counteracted the upregulation of all Preg target genes (except *gilz* in case of Ret. acid). Surprisingly, elevated induction of mRNA levels of *hsd11b2*, *gilz* and *cyp3a65* (in case of Ret. acid) were found in larval co-exposure to Dex, rather than the repression expected based on the GRIZLY data (and the gene expression changes observed under Preg stimulation). Only *cyp1a1* was also decreased by Ret. acid/ Aci + Dex, consistent with the downregulation of this xenometabolic marker by Ret. acid/ Aci + Preg. Apparently, for these genes, Ret. acid and Aci interfered with Dex-induced GC signaling partly via different mechanisms than with Preg treatment.

4.8.3 Potential mechanisms of glucocorticoid signaling disruption by retinoids

1) Interference with GR signaling

The signal inhibition of Ret. acid and Aci in all three GRIZLY assays suggests a direct impact of the compounds on GC signaling. As described in section **4.8.1**, retinoid interactions with GR pathways, leading to potentially reciprocal effects on receptor activity and mRNA/ protein expression, have been reported in literature (Aubry and Odermatt 2009; Bonhomme et al. 2014; Brossaud et al. 2013; Lefebvre et al. 1999; Tóth et al. 2011; Yamaguchi et al. 1999). Via non-classical pathways, either the retinoids themselves may have interfered with GC signaling, or the RAR/ RXR heterodimer decreased the GR activity via receptor crosstalk with the liganded GR (see section **4.4.2.5**).

For instance, Brossaud et al. (2013) described that Ret. acid amplified the GC-evoked homologous downregulation of GR expression via a negative GR feedback loop *in vitro*, when they co-treated hippocampal neurons (HT22) with Dex and Ret. acid. They also found that Dex and Ret. acid both interact through regulations of RARβ and GR and that the two compounds can act synergistically and oppositely on their own and the reciprocal receptor mRNA and protein expression. Also for the results of the present study, an amplified homologous downregulation (section **4.4.2.4**) of the GR by the retinoids would be a possible mechanism leading to the observed effects in the GRIZLY assay. Maybe, Ret. acid and Aci accelerated or intensified this negative feedback loop, synergistically with Dex / Cort, and thereby reduced the GRα expression, whereupon the GRα : GRβ ratio decreased and the activity of remaining GRα could be further attenuated (section **4.4.2.3**).

2) Disruption of Cortisol biosynthesis

In addition to direct interactions with GR signaling, disrupted Cort biosynthesis in larvae co-exposed to Ret. acid/ Aci + Preg is suggested according to the present UPLC-MS/MS data (section **4.4.1.1**). Reduced Cort production in larvae could moreover explain the decreased mRNA levels of *hsd11b2*, *gilz*, *fkbp5* (only with Ret. acid), *cyp3a65* and *cyp1a1*, which was observed under the same treatment conditions.

In Dex-treated larvae, reduced Cort production would not have been detectable by means of the applied UPLC-MS/MS system due to sensitivity issues. If steroidogenesis was indeed disrupted with Ret. acid/ Aci + Dex, and less Cort was produced, this would not have altered Dex signaling in the larvae. Thus, no conclusion can be drawn from these data about the retinoid effect on the endogenous Cort production with Dex treatment.

However, the hypothesis of a disrupted Cort production due to retinoid treatment would be consistent with the above mentioned study by Páez-Pereda et al. (2001), where the researchers observed that Ret. acid inhibited the production of ACTH and corticosterone *in vivo*, in ACTH-secreting tumors in mice and *in vitro*, in human pituitary ACTH-secreting tumor cells and in a small-cell lung cancer cell line. As described in section **1.2.1**, ACTH regulates the synthesis of GCs and, thus, a decreased ACTH production could have led to the decreased Cort levels within the present study. Also clinical studies were conducted to assess the effect of Ret. acid on ACTH production in Cushing's disease, but the outcomes are diverse, with either increases or decreases in *pomc* (gene encoding the proopiomelanocortin (POMC) protein, a precursor of ACTH) transcription and the underlying mechanisms are not completely understood (Lau, Rutledge, and Aghi 2015). Thus, the findings of this thesis work suggest that the effects of Ret. acid on the production of Cort are conserved in zebrafish, but the molecular mechanisms are unclear. Possibly, as a retinoid-amplified homologous downregulation of GRα can be suspected to imply a negative feedback loop on the endogenous GC production (via ACTH), this might be an explanation for the decreased Cort levels in larvae.

3) Indirect interactions with glucocorticoid signaling

Also indirect compound interactions with GC signaling could have occurred in co-treatment of the retinoids with Dex/ Preg. For instance the opposing effects of Dex/ Preg + retinoid on the

expression of *cyp3a65* may suggest diverging xenometabolic effects (section **4.1.4**) under the different treatment conditions. Although the expression of numerous CYP enzymes can be regulated by the GR (Dvorak and Pavek 2010), most members of the CYP3A family are predominantly regulated by the PXR (Bainy et al. 2013; Kubota et al. 2015; Lehmann et al. 1998; Tolson and Wang 2010) Accordingly, also the *cyp3a65* zebrafish gene is a candidate target of the PXR (Bainy et al. 2013; Kubota et al. 2015). Thus, receptor crosstalk between the GR and the PXR can occur (Pascussi et al. 2000), potentially regulating the transcription of *cyp3a65* ligand- and context-specifically. In accordance with this idea, several coactivators for steroid hormone signaling, e.g. steroid receptor coactivator 1 (SRC-1) and glucocorticoid receptor interacting protein 1 (GRIP-1) have been reported to play pivotal roles in tissue specific PXR target gene induction (Tolson and Wang 2010). Moreover, the expression of *cyp3a65*can also be regulated GR independently, for instance via the PXR alone, or via receptor interactions between the PXR and the RXR (Moore and Kliewer 2000), which can activate xenobiotic response element (XRE) sequences within the promotor region of the *cyp3a65* gene (Tseng et al. 2005).
In order to consider these findings for the interpretation of the *cyp3a65* expression within the present study, other transcription factors may have altered the mRNA level of *cyp3a65* in addition or alternative to the GR, and GR/PXR or PXR/RXR crosstalk (section **4.4.2.5**) possibly played a role therein. These interactions were probably diverse between the different co-treatments and led to context- and ligand-specific responses such as downregulation of the target gene in co-treatment of the retinoids + Preg but to an elevated mRNA level with Dex + Ret. acid.

Similarly, the observed downregulation of the second xenometabolic marker gene, *cyp1a1*, by Ret. acid/ Aci + Dex/ Preg may also be attributed to retinoid interference with other nuclear receptors, such as the AhR. For instance, retinoids have been described to repress the pathway of AhR-mediated induction of *cyp1a1* through the silencing mediator for retinoid and thyroid hormone receptors (SMRT) corepressor (Fallone et al. 2004), which could explain the decreased mRNA levels within the present study.

4.8.4 Next steps

Regarding direct interference with GR signaling, the transcriptional activity of GRα appears to play a major role therein, which makes evaluation of GRα/GRβ levels and their ratio a reasonable starting-point to pursue mechanisms of retinoid and GC signaling interactions.

In order to follow up on retinoid interference with the GC biosynthesis pathway, the HPA axis status can be further investigated under Ret. acid/ Aci + Dex/ Preg treatment, for instance via *in situ* hybridization assessing the ACTH/ *pomc* expression in the zebrafish larvae. Also, zebrafish models for Cushing's disease, as recently developed by Liu (2011) and colleges, could serve as a valuable tool to trace this effect. Moreover, translational research with this system might provide essential knowledge on retinoid therapies for the treatment of Cushing's disease.

For further investigations of nuclear receptor crosstalks (possibly involving RAR, RXR, GR, PXR and AhR) under Dex/ Preg + retinoid co-treatment, target gene expression analysis in dependency of different retinoid concentrations, as well as receptor binding studies, can certainly provide useful information. However, a more detailed understanding will require a comprehensive mapping of interactions between putatively involved nuclear receptors and their activities in the regulation of key genes of the target regulatory networks.

4.9 Progestins

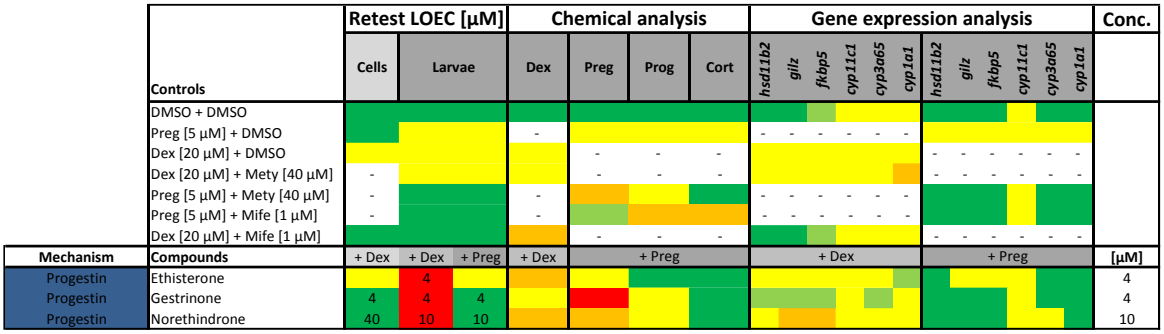

Figure 49: Progestins (extract from Figure 45)

4.9.1 The biological functions of progestins and their mechanisms of action

Ethi, Nor and Gestri belong to the steroid hormone group of progestins. Progestins are synthetic progestogens which were designed to be potent PR agonists. Thus, in cells of target organs, they are expected to mediate their effects predominantly by transcription regulation of specific target genes via binding to and regulating the activity of the PR. Most progestins are structurally very similar to Prog, 17α-hydroxyprogesterone or testosterone, as they were first derived from testosterone and were then chemically modified in order to enhance their progestogenic properties and to develop synthetic drugs which mimic the function of Prog, the major endogenous progestogen (Africander et al. 2011; Bray et al. 2005; Graham and Clarke 1997; Louw-du Toit et al. 2017).

Progesterone

Prog is a key hormone in the regulation of female reproduction, which involves the release of mature oocytes, maintenance of pregnancy, milk protein synthesis and sexual behavior in PR-expressing organs including uterus, ovary, mammary gland, brain and pituitary gland. Targeting these biological functions, progestins are used in reproductive medicine with manifold applications for hormone replacement therapy (HRT), contraception and treatment of reproductive disorders or cancer (Africander et al. 2011; Bray et al. 2005; Graham and Clarke 1997; Louw-du Toit et al. 2017).

Ethisterone

The oldest and first marketed orally active compound with progestogenic activity is Ethi, one of the three progestins identified within this study to interfere with GC signaling. Ethi was structurally directly derived from testosterone and although minor structural changes increased

the binding affinity of Ethi for the PR over that for the AR, its progestogenic effects were still likely to be accompanied by androgenic side-effects (Inhoffen and Hohlweg 1938). Nowadays, newer generations of progestins replace Ethi for applications in reproductive medicine. Among those is the group of 19-nortestosterone progestins, structurally derived from Ethi, to which Gestri and Nor belong (Djerassi 1992; Louw-du Toit et al. 2017).

Gestrinone

Gestri is described as a mixed PR agonist and a selective progesterone receptor modulator (SPRM) (Bromham et al. 1995). According to Tamaya et al. (1986), who conducted a study on human endometrial cytosol, Gestri binds with moderate affinity to the PR, but it possibly occupies "*almost all specific binding sites of steroids in the steroid target cells in spite of the presence of endogenous steroids*". One earlier *in vitro* receptor binding study from Raynaud et al. (1980), where steroid hormone receptors were obtained from diverse rodent tissue homogenates, found a higher relative binding affinity of Gestri for the AR and the GR than for the PR (AR > GR > PR > MR). Although *in vitro* binding studies have to be evaluated carefully, as further discussed in the following paragraphs, these observations imply a general potential for Gestri to directly interfere with GR binding, which may be considered for the discussion of the present results.

Norethindrone

The progestogenic activity of Nor (also termed Norethisterone) was described to be about 20-fold higher than that of Ethi (Djerassi 1992). However, due to its structural ability to bind the AR, weak androgenic effects can also be expected. Moreover, Nor has a weak estrogenic potential, as a small portion of the progestin can be metabolically converted into the synthetic estrogen Ethinylestradiol (Kuhnz et al. 1997; Paulsen et al. 1962). Interestingly, according to Ronacher et al. (2009), Nor has no transactivation and transrepression potency (tested on NF-κB or AP-1) for the GR. They showed furthermore that Nor has a very high binding IC50 (1688 +/- 300 nM) to the GR in transfected COS-1 cells.

4.9.2 The effects of progestins within the present study

In the GRIZLY assay, Ethi, Nor and Gestrisuperactivated Dex-induced signaling *in vivo*. By contrast, only Gestri and Nor inhibited Dex-induced signaling *in vitro* and Preg-induced signaling *in vivo*. Chemical analysis revealed trends for elevated Dex levels in larvae co-treated with Dex + Ethi and Dex + Nor. Furthermore, all three compounds led to reduced Cort concentrations. Ethi treatment additionally decreased Prog levels, while for Gestri and Nor, Prog was unchanged. Ethi did not change Preg levels, which increased in larvae co-treated with Nor and Gestri. The qPCR data are ambiguous for the progestins in co-treatment with Dex, as only with Nor a trend is visible for two target genes (*gilz* and *fkbp5*) to be upregulated, which would be in accordance with the outcome of GRIZLY 2. In co-exposure with Preg, all three compounds downregulated several of the target genes, supporting a reduced GC signaling by Cort.

Although the opposite responses between the different GRIZLY approaches were rather unexpected, the follow-up experiments helped to confirm these findings in part. The UPLC-MS/MS and qPCR results furthermore provided additional starting points for considerations about the – apparently complex – mechanisms of action possibly underlying the compound effects. Several are depicted as follows:

1) Direct interactions with GR signaling

As implied by their structural characteristics, direct interactions of progestins with GRs are generally possible and may, thus, have contributed to the observed effects of Ethi, Gestri and Nor on Dex- and Preg-induced GC signaling in the GRIZLY assay. In GRIZLY 1, possibly high concentrations of both, Dex and the progestins would reach the cells, and any progestin binding could lower the total signal, suggesting a partial antagonism of the progestins under these treatment conditions (a context-specific effect; section **4.3.2**).

In larvae, the physiological environment might be different, which could lead here to partial agonistic effects of the progestins. Also, as it was assessed earlier (in section **3.5.1** and discussed in section **4.1.1**), Dex enters the larvae at much low concentrations than Preg. Maybe, according to the structural similarity between Preg and the progestins, the relative concentrations of Ethi, Gestri and Nor might also be higher in larvae than that of Dex, which may have made context-specific (section **4.3.2**), synergistic effects of the progestins on GR activation possible in GRIZLY 2. Considering that Gestri was described in literature to possibly exhibit binding affinity for the GR (Raynaud et al. 1980; Tamaya et al. 1986), a direct GR antagonism of this compound may be supposed to underlie the observed inhibition in GRIZLY 1 and a synergistic GR agonism the superactivation in GRIZLY 2. Also for Nor, although no activity on the mammalian GR was described (Africander et al. 2011; Ronacher et al. 2009), for the zebrafish receptor, a potential direct ant/agonism of the compound could still have had a relevant impact on the outcome of the GRIZLY assay. The reduced Cort levels found for Gestri, Nor and Ethi in co-exposure with Preg are likely to explain signal inhibition by Gestri and Nor in GRIZLY 3. In case of Ethi, the compound may have had a stronger partial agonistic activity than the other two progestins, which possibly even counteracted the downregulation of reporter gene expression in GRIZLY 3 that could have been expected from the reduced Cort production.

2) Superactivation in the presence of glucocorticoids

Interestingly, the experiments in which larvae were exposed to Ethi, Nor and Gestri in absence of Dex or Preg did not show an agonistic potential of any of the three progestins alone (**Figure 33 a**). Also experiments with other progestins, which were not contained in the FDA library, such as Levonorgestrel [10-50 µM] and Melengestrol acetate [10-50 µM], showed no direct activation of GC signaling, but superactivation in combination with Dex (see appendix, **Supplementary Figure IV**). Apparently, the presence of Dex or of directly administered Cort (**Figure 33 b**) was necessary to superactivate GC signaling. Potentially, ligand binding of the GCs induced conformational changes at the GR which then allowed also the progestins to agonize the GR. Alternatively, these results could indicate that a cofactor environment depending on the GC-liganded GR at the reporter gene promotor (context-specifically; section **4.3.2**) may enable for

other transcription factors to bind (Weikum et al. 2017), which could in turn allow for receptor crosstalk between the GR and other nuclear receptors, such as the PR (see examples for receptor crosstalk in section **4.4.2.5**). Possibly consistent with the "hit and run" (Keeton et al. 2002)and "assisted loading" (Voss et al. 2011) models of GR signaling, initial chromatin binding of the activated GR may have triggered chromatin remodeling in the regulatory sequence. This could have exposed a higher number of DNA binding sites and facilitated the attachment of additional factors and possibly other nuclear receptors, which reinforced the activation of the GC target gene (Cohen and Steger 2017; Goldstein et al. 2017; Petta et al. 2016).

3) Changes in xenometabolism

The UPLC-MS/MS measurements showed elevated Dex levels in the larvae treated with Dex + Ethi and Dex + Nor, which possibly underlie changes in Dex-uptake and -metabolism (section **4.1.4**). Thus, the higher amount of Dex could have contributed to the co-activating effect of Ethi and Nor observed in GRIZLY 2.

4) Disruption of cortisol biosynthesis

Chemical analysis data suggest inhibition of Preg-induced biosynthesis of Cort as a potential mechanism of action of all three compounds (section **4.4.1.1**). In particular, Gestri and Nor induced the same steroid level profile as the reference inhibitor Mety, thus, a common mechanism of action could be suspected for Gestri, Nor and Mety. As Mety specifically inhibits 11β-hydroxylase, this enzyme is a good candidate to be also a point of disruption by Gestri and Nor. In accordance with that, the gene expression changes induced by Gestri, Nor and Mety across the different targets are also comparable, which would equally be consistent with common mechanisms of action.

Ethi may have disrupted the biosynthesis pathway earlier than Gestri and Nor, as Prog levels are reduced in addition to Cort (for details of the steroidogenesis pathway see **Figure 3**). Inhibition of 3β-HSD, the enzyme that converts Preg and 17α-hydroxypregnenolone into Prog and 17α-hydroxyprogesterone would be a possible explanation for this steroid level profile. Preg might not accumulate as Preg and 17α-hydroxypregnenolone can still be metabolized into downstream androgens when 3β-HSD is inhibited.

5) Lower concentrations of Norethindrone and Gestrinone superactivate glucocorticoid signaling

During retests with lower concentrations of Nor and Gestri, which were conducted in order to assess the concentration-dependency of their effects (**Figure 34**), both compounds superactivated Dex signaling in all concentrations. Surprisingly, in co-treatment with Preg, Nor (significant at 0.25-1 µM) and Gestri (a trend at 0.25 µM) also showed co-activating potential in the lowest concentrations.

Possibly, a higher concentration of Nor (>2 µM) and Gestri (>0.25 µM) was necessary to disrupt the Preg-induced Cort production, while lower concentrations still enabled the endogenous conversion of Preg into Cort. In this case, the GCs liganded the GR and activated GC signaling.

Assuming from the increased GRIZLY response, Nor (and maybe Gestri) could then interfere with GC signaling, which led to the observed superactivation.
This, in turn, could rely on several of the already mentioned mechanisms. The progestins may have directly interfered of with GC signaling, for instance via the above considered GC-induced changes of either the GR conformation (may enable partial GR agonism for progestins) or the chromatin structure within the promotor region of target genes (could facilitate chromatin binding of additional factors, e.g. the progestin-liganded PR). Alternatively, the superactivation could result from compound-mediated changes in drug uptake and -metabolism, as it was suspected already for Nor + Dex treatment. The larvae-internal amount of Preg may have been elevated, which could have led to higher Cort levels and enhanced GC signaling.

Summary: multilayered effect mechanisms
To summarize these considerations, several multilayered effect mechanisms possibly apply for the compounds. These, in turn, may have been context-specifically influenced by diverse general (section **4.3**) and specific (section **4.4**) mechanisms of GC signaling disruption. In the next sections the considered mechanisms will be discussed with regard to the available literature describing effects of progestins in general and of Ethi, Nor and Gestri specifically on GC signaling.

4.9.3 Potential mechanisms of glucocorticoid signaling disruption by progestins

Direct interactions with GR signaling
According to the available literature, studies investigating the three discussed progestins are very limited, in particular animal or clinical studies elucidating their effects *in vivo* are rare (Louw-du Toit et al. 2017). Especially for Ethi, the oldest of the three progestogenic compounds, this circumstance may be explained by the rapid evolution of newer generations of progestins for medical applications, which are in the focus of most publications from the last decades. However, some common information about their principal progestogenic mechanisms is important for the evaluation of the compound effects observed in the present study. In general, for most if not all progestins, their entire mechanisms of action are not completely understood. Like for all steroidogenic ligands, their binding affinities and agonistic or antagonistic potencies for a specific receptor, as well as their biological activities are context-specific (section **4.3.2**) (Africander et al. 2011; Hapgood et al. 2004; Heitzer et al. 2007; Inhoffen and Hohlweg 1938; Louw-du Toit et al. 2017; Weikum et al. 2017).

Furthermore, a high structural similarity of the PR to the AR especially, but also to the MR and the GR, allows progestogens in principle *per se* to bind also to these other membersof the nuclear receptor superfamily. The molecular modifications of progestins targeted higher efficacies and provided increased bioavailability and stability of the compounds compared to natural Prog. However, these structural changes also affected their pharmacokinetics, relative binding affinities and potencies not only for the PR, but also for AR, MR and GR, providing each progestin with a potentially unique effect spectrum (Africander et al. 2011; Bray et al. 2005; Louw-du Toit et al. 2017).

Thus, the variety of progestins shares several progestogenic activities with each other and with Prog, but their structural diversity comes also along with variable and individual patterns of hormonal activities, partly due to binding with different affinities to one or several members of the steroid hormone receptor group, possibly agonizing or antagonizing them. Although these interactions do not always induce a downstream signaling response, they can mediate ligand-specific progestogenic activities, as well as numerous side-effects. The unwanted effects of progestins are considered to be partly mediated by their binding to other steroid hormone receptors in non-target tissues. For instance, increased risks for breast cancer, negative effects on immune functions, cardiovascular complications, higher blood pressure, weight gain and reduced bone density belong to the risks which may accompany progestin medications and are effects that are functionally closely associated with progestin interference with GRs and MRs (Africander et al. 2011; Inhoffen and Hohlweg 1938; Louw-du Toit et al. 2017; Schindler et al. 2003).

As an example, the most prominent, first-generation progestin, Medroxyprogesterone acetate (also a 19-nortestosterone derivative, like Gestri and Nor), was described to bind to the GR even with higher affinity than Cort(Africander et al. 2011) and was shown to exert GC-like activity (as an agonist or partial agonist), possibly by interfering with immune and cardiovascular functions and affecting bone density (Bamberger et al. 1999; Ishida, Ishida, and Heersche 2002; Kontula et al. 1983; Koubovec et al. 2005; Kurebayashi et al. 2003; Ronacher et al. 2009; Saitoh et al. 2005).

Disruption of Cortisol biosynthesis

Also, progestins are able to disrupt the endogenous steroid production(section **4.4.1.1**) in the organism. Due to their structural similarity with Prog, 17α-hydroxyprogesteroneand testosterone, they can compete not only with other ligands for binding to steroid hormone receptors, but also with endogenous metabolites for binding to steroidogenic enzymes. This competitive inhibition can disrupt the steroidogenesis and provoke feedback mechanisms via the HPA axis (for reviews see Africander et al. 2011 and Louw-du Toit et al. 2017). The reduction of Preg-induced Cort levels seen in co-treatment with all three progestins may, therefore, well be a consequence of such an inhibition of steroidogenic enzymes.

These mechanisms depend highly on the biological environment, including availability of enzymes and steroidogenic metabolites, as well as on the route of administration and the concentration of the respective progestin. Accordingly, other studies investigating disruptions of the Cort biosynthesis pathway or inhibited function of steroidogenic enzymes by progestins were certainly conducted under different physiological conditions, but some publications are here considered for further discussion.

Interestingly, very few clinical studies on humans are available which assess progestin effects on endogenous steroid hormone levels, although these pharmaceuticals are developed for human medical applications. In one study, women administered with Medroxyprogesterone acetate showed decreased levels of endogenous Prog, 17α-hydroxyprogesterone and Cort, which were explained by progestin-induced inhibition of the HPA axis (Aedo et al. 1981; van Veelen et al.

1984). Also, decreased Cort levels were found in women using Levonorgestrel for contraception (Toppozada et al. 1997).
Apart from such few exceptions, the available literature mainly consists of animal and *in vitro* studies, which can contribute to the understanding of progestogenic (side-) effects, and may help to narrow down the putative points of Cort biosynthesis disruption.

1) 3β HSD, 17α-hydroxylase and 17, 20-lyase

In one *in vitro* study by Arakawa et al. (1989), the effects of Nor, Gestri, Levonorgestrel (also a 19-Nortestosterone derivative and, thus, structurally very similar to Nor and Gestri) and other progestins (Danazol, Desogestrel and 3-keto-desogestrel) on rat ovarian steroidogenic enzymes 3β-HSD, 17α-hydroxylase and 17, 20-lyase were assessed. They found that all progestins inhibited 3β-HSD and 17, 20-lyase, with Gestri showing an especially distinct suppression of 3β-HSD (Ki = 3.0 µEM) and of 17, 20-lyase (30 µEM). This correlates with the results of the present study, where elevated Preg and reduced Cort levels were found in zebrafish larvae treated with Preg + Nor and Preg + Gestri. For larvae treated with Preg + Ethi, Preg levels were not altered, but Prog and Cort were reduced. Even though the disruption of Preg-stimulated Cort production is not one hundred percent comparable with steroidogenesis inhibition at endogenous steroid levels, these steroid level profiles may suggest that all three progestins inhibited 3β-HSD and/or 17,20-lyase within the present study and thereby disrupted the biosynthesis of Cort.

Also, decreased mRNA expression of genes encoding 17,20-lyase, 17α-hydroxylase (both *cyp17a1*) and 3β-HSD (*hsd3b1*) were found by Overturf et al. (2014), who conducted an *in vivo* study in fish and exposed fathead minnows (*Pimephales promelas*) to Levonorgestrel. However, they also found reduced levels of Preg, Prog, 17α-hydroxyprogesterone and testosterone, which is different from the results of the present study, where Preg was induced while Prog was not affected and Cort was reduced by Gestri and Nor. As considered above, within the present study, the Cort production was stimulated in larvae by Preg treatment, which may lead to different metabolite accumulations upstream of Cort than the disruption of Cort production at endogenous levels. Accordingly, despite this difference, 17,20-lyase, 17α-hydroxylase and 3β-HSD may still have been affected within the present study, but also another steroidogenic enzyme could have been inhibited in the zebrafish larvae. As the steroid levels of larvae co-treated with Preg and either Nor, Gestri or Mety are identical to each other, disruption of the specific target enzyme of Mety, 11β-hydroxylase, appears more likely to underlie the effect of Gestri and Nor than inhibition of 17,20 lyase, 17α-hydroxylase or 3β-HSD. However, for Preg + Ethi, inhibition of 3β-HSD and maybe also 17α-hydroxylase constitutes still a possibility supported by the metabolite measurements.

2) 11β-hydroxylase

Further supporting the hypothesis that Gestri and Nor might have disrupted 11β-hydroxylase within this thesis work, one other fish study showed inhibition of several gonadal steroidogenic enzymes of carp (*Cyprinus carpio) in vitro*, upon cell treatment with Nor and other progestins (Levonorgestrel, Cyproterone acetate). Here, all progestins inhibited 17, 20-lyase, and Nor (as

well as Cyproterone acetate) furthermore inhibited gonadal 11β-hydroxylase (Fernandes et al. 2014). Although their effect was observed *in vitro* and only for gonadal 11β-hydroxylase, these findings may imply that the activity of adrenal 11β-hydroxylase in fish can also be disrupted by Nor *in vivo*.

3) 11β-HSD2

In another *in vitro* study, on human H295R adrenal cells, treated with Nestorone (19-Norprogesterone derivative) and Drosperineone (derivative of MR antagonist Spironolactone), also reduced Cort levels were observed with both progestins. This effect was accompanied by elevated Cortisone levels in case of Nestorone, suggesting an increased activity of the responsible enzyme 11β-HSD2 (Louw-du Toit et al. 2017).

Considering an involvement of 11β-HSD2 in the reduction of Cort levels within the present study, all tested compounds significantly decreased the Preg-induced mRNA expression of *hsd11b2*. This correlates with their common inhibitory effect on Preg-induced GC signaling, as *hsd11b2* is a GR target gene (Alderman and Vijayan 2012) and, thus, inhibition of GC signaling resulted in reduced transcription of *hsd11b2*. Accordingly, the present results give no indication for an increased activity of 11β-HSD2 by any of the tested compounds compared to the negative control (Preg + DMSO), making this explanation for the reduced Cort levels unlikely. Rather, they further support inhibition of upstream enzymes involved in the GC biosynthesis pathway as the origin for the decreased amount of Cort.

4) 3β-HSD - a common progestin target

Interestingly, Louw-du Toit et al. (2016) also found Nestorone, Nomegestrol acetate and Drospirenone to act upstream and to reduce the biosynthesis of steroid hormones by inhibiting the activity of 3β-HSD *in vitro*in (non-steroidogenic) COS-1 cells expressing human 3β-HSD. Inhibition of human 3β-HSD was also shown for Medroxyprogesterone acetate, as described for *in vitro* competitive binding studies by Lee, Miller, and Auchus (1999), and again by Louw-du Toit et al. (2017).

According to the cited studies, 3β-HSD is a common target of numerous progestins, disrupting the Cort biosynthesis within completely different study approaches. This knowledge highlights that progestins, albeit their precise structural differences and the diverse biological and physiological environments their effects were investigated in, still have some common targets. Therefore, animal and cell culture studies clearly provide essential information about the effect mechanisms of this pharmacological group, although they may not always reflect the compound effects in humans, as emphasized also by Louw-du Toit et al. (2017) and Miller & Auchus (2011).

Indirect interactions with glucocorticoid signaling

Beside the above mentioned direct interactions with diverse steroid hormone receptors (section **4.4.2.5**) or their potential to disrupt the production of GCs, progestins were also described to interfere with several biological processes, which can equally lead to numerous side-effects accompanying their progestogenic effects. For instance, depending on their concentrations,

route of administration and target organism, progestins can alter drug pharmacokinetics, metabolism and bioavailability (as reviewed by Africander et al. 2011 and Louw-du Toit et al. 2017), which was assumed to underlie the observed superactivation of Ethi and Nor on Dex-induced GC signaling according to the elevated Dex concentration found by chemical analysis.

4.9.4 Next steps

Within the present study, potential side-effects of progestogenic treatment on diverse physiological processes, covering possible mechanisms of direct interaction with other steroid hormone receptors, changes in drug pharmacokinetics, -metabolism and -bioavailability, as well as disruption of the endogenous steroid hormone production were observed in zebrafish larvae exposed to progestins. As, for instance, progestin treatment of zebrafish larvae (Preg + Ethi) decreased the endogenous concentrations of Prog and Cort just as well as it was found in women upon medical progestin (Medroxyprogesterone acetate) administration (Aedo et al. 1981; van Veelen et al. 1984), this study highlights that although the (side-)effects of progestins rely on various parameters, zebrafish larvae are well-suited organisms to investigate their pharmacological effects *in vivo*.

Regarding the effects of Ethi, Nor and Gestri within this thesis work, additional experiments will be necessary to validate the hypothesized mechanisms of action. Further experiments using various concentration combinations of the different ligands in both cell culture and larvae, as well as quantification of uptake of the progestins to map out the effective concentrations in the larvae, may help to solve these issues.

In order to assess direct interactions of progestins with GR signaling, the compound behavior will be surveyed in cells, in application of diverse concentrations of Dex/ Cort + progestins. In the next step, zebrafish nuclear receptor binding assays can certainly provide more substantiated information about the progestin potential to ant-/agonize the GR or to induce nuclear receptor crosstalk under the applied exposure conditions.

The assumed changes in drug uptake and metabolic activity due to progestin treatment by Nor and Ethi in combination with Dex can be addressed by a more detailed gene expression analysis, where additional marker genes for more specific xenometabolic enzymes can be measured. Also, via chemical analysis, metabolites of Dex could be quantified, which may provide more information about the drug turnover within the larvae.

The hypothesis that Ethi, Nor and Gestri disrupted the Cort biosynthesis pathway will also be followed up by chemical analysis, where more metabolites involved in steroidogenesis (see Figure 3) will be measured, which should facilitate to pinpoint a point of biosynthesis disruption and will help to validate the suggestions for affected enzymes. Following these efforts, further validation steps will include the application of specific inhibitors of the respective enzymes and enzyme activity measurements.

4.10 Synthetic ligands for androgen receptor and mineralocorticoid receptor

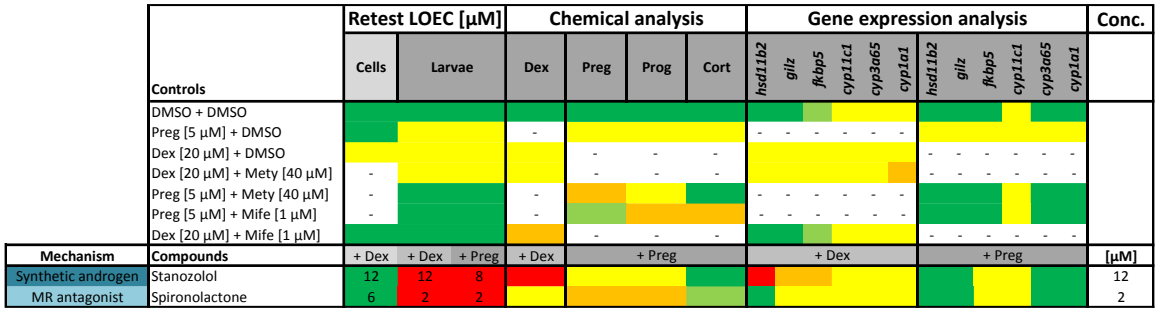

Figure 50: Synthetic ligands for AR and MR (extract from Figure 45)

The two compounds Stano and Spiro showed similar effects within the present study. Both are synthetic steroids, but they are designated to act on different targets. While Stano is a synthetic AR agonist and Spiro predominantly acts as an MR antagonist, neither of the two compounds would be necessarily expected to activate GC signaling. Surprisingly, within the GRIZLY assay, both steroids significantly co-induced the Dex-and Preg-mediated signal in larvae.

4.10.1 Stanozolol

4.10.1.1 The biological functions of Stanozolol and its mechanisms of action

Stano is a synthetic anabolic-androgenic steroid which is structurally derived from dihydrotestosterone (DHT), a natural androgen(Boada et al. 1999). Due to its anabolic and performance-enhancing properties, the usage of Stano is not allowed in sports competitions of humans and animals (Kicman 2008). In medicine, the synthetic androgen predominantly finds application for the treatment of anemia (Resegotti et al. 1981; Yuan, Liu, and Huang 1998).

The potential of Stanozolol to disrupt the Cortisol biosynthesis pathway

Already 30 years ago, Lambert et al. (1986) conducted an *in vitro* study in guinea-pig adrenal cells, where they assessed compound effects of the secretion of Cort from ACTH-stimulated cells. They found that Stano [50 µM] (and other compounds such as Etomidate, Metyrapone, Megestrol acetate, Cyproterone acetate, Danazol) interfered with the biosynthesis of Cort. Three years later, it was shown by another group that Stano is a competitive inhibitor of CYP17A1, as the anabolic androgen inhibited the activity of 17α-hydroxylase and 17,20-lyase in pigs, with IC_{50} values of 2.9 µM and 0.74 µM, respectively (Nakajin et al. 1989). Inhibitory effects on the steroidogenic activity of CYP17A1 were also observed for other synthetic androgens (such as testosterone acetate) and for anabolic steroids (as for instance Mestanolone and Furazobol) in diverse studies (for review see R. Salvador et al. 2013).

Unlike natural androgens, Stano cannot be aromatized into estrogens due to the 17α-alkyl group, which was added to the compound by synthetic modification. Thus, it possesses no estrogenic activity (Kicman 2008). However, Stano can still induce the expression of aromatase, which in turn increases the conversion of endogenous androgens into the natural estrogen Estradiol, as it was for instance observed in a human breast cancer cell line (MCF-7) by Sirianni

and collegues(2012). Based on this finding, the researchers further confirmed an estrogenic activity of Stano by showing that the compound was capable to induce the expression of the ER target gene CCND1 (cell cycle regulator cyclin D1), which encodes a key protein for the control of breast cancer cell proliferation. Therefore, Stano can possibly disrupt the steroidogenesis pathway by interfering with several enzymes.

The potential of Stanozolol to interfere with GR signaling

Like numerous anabolic androgens, Stano may have anticatabolic effects that are suspected to rely on compound interference with GR target gene expression (Bates, Chew, and Millward 1987; Kicman 2008). However, anabolic androgens normally possess a very low GR binding affinity and, thus, have no important GR antagonistic potential (Hickson et al. 1990). Interactions with GR signaling via mechanisms other than receptor antagonism have been described for instance by Fernández and collegues (1995), who observed that in contrast to natural androgens Stano and Danazol (both androgenic 17α-alkyl derivatives) were able to interact with steroid binding sites in rat liver microsomes that are capable to bind various GCs and also Prog. The researchers showed *in vivo* that Stano significantly reduced the binding capacity of radioactively labeled [3H] Dex and demonstrated that Stano, Danazol and Methyltestosterone mediated a significant increase in the concentration of the GR.

4.10.1.2 The effects of Stanozolol within the present study

Within the present study, Stano superactivated GC signaling in both *in vivo* assays but inhibited the Dex-induced signal *in vitro*. For larvae treated with Dex + Stano, chemical analysis revealed that the amount of Dex was strongly increased. In correlation to that, also the expression of the three Dex target genes *hsd11b2*, *gilz* and *fkbp5* was induced by the androgen. In contrast, when larvae were co-treated with Preg + Stano, reduced Cort levels were measured by UPLC-MS/MS. Also, the expression of all Cort target genes except *fkbp5* was significantly decreased. According to this, it appears likely that Stano may have disrupted the biosynthesis of Cort (section **4.4.1.1**), which possibly resulted in a decrease of target gene expression, but that another mechanism, which led to superactivation on the isolated 4 x GRE elements and possibly affected *fkbp5* expression, took place in parallel.

4.10.1.3 Potential mechanisms of glucocorticoid signaling disruption by Stanozolol

Disruption of Cortisol biosynthesis

According to the cited literature, Stano may have interfered with the steroidogenesis pathway via interactions with several of the involved enzymes (section **4.4.1.1**). It could have inhibited 17α-hydroxylase via competitive binding and thereby blocked the production of Cort (Lambert et al. 1986; Nakajin et al. 1989). As described already for the progestins (section **4.9.3**), Stano could possibly compete with Preg and Prog for enzyme binding due its structural similarity with the two progestogens and, thus, decrease the amount of active 17α-hydroxylase available for the downstream synthesis of Cort.

Another option is that Stano induced the activity of aromatase, as it was described by Sirianni and collegues (2012) and, thereby, increased the production of estrogens (Estradiol) from

endogenous androgens in larvae. As it will be discussed below (in chapter **4.11)** in more detail, estrogens are also potential disruptors of the Cort biosynthesis pathway.

Superactivation of the 4 x *GRE:Luc* reporter *in vivo*

Regarding the observed superactivation of the 4 x *GRE:Luc* reporter, it may be possible that the Stano-liganded AR interacted context-specifically (section **4.3.2**) with the (isolated) 4 x GRE in the transgenic larvae due to close similarity between the hormone responsive elements (HREs) of the two receptors. This potency for overlapping specificities between these two steroid hormone receptors to bind to the transgenic reporter construct leaves space for speculations. Three imaginable scenarios are discussed below:

Option 1

The AR can activate transcription via the the 4 x GRE with more potency than the GR, and induce the expression of the luciferase reporter gene, which leads to a stronger bioluminescent signal than the Dex- or Preg-liganded GR. However, experiments assessing the ability of androgens to activate the 4 x GRE reporter when they were exposed alone showed no signal induction. This lack of induction was observed for Stano in larvae (**Figure 33a**), for DHEA (endogenous androgen) in cells (see appendix, **Supplementary Figure V**) and for Methyltestosterone, Trenbolone (both synthetic androgens) and 11-Ketotestosterone (major endogenous androgen in fish) in larvae and cells (data not shown). As already described in the results part (**3.2.2.2**), the observed superactivation by Stano (and Gestri, Nor, Ethi and Spiro) apparently needs the presence of a GR agonist. Therefore, option 1 is rather unlikely.

Option 2

The GC-liganded GR activated the artificial 4 x GRE, but the presence of Stano may have stabilized the GR. For instance, Stano could have prevented homologous downregulation of GRα which can be expected upon GC treatment and which, in this study, may be assumed to underlie the normally observable signal decrease in the GRIZLY assay after 10 - 14 h in GC-exposed larvae (see section **4.4.2.4**). As it was clearly visible from the bioluminescent traces of Stano + Dex (**Figure 32 s**), the induced signal remained on a plateau between 10 and 14 h and then, instead of the normal decrease, a second and stronger increase was observed. The For Stano + Preg, not such a distinct plateau was observable between 10 and 14 h but instead; the signal appeared to continuously increase up to 16 - 18 h and then remained on a constant level until the end of the measurement (**Figure 32 t**). Both kinetics may suggest an extendedtranscriptional activation of the receptor on the luciferase reporter gene via the 4 x GRE. Potentially, prevention of homologous downregulation or even upregulation of GRα by Stano increased the amount of active receptors; an idea that would be supported by the increased GR concentrations observed by Fernández et al. (1995) in rat liver microsomes upon incubation with Stano + [3H] Dex.

Option 3

Although Stano superactivated Dex- and Preg-stimulated GC in the GRIZLY assay, this overstimulation was not mirrored for Preg + Stano in the UPLC-MS/MS and qPCR experiments.

Instead, reduced Cort production was measured in Preg-treated larvae upon co-exposure with Stano, accompanied by decreased GC target gene expression. As 26% of the control amount of Cort (Preg + DMSO, 100 %) were produced with Preg + Stano, the Cort production was not completely blocked and the remaining amount probably sufficed to induce transcriptional activity at the 4 x GRE. This consideration leads to option 3, where the GC-liganded GR regularly activated the 4 x GRE, but the Stano-liganded AR took over and further activated the 4 x GRE when either GRα started to decrease (probably due to homologous downregulation) or when the available amount of Cort was limiting in case of Preg + Stano. Thereby, an extended and overinduced GRIZLY signal could be expected, as it was visible in **Figure 32 s** + **t**.
A possible explanation for this observation might be that the liganded AR binds to the artificial 4 x GRE and thereby induces the expression of the luciferase reporter gene. As it was discussed above for the group of progestins (section **4.9.2**), initial chromatin binding of the activated GR may have induced chromatin remodeling in the regulatory sequence, whereby the accessibility of DNA binding sites for other nuclear receptors such as the AR could have been increased (Cohen and Steger 2017; Goldstein et al. 2017; Petta et al. 2016).

Suppression of the 4 x *GRE:Luc* reporter *in vitro*
Surprisingly, Stano showed no superactivation in the GRIZLY assay in cells as it showed larvae. In GRIZLY 1, it inhibited GC signaling, although some interaction between the artificial 4 x GRE, GR, Stano and AR could equally have been possible *in vitro*. As Stano is orally active and its first-pass metabolism within the liver is sterically hindered by the 17α-alkyl group (Kicman 2008), no metabolic activation within the larvae is likely to explain these discrepancies and a similar compound behavior *in vitro* and *in vivo* could be expected. However, also here, the different physiological context (section **4.3.2**) between *in vitro* and *in vivo* exposure may have played an important role, which possibly enabled an antagonistic effect of Stano on the GR, leading to inhibition of 4 x *GRE:Luc* reporter gene transcription. Possibly, due to the more direct chemical exposure in cells than in larvae, much higher compound concentrations may reach the cells which maybe allowed for competitive binding of the androgen to the GR, leading to receptor antagonism *in vitro*.

4.10.1.4 Next steps

In order to follow up on the considered mechanisms of GC signaling disruption by Stano, measurements of the expression levels of both GR isoforms will help to substantiate the hypothesis of an inhibited homologous downregulation or an induced expression of GRα by Stano (option 2). In case that option 2 cannot be confirmed, transcriptional activation of the 4 x *GRE:Luc* reporter via the AR (option 3) may be true. To validate this, co-treatment experiments of transgenic larvae with Dex/ Preg + Stano in combination with a specific AR antagonist (such as Flutamide) could be useful, as the GRIZLY signal should be reduced by the antagonist if the AR plays a role (when compared to the Dex/Preg + Stano control). In addition, ChIP (Chromatin ImmunoPrecipitation) experiments with the reporter sequence will help to identify the receptors and cofactors that bind to the GRE element under the applied treatment conditions. By means of this, it can be verified if the AR is indeed involved in the observed effect

and it can possibly be pinpointed which cofactors contributed to the superactivation of the 4 x *GRE:Luc* reporter.

4.10.2 Spironolactone

4.10.2.1 The biological functions of Spironolactone and its mechanisms of action

Spiro is a synthetic steroid and a prodrug that has been marketed since 1959 for several medical applications including the treatment of high blood pressure, heart failure, low potassium, transgender hormone therapy, edema, hepatic cirrhosis and kidney disease. It is a member of the steroidal group of spirolactones, to which also Canrenone, Canrenoate (both major metabolites of Spiro), Potassium Canrenoate (Canrenoate-K; also a prodrug), Eplerenone and Drospirenone belong. All spirolactones are antagonists of the MR and some also possess progestogenic and/ or antiandrogenic properties. Spiro antagonizes the MR with high potency (Fagart et al. 2010; Kolkhof and Bärfacker 2017) and can also interfere with AR (anti-androgen; mechanisms include relatively strong AR antagonistic potential) (Bonne and Raynaud 1974; Luthy, Begin, and Labrie 1988; Rathnayake and Sinclair 2010), ER (mixed agonist and antagonist) (Levy et al. 1980), PR (agonist) (Sabbadin et al. 2016), GR (antagonist) (Campen and Fanestil 1982; Couette et al. 1992; Kolkhof and Bärfacker 2017) and PXR (agonist) (Cheng et al. 1976; Pelkonen et al. 1998) activity. However, after administration, the prodrug Spiro is converted *in vivo* and exerts its pharmacological effects majorly via its three major active metabolites: 7α-thiomethyl-spironolactone (TMS), 6β-hydroxy-7α-thiomethyl-spirono-lactone (HTMS) and Canrenone (Gardiner et al. 1989; Garthwaite and McMahon 2004; Karim 1978; Kolkhof and Bärfacker 2017), with TMS being the major metabolite (Karim 1978). Thus, the effect profiles for Spiro on the respective nuclear receptors may differ *in vivo*, depending on the metabolization state of the drug (Gardiner et al. 1989; Garthwaite and McMahon 2004; Karim 1978; Kolkhof and Bärfacker 2017).

The potential of Spironolactone to interfere with GR signaling

As specified above, Spiro can interact with many types of nuclear steroid hormone receptors, but the affinity for a certain receptor may be different for its metabolites. The pharmacological *in vivo* effects of Spiro are described in numerous publications, but unfortunately it is often not clear if the parent or an intermediate product exerts the observed effects (Kolkhof and Bärfacker 2017).

Campen & Fanestil published 1982 that Spiro showed a threefold higher affinity for the GR than Dex in rat hepatoma tissue culture (HTC) cells and that it was a potent full antagonist of Dex-induced tyrosine aminotransferase (TAT) enzyme activity in whole rat cells. Also *in vivo*, the researchers showed that Spiro completely inhibited Dex-induced enzyme activity in adrenalectomized rats. They concluded from these findings that Spiro is a GR antagonist but that it lacked GR agonist activity. This is in accordance with other publications assessing the effects of Spiro, where antagonism (Couette et al. 1992; Kolkhof & Bärfacker 2017), but not agonism of the compound was described for the GR.

The potential of Spironolactone to disrupt the Cortisol biosynthesis pathway

Spiro and/ or its metabolites can inhibit diverse steroidogenic enzymes including 11β-hydroxylase, 17α-hydroxylase and 17,20-lyase (Cheng et al. 1976; Colby 1981; Helfer, Miller, and Rose 1988; Menard et al. 1974; Rourke et al. 1991). For instance, Spiro inhibited the activity of adrenal 17α-hydroxylase in guinea pigs and dogs (Menard et al. 1974). Also, Kossor and collegues (1991) observed in guinea pig adrenal microsomes that Spiro is converted by 17α-hydroxylase into the reactive metabolite 7α-thio-spironolactone. The researchers further described a mechanism-based inhibition of 17α-hydroxylase within this metabolization step as Spiro binds competitively to the enzyme and thereby reduces its availability for functions within the steroidogenesis pathway.

More recent publications are available describing the effects of the Drospirenone, a member of the spirolactone group that was derived from Spiro and which is a potent PR agonist. For instance, as already mentioned in the chapter discussing progestin effects (section **4.9.3**), in one study, Drospirenone increased the levels of Preg, Prog and 17α-hydroxyprogesterone in an adrenal cell line (H295R) and reduced the activity of CYP17A1 (17α-hydroxylase, 17,20-lyase) and 3β-HSD in COS-1 cells transfected with human CYP17A1 or 3β-HSD (Louw-du Toit et al. 2017). In *in vitro* experiments, Drospirenone was shown to inhibit 17,20-lyase in carp gonads (Fernandes et al. 2014). Furthermore, in a human study with women carrying an implant containing Drospirenone, reduced ratios of 17α-hydroxyprogesterone to Prog were measured and reduced activities of 17α-hydroxylase and 17,20-lyase were reasoned by De Leo and collegues(2007), which correlates with the results from the above mentioned *in vitro* studies.

4.10.2.2 The effects of Spironolactone within the present study

In the GRIZLY assay, Spiro showed the same effects as Stano, as it inhibited GC signaling *in vitro*, but superactivated the Dex- and Preg-induced signal *in vivo*. Chemical analysis revealed no alteration of the larvae-internal Dex concentration, but in Preg-treated larvae, the amount of Cort was slightly reduced while Preg and Prog levels tended to be elevated. Thereby, a disrupted Cort biosynthesis was suggested.

The qPCR data for Dex-treated larvae showed that Spiro decreased solely the expression of *hsd11b2*, which is in contrast to the co-inductive effect within GRIZLY 2. However, although the *hsd11b2*mRNA levels were significantly reduced compared to the negative control (Dex + DMSO) due to low variations between the replicates, the effect was not very pronounced,with a gene expression ratio of 0.86.

The qPCR results for larvae treated with Preg + Spiro revealed the same pattern as for Preg + Stano, the mRNA levels of all Cort target genes were reduced, except for *fkbp5*, which was not altered. The expression ratio of *hsd11b2* (0.62) and *gilz* (0.69) was significantly and moderately decreased, possibly due to the reduced Cort production. The expression ratios of the two xenometabolic marker genes *cyp3a65* (0.25) and *cyp1a1* (0.34) showed stronger suppression by Spiro, suggesting an inhibitory effect of the compound on xenometabolic enzymes. Regarding the non-reduced expression of *fkbp5*, another mechanism of action might

have compensated for the lacking Cort, as it was hypothesized above for Stano. This mechanism might also be involved in the superactivation of GC signaling in GRIZLY 2 and GRIZLY 3.
In summary, the effects of Spiro among the different assays are heterogenous, which suggests complex interactions of Spiro and/ or several metabolites with the GC system, possibly involving different mechanisms of action which may compensate themselves.

4.10.2.3 Potential mechanisms of glucocorticoid signaling disruption by Spironolactone

As Spiro can be endogenously converted into diverse active metabolites, it is not clear if the Spiro-triggered responses within the present study were induced by the compound itself or by one of its metabolites (e.g. TMS, HTMS, Canrenone). However, the differing response of cells and larvae to Spiro treatment may indicate that the compound was metabolized *in vivo* and, thus, acted via different mechanisms in larvae than in cells.

GR antagonism *in vitro*
The concentration-dependent signal inhibition in GRIZLY 1 and the above mentioned literature describing GR antagonism by Spiro (Campen and Fanestil 1982; Couette et al. 1992; Kolkhof and Bärfacker 2017) allow me to assume that the compound antagonized the GR *in vitro*. It appears likely that Spiro was not converted into metabolites in the cells, due to lacking metabolic enzymes, and had itself, as a prodrug, an antagonistic effect on GR signaling. This antagonism possibly occurred context-specifically (**4.3.2**) within this cellular environment, where a relatively high amount of Spiro may have entered the cells.

***In vivo* transformation into an active metabolite with different effect mechanisms**
In larvae, Spiro may have been converted into a metabolite with a different behavior, which led to the superactivation of GC signaling according to the results of GRIZLY 2 and GRIZLY 3. The above cited literature implying interactions of Spiro and/or its metabolites with diverse nuclear receptors allow speculations about partial agonism of the metabolite on one of these receptors to be involved in the *in vivo* effect.
Interestingly, the active metabolite Canrenone was also a compound contained in the FDA library (screen concentration 11.7 µM), but its effects did not reach statistical significance due to the stringent cut-off criteria of $p < \alpha < 0.0001$ and the compound was therefore not considered a screen hit. However, Canrenone strongly superactivated GC signaling in both *in vivo* screens, in co-exposure with Dex (Mean rel. AUC = 386.25; p-value = 0.004) and with Preg (Mean rel. AUC = 297.46; p-value = 0.009). *In vitro*, it had no effect (Mean rel. AUC = 127; p-value = 0.08) in the GRIZLY assay. These findings strongly support my argumentation that Spiro was not metabolized *in vitro* and inhibited GC signaling in the cells as a prodrug (via GR antagonism), while it was metabolized *in vivo* (metabolic activation, section **4.3.1**), where the metabolite induced superactivation in GRIZLY 2 and GRIZLY 3. From the close similarity of the *in vivo* effects measured for Spiro and Canrenone, it appears likely that Spiro was converted into the active metabolite Canrenone in the larvae, which exerted the GRIZLY effect.

Superactivation of the 4 x *GRE:Luc*reporter *in vivo*

Regarding the *in vivo* effects of Spiro treatment, as before for Stano, different options can be proposed to explain the superactivation in the GRIZLY assay and the heterogeneous behavior inGRIZLY, UPLC-MS/MS and PCR experiments:

Option 1

Possibly, the active metabolite of Spiro was able to bind to the GR and to activate the 4 x GRE reporter construct, once the chromatin structure was opened by Dex or Cort (as it was also considered for the progestins (section **4.9.2**) and for Stano (section **4.10.1.3**). Apparently, this was not possible within the promotor regions of the selected endogenous target genes, as they were not transcriptionally upregulated by Spiro. Maybe, as it was already discussed for the superactivating effect of Stano in the *in vivo* assays (**4.10.1.3**; option 2 and 3), the observed overinduction of the GRIZLY signal is enabled within the regulatory region of the 4 x *GRE:Luc* reporter due to context-specific (section **4.3.2**) availabilities of coactivators and corepressors, but not within natural GREs, which lead to the dissimilar outcomes of transcriptional activity within the 4 x *GRE:Luc* reporter and the examined endogenous target genes of Dex and Cort.

Option 2

Alternatively, the metabolite of Spiro was able to activate the 4 x GRE reporter construct and endogenous GC target gene expression, but on Dex target genes this was not identified due to different mechanisms of action, counteracting the inductive effect context-specifically (**4.3.2***).* The reduced Cort production and the downregulation of Preg- but not of Dex-target genes support such opposing effects, which will be discussed in detail within the next paragraphs.

Disruption of Cortisol biosynthesis

The reduced Cort levels in larvae treated with Preg + Spiro that were measured by UPLC-MS/MS are in accordance with the literature mentioned above (Cheng et al. 1976; Menard et al. 1974; Rourke et al. 1991; Helfer et al. 1988; Kossor et al. 1991; Louw-du Toit et al. 2017; Fernandes et al. 2014; De Leo et al. 2007), where Spiro (or an active metabolite of it) was described to interfere with steroidogenic enzymes. Spiro or the metabolite most likely disrupted the biosynthesis of Cort (section **4.4.1.1**) in Preg-treated larvae within the present study. The steroid level profile indicates an accumulation of Preg and Prog, which may narrow the point of inhibition down to 17α-hydroxylase (see the steroid biosynthesis pathway in **Figure 3**). The findings from Kossor and collegues (1991) mentioned above support the idea that Canrenone is the active agent and that 17α-hydroxylase is the point of steroidogenesis disruption. However, also additional inhibitory effects on other steroidogenic enzymes, i.e. on 11β-hydroxylase could be possible.

However, Spiro may also have altered the larvae-internal levels of Preg, Prog and Cort via another mechanism. As one of the major mechanisms of action described for Spiro and its metabolites is MR antagonism (Fagart et al. 2010; Kolkhof and Bärfacker 2017), it could be expected that MR antagonism (similar to GR antagonism) initiates a positive feedback loop via the HPA axis (at least in mammals), which induces the *de novo* Cort production in order to

produce more ligands that can activate the MR. Accordingly, an increased Cort level could be anticipated in the larvae, which would provide a possible explanation for the observed superactivation in both *in vivo* assays. As instead the opposite effect, a tendency for reduced Cort was found in larvae treated with Preg + Spiro, the impacts of a decreased Cort biosynthesis due to inhibition of steroidogenic enzymes and of a stimulated *de novo* Cort production via the HPI-axis may possibly have compensated each other. This interplay may have led to the observed increased levels of Preg and Prog and the decrease of Cort, which was less pronounced than it was observed with other putative inhibitors of the steroid biosynthese pathway within this study (e.g. Mety, Gestri, Nor).

In larvae treated with Dex + Spiro, changes of the endogenous Cort level could not be detected due to sensitivity limitations of the UPLC-MS/MS system. However, here, an induced Cort production due to MR antagonism would probably be counteracted by the effect of Dex on the GR, as GR agonism is known to decrease the endogenous Cort production via a negative feedback mechanism. As an outcome, in larvae treated with Dex + Spiro, the Cort level would probably not be noticeably altered.

Effects on target gene expression

The trend for reduced Cort production in larvae treated with Preg + Spiro probably explains the decreased mRNA levels of the Cort target genes *hsd11b2*, *gilz*, *cyp3a65* and *cyp1a1*. The only target that was not altered by Spiro is *fkbp5*, which might be explained by the fact that this gene was the most sensitive of the Cort targets, whose expression was possibly strongly upregulated, even with a reduced amount of Cort.

For Dex + Spiro, the qPCR results are less easily interpretable, as *hsd11b2* was the only downregulated target, with a significantly but not very pronounced decreased gene expression ratio of 0.86. It may be considered that Spiro affected the steroidogenesis pathway in Dex-treated larvae similarly to what was assumed with Preg, but as the steroid levels were not quantifiable under these treatment conditions, no clear indications are given. Still, the downregulation of *hsd11b2* may suggest a decreased Cort production, as a reduced synthesis of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) might result from lower Cort mediated stimulation of expression.

As described in section **4.4.1.2** (Ferrari 2010; Leckie et al. 1998; Mune et al. 1995; White et al. 1997), 11β-HSD2 plays an important role for GC and mineralocorticoid signaling, because the enzyme prevents MR activation by Cort within mineralocorticoid target tissues. If 11β-HSD2 is inhibited, the ratio of Cort to Cortisone increases and the half-life of Cort may be prolonged, which would lead to Cort-induced mineralocorticoid effects, as it was observed in humans (Ferrari 2010). Downregulation of *hsd11b2* is the only common feature between the gene expression patterns from larvae treated with Dex + Spiro and Preg + Spiro and none of the other GRIZLY superactivators found in the FDA library significantly suppressed the Dex-induced transcription of *hsd11b2* (only Gestri showed a trend). Hence I wondered if the effect on *hsd11b2* might be specific for Spiro treatment, wherein MR antagonism possibly plays a role.

4.10.2.4 Next steps

According to the heterogeneous effects of Spiro, complex compound and metabolite interactions with the GC system were suggested and discussed above, but substantial future work will be necessary to investigate the underlying mechanisms of action. In the first step, larvae and cell exposure to important active metabolites of Spiro (such as Canrenone, TMS, HTMS,) and assessment of their effects within GRIZLY, UPLC-MS/MS and qPCR experiments will enable to allocate the effect origin. Possibly, certain mechanisms of action can be assigned to single metabolites.

Once the metabolites responsible for the discussed mechanisms are identified, GR activity can be measured under diverse treatment combinations by *in vitro* receptor binding studies. In addition, ChIP experiments of the 4 x GRE reporter gene sequence can be conducted, which will enable to identify the receptors and cofactors that bind to the sequence under diverse treatment combinations.

In order to pursue if steroidogenic enzymes are affected, the production rate of Spiro metabolites can be quantified relative to that endogenous metabolites by UPLC-MS/MS and the enzyme activity of selected enzymes can be quantified spectrophotometrically. For instance, in order to follow up on a potential competitive inhibition of 17α-hydroxylase by Spiro, the activity of 17α-hydroxylase could be assessed by comparing the endogenous metabolization rate for Preg to 17α-hydroxypregnenolone with that of Spiro to 7α-thio-spironolactone.

Furthermore, as the applied UPLC-MS/MS technique was not sensitive enough to quantify Cort levels in Dex-treated larvae, alterations of the endogenous Cort production by Spiro could be measured in future experiments via gene expression analysis of the *pomc* gene, where upregulation of the genewould indicate elevated ACTH activity and induced Cort production.

4.11 Natural estrogens and androgens

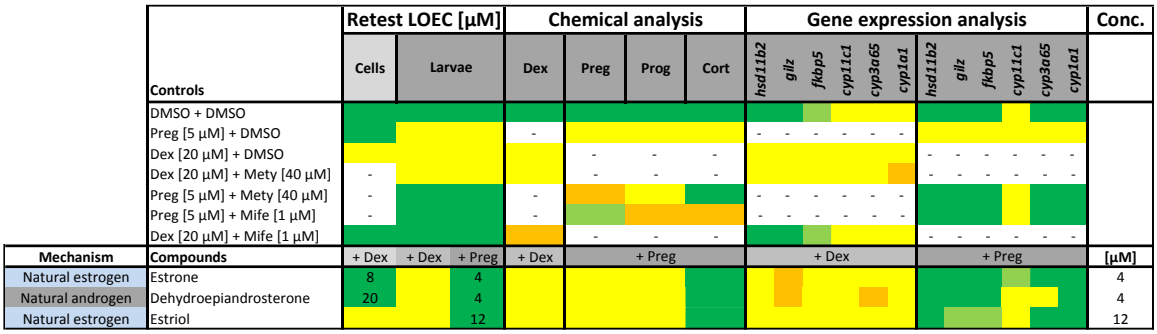

Figure 51: Natural estrogens and androgens (extract from Figure 45)

4.11.1 The biological functions of Estrone, Estriol and dehydroepiandrosterone (DHEA) and their mechanisms of action

Estrone and Estriol

Estrone (E1) and Estriol (E2) are natural estrogens with relatively weak agonistic potentials for the ER, when compared to the third and most potent natural estrogen Estradiol (E3). In humans, distinctly lower binding affinities and transactivation capacities of Estriol relative to Estradiol

were found *in vitro*for ERα (11.3% and 10.6%) and ERβ (17.6% and 16.6%). For Estrone, even lower relative binding affinities and transactivation capacities for ERα (4% and 2.6%) and ERβ (3.5% and 4.3%) were measured in the same study (Escande et al. 2006). However, Estrone, but not Estriol, can be metabolized into Estradiol (for an overview of the steroid hormone biosynthesis pathway see **Figure 3**), which mediates most of the estrogenic *in vivo* potency of Estrone (Kuhl 2005). Both compounds can be applied in hormone replacement therapies for menopausal symptoms, but nowadays, Estrone has been replaced by more potent estrogens. (Nabulsi et al. 1993; Ross et al. 2000)

DHEA

DHEA is a natural androgen that serves as a hormonal biosynthetic precursor without an active role on its own. It is metabolized by 3β-HSD and additional specific steroidogenic enzymes into other androgens (e.g. Testosterone, the major androgen in humans; Dihydrotestosterone (DHT) and androstenedione)or into estrogens (Miller 2002). In the latter case, DHEA is first converted into Estrone, then into Estradiol (see **Figure 3**), which is why it finds also medical application in hormone replacement therapies for menopause (Labrie et al. 1998). Additionally, it is occasionally prescribed for the treatment of adrenal insufficiency in combination with the standard medication of Cort and synthetic mineralocorticoids (e.g. Fludrocortisone) in order to restore the steroid levels normally produced by the zona reticularis (Alkatib et al. 2009; Allolio et al. 2007; Gurnell et al. 2008).

The potential of estrogens and DHEA to interfere with GR signaling

For DHEA, antiglucocorticoid properties are often described in the available literature. For instance, research investigating the posttraumatic stress disorder (PTSD), a disease that is associated with HPA axis and immune function alterations, revealed a close correlation between endogenous levels of Cort and DHEA (Olff et al. 2007). In this disease, increased DHEA and decreased Cort levels, in combination with an induced production of the proinflammatory cytokines TNF-α and IL-6 are often found in human patients (Gill, Vythilingam, and Page 2008). In human depressed patients, decreased Cort : DHEA ratios (when compared to healthy controls) can be measured, which result from the antiglucocorticoid effects of DHEA, as the androgen precursor protects the organism from adverse effects of Cort overexpression (Young, Gallagher, and Porter 2002). However, the molecular mechanisms underlying the antiglucocorticoid properties of DHEA appear to be not yet completely understood. Although Kalimi et al. (1994) described more than two decades ago that DHEA mediates its antiglucocorticoid effects by modulating the GR, as they observed downregulation of GR expression in rat liver upon DHEA treatment, publications investigating this mechanism of action in more detail appear to be limited.

Quite recently, the group of Pinto and collegues (2015) found out that DHEA dose-dependently induced the mRNA expression of GRβ, while GRα levels remained constant in a THP1 monocytic cell line. They concluded that the antiglucocorticoid effect of DHEA on the expression of proteins related to GC signaling may be caused by the decreased GRα : GRβ ratio that inhibits GRα activity (as it was described in section **4.4.2.3**).

Also Estrogens have been characterized to interfere with GR signaling in mammals (Heitzer et al. 2007). For instance, it was observed in a human model breast cancer cell line (MCF-7) that treatment with the ER agonists Estradiol, Diethylstilbestrol and Genistein modulated transcriptional regulation of GR expression. The ligand-bound ER inhibited the induction of mRNA and protein levels of the GR. The researchers concluded from these and from additional data that ER agonists downregulate the GR via the proteasomal degradation pathway (descibed e.g. by Kinyamu & Archer 2003 and Calligé & Richard-Foy 2006), which is a mechanism related to posttranslational modification (section **4.4.2.2**). Also, interference of estrogens with the activity of 11β-HSD2 (section **4.4.1.2**) has been reported for several *in vivo* and *in vitro* studies, suggesting this enzyme to be a main regulator of estrogen-mediated disruption of GC signaling (Tremblay et al. 1999).

The potential of estrogens and DHEA to disrupt the Cortisol biosynthesis pathway

It was already described for the progestins (section **4.9.3**), for Stano (section **4.10.1.3**) and for Spiro (section **4.10.2.3**), which are structurally very similar to endogenous progestogens or androgens, that they can competitively bind to enzymes within the steroidogenesis pathway and decrease the enzyme functionality for the production of Cort (Africander et al. 2011 and Louw-du Toit et al. 2017). Likewise, also DHEA can possibly inhibit the Cort synthesis, as the androgen is naturally converted into Androstenedione by 3β-HSD, the key enzyme involved in the biosynthesis of practically all steroids (see **Figure 3**). Thus, if this enzyme is majorly occupied by DHEA, the conversion of Preg into Prog and of 17α-hydroxypregnenolone into 17α-hydroxyprogesterone by 3β-HSD may be reduced, which can disturb all downstream synthesis steps.

Similarly, also the estrogens could competitively bind to steroidogenic enzymes and thereby disrupt the Cort biosynthesis. For instance, one research group investigated the impact of flavonoid phytochemicals on the production of Cort and on the activity of several steroidogenic enzymes (Ohno et al. 2002, 2004). They found out that several flavonoid phytochemicals, among those genistein, a phytoestrogen and a potent ER agonist, significantly decreased the synthesis of Cort in human adrenocortical H295R cells. Furthermore, Genistein and other flavonoids significantly inhibited the activity of 3β-HSD and 21-hydroxylase, two enzymes which are necessary for the conversion of Preg to Cort (see **Figure 3**). Genistein and Daidzein were shown to be competitive inhibitors of 3β-HSD. Also other steroidogenic enzymes were blocked by some flavonoid phytochemicals, for instance 6-hydroxyflavone inhibited the activity of CYP17A1 and 11β-hydroxylase.

Likewise, the estrogenic potential to inhibit 3β-HSD was described by another study, in which Estradiol significantly reduced the activity of this enzyme and, thus, prevented the metabolization of DHEA into Androstenedione within the brain of adult zebra finch (songbird; *Taeniopygia guttata*) (Pradhan, Yu, and Soma 2008).

4.11.2 The effects of Estrone, Estriol and DHEA within the present study

Within the present study, all three natural sex hormones inhibited Preg-induced GC signaling *in vivo*. Estrone and DHEA furthermore decreased the activity of Dex in GRIZLY 1, but at higher concentrations than which were significantly effective in GRIZLY 3 [each 4 µM]. In GRIZLY 1, Estrone [8 µM] reduced the signal down to 50 - 80% and DHEA [20 µM] led to 70 - 95% of maximal GC signaling activity, as it was described for **Figure 36 b** and **f**.
Their impact in GRIZLY 3 was more pronounced, as here both compounds inhibited Preg-induced signaling more or less completely, with no remaining signal intensitiy with Estrone (**Figure 36 d**) and 5 - 35% of the maximal activity with DHEA (**Figure 36 h**). In order to demonstrate a clear concentration-dependency of their inhibitory effects, retests with lower concentrations of both compounds were conducted, which showed a LOEC of 0.25 µM for Estrone and of 4 µM for DHEA (both in in appendix, **Supplementary Figure II**). The inhibitory effect of Estriol in GRIZLY 3, although significant, was not that pronounced, as the estrogen only led to a signal decrease down to 50 - 80% of the maximal activity of Preg (**Figure 37 h**), even though it was applied in a three-fold higher concentration [12 µM] than Estrone and DHEA.

4.11.3 Potential mechanisms of glucocorticoid signaling disruption by of Estrone, Estriol and DHEA

In vivo
Interestingly, the extent of signal inhibition by each compound correlates well with the data obtained by UPLC-MS/MS experiments. All three substances significantly disrupted the biosynthesis of Cort (section **4.4.1.1**), but while with Estrone only 3% of the total amount of Preg-derived Cort (Preg + DMSO control = 100%) were produced, and with DHEA only 5%, Estriol still allowed 57% of the maximal amount of Cort to be synthesized. From this correlation, a dependency of the strength of the GRIZLY 3 response (Preg-induced signal inhibition: Estrone [4 µM] > DHEA [4 µM] >>> Estriol [12 µM]) on the respective fraction of produced Cort (Inhibition of Cort production: Estrone [4 µM] > DHEA [4 µM] >>> Estriol [12 µM]) is clearly noticeable. This correlation strongly suggests a disrupted steroidogenesis to be the mechanism underlying the signal inhibition in GRIZLY 3 by Estrone, Estriol and DHEA. DHEA potentially competed with Preg for the binding and the enzymatic activity of 3β-HSD, whereby the Cort biosynthesis pathway was disrupted. Estrone (or Estradiol, as Estrone could have been metabolized into this more potent estrogen) and Estriol may also have inhibited 3β-HSD, or potentially also other enzymes such as 21-hydroxylase, like it was already described for Genistein and Estradiol (Ohno et al. 2002, 2004; Pradhan et al. 2008).

Also qPCR analysis data correlate highly with GRIZLY 3 and UHPLC-MS/MS results, as Estrone treatment resulted in the strongest, DHEA in a slightly weaker and Estriol in the weakest downregulation of Preg-induced target genes (Estrone [4 µM] > DHEA [4 µM] >>> Estriol [12 µM]), as visible in detail in **Figure 44.I**(with *cyp3a65* being the only exception, as its expression was comparably low with Estrone and Estriol, but not suppressed by DHEA).

A possible reason for the different effect magnitudes of Estrone, DHEA and Estriol may be that Estrone and DHEA were converted into the more potent estrogen Estradiol (metabolic activation, section **4.3.1**), which may have exerted the disruption of Cort biosynthesis, whereupon the Preg-stimulated GRIZLY signal and target gene expression was suppressed. Estriol, in contrast, could not be metabolized to Estradiol and had, thus, a weaker inhibitory effect due to the lower potency of this estrogen. Alternatively, also without metabolic transformation, natural Estrone, DHEA and Estriol may have had different potencies to disrupt the steroidogenesis pathway. Moreover, they could have been taken up by the larvae to different degrees.

In vitro

Estriol, the compound with the apparently lowest capability to disrupt the synthesis of Cort *in vivo*, was also the only candidate out of the three sex steroid hormones which had no effect in GRIZLY 1. As the other two substances reduced GC signaling *in vitro* at higher concentrations, they appear to have partly antagonistic properties on the GR, possibly due to a particular cellular context (section **4.3.2**), which may have enabled a specific mechanism of GR signaling disruption (section **4.4.2**).

Similarly to this, inhibition of GC signaling in GRIZLY 1 was observed before for several other ligands of steroid hormone receptors within the present study, as also Nor, Spiro and Stano inhibited GC signaling *in vitro* at higher concentrations and Gestri at the same concentration that had co-activating effects *in vivo* in GRIZLY 2. It may be assumed that all these steroidal ligands (including GCs) are generally available in higher concentrations *in vitro* than *in vivo*, as an increased uptake of the compounds by the cells is likely, due to a lower number of transport barriers to pass and more limited metabolic capabilities of cells compared to the whole organism by the cells. Accordingly, due to this specific cellular context (section **4.3.2**), there might be a higher chance for the steroids to unspecifically bind to the GR, whereby they could partly compete with GCs or otherwise cause the observed (partial) antagonistic effects. However, it has to be noted the antagonism of each compound is still relatively weak when compared to the potent GR antagonist Mife.

The higher antagonistic potential of Estrone over DHEA and Estriol (Estrone [8 µM] > DHEA [20 µM] >>> Estriol [-]) *in vitro* may also suggest that within the *in vivo* experiments of this study, a variable uptake of the compounds or diverse potencies of the natural steroid hormones to interfere with GC signaling (i.e. to disrupt the Cort biosynthesis) may rather underlie the different effect strengths of the compounds than a potential metabolization of Estrone and DHEA into Estradiol, which is restricted to steroidogenic tissues (i.e. the adrenal gland) and could, thus, not have occurred within the AB.9-*GRE:Luc* cells.

4.11.4 Future steps

The present results indicate that Estrone, Estriol and DHEA disrupted the biosynthesis of Cort. In order to further validate this hypothesis, it is necessary to assess if Estrone and DHEA exerted their effects themselves or whether the compounds were converted into Estradiol or other

metabolites, which were responsible for the *in vivo* activity. For this, Estradiol will be co-treated with Dex/ Preg and the effects of these exposure combinations in GRIZLY, UPLC-MS/MS and qPCR experiments will be compared with the results for Estrone and DHEA. Furthermore, the endogenous levels of Estradiol under all exposure conditions will be assessed via UPLC-MS/MS. Once the effect origin is clarified, also the precise point of inhibition can be located by chemical analysis, testing for accumulation and deficiency of metabolites directly serving as substrates and products of a specific steroidogenic enzyme (e.g. accumulation of DHEA and deficiency of Androstenedione would indicate inhibition of 3β-HSD). In addition, the activity of the presumably affected enzyme can be quantified, for instance in application of a photometrical *in vivo* assay. Moreover, regarding the putative partial GR antagonism by Estrone and DHEA *in vitro,* receptor binding studies and measurements of GRα : GRβ levels should help to verify this speculation.

4.12 Conclusion and future perspectives

The GRIZLY assay served as a reliable, easy and fast tool to identify compounds that interfere with GC signaling via diverse pathways and enabled to select from a large library of substances a few candidates of high interest. The parallel application of both the cell and larvae system allowed furthermore to differentiate between *in vitro* and *in vivo* effects, while the comparison between direct and indirect stimulation of GC signaling *in vivo* served the pre-categorization of underlying effect-mechanisms and suggested promising follow-up studies. By means of concentration-dependent retesting, the identified hits were confirmed and high reliability of the GRIZLY results was demonstrated. According to the first aim of this study, the GRIZLY assay has been proven to be a suitable tool for the detection of GC signaling inhibitors. Moreover, it enables also the detection of superactivators, i.e. compounds which can increase GC signaling, which constitutes an additional useful application of the GRIZLY assay.

The tiered testing approach was a good strategy to systemically extract the candidates of interest from a large amount of substances. Identified screen hits were confirmed by concentration-dependent retesting. Verified inhibitors and superactivators of the GRIZLY signal were then subjected to chemical and gene expression analysis in order to gain more information about their putative mechanisms of GC signaling disruption.

The UPLC-MS/MS system robustly detected and validated inhibition of the Cort biosynthesis by several compounds and helped to narrow down the point of disruption within the steroidogenesis pathway. Thus, it was a very suitable technique to achieve the second aim of this study, the identification of GC biosynthesis disruptors. Chemical analysis moreover allowed to identify changes in larvae-internal levels of Dex and Preg, which gives indications for potentially disrupted drug uptake or -metabolism being possibly the reason for several observed compound effects.

Gene expression analysis was beneficial regarding numerous aspects of the effect evaluation. It served as an additional validation for the UPLC-MS/MS data, and the qPCR experiments

revealed new information about compound effects on target gene expression. The obtained effect spectra expand the information on compound effects and are a highly useful readout for future investigations, as they mirror well the potential effect complexity of a drug or pollutant. For some compounds, where the spectrum showed consistent effects within all experiments, one major mechanism of action is strongly indicated, such as GR antagonism in case of Mife or GC biosynthesis disruption in case of Estriol. For other drugs, in contrast, multilayered effects were indicated, suggesting several combinational or counteracting mechanisms to occur simultaneously. Furthermore, the effect spectra allowed to identify clusters among molecular effects of GC signaling disruptors and revealed patterns common to chemical classes of compounds. Thus, it enabled the achievement of the third aim of this study, to identify compounds with - so far - unknown potential to interfere with GC signaling and with unknown mechanisms of action. The obtained results from the tiered testing approach helped me to narrow down the mechanisms of action possibly underlying the compound effects and gave me ideas which future experiments will be necessary to characterize their mechanisms in detail.

Regarding future applications of the GRIZLY assay for pharmaceutical and environmental risk evaluation, especially the possibility for whole-organism assessment in zebrafish larvae provided by the assay is a huge advantage when investigating drug effects. The compound bioavailability, metabolism, pharmacokinetics and interference with endogenous synthesis pathways are implicated in the effect outcomes, as observed within the current study, where the compound effects and their interactions with other drugs (natural prehormone Preg and synthetic GC Dex) could be assessed regarding their impacts not only on steroid hormone receptor signaling, but also on drug pharmacokinetics, drug metabolism, steroidogenesis and target gene transcription.
Thus, *in vivo* studies in zebrafish larvae, like the GRIZLY assay, enable us to understand the manifold effect mechanisms of pharmaceuticals in the whole organism. This will help to further elucidate their - possibly severe - side effects and may increase the comprehension of cell- and tissue-specific intracellular responses to drugs and their relative benefit to risk ratio.

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Appendix

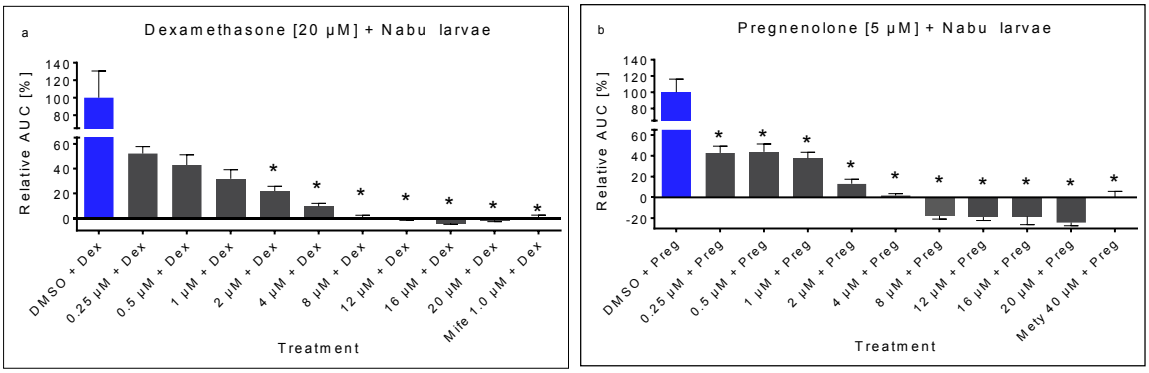

Figure I: Concentration-dependency of the inhibitory effect of Nabu on Dex- (**a**) and Preg (**b**)-induced GC signaling. The LOEC of Nabu was determined at 2 µM in co-treatment with Dex and at 0.25 µM in combination with Preg.

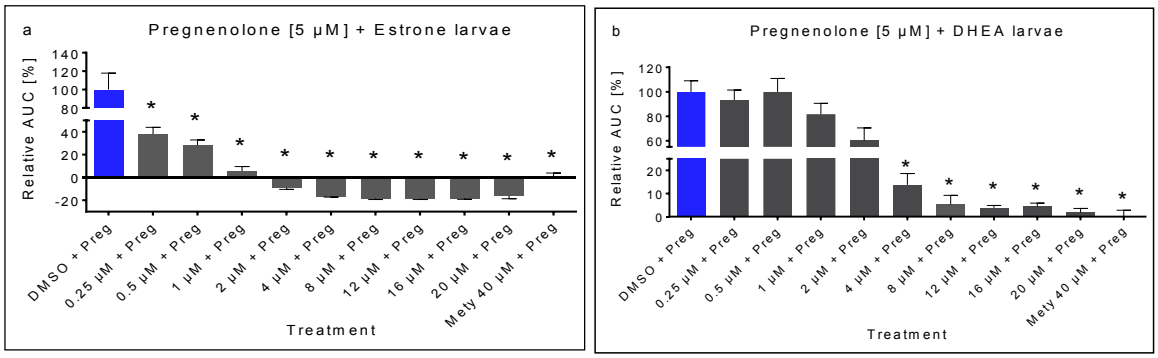

Figure II: Concentration-dependency of the inhibitory effect of Estrone (**a**) and DHEA (**b**) on Preg-induced GC signaling. The LOEC of DHEA was determined at 4 µM and that of Estrone at 0.25 µM.

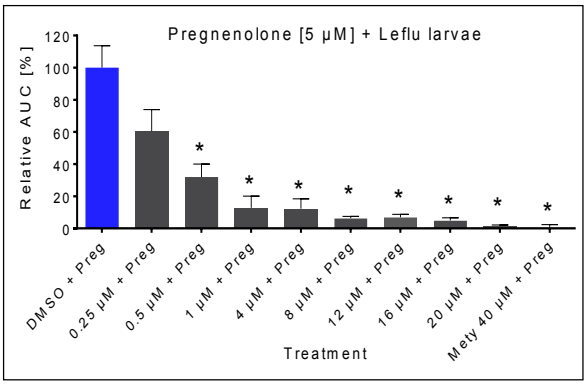

Figure III: Concentration-dependency of the inhibitory effect of Leflu on Preg-induced GC signaling. The LOEC was determined at 0.5 µM.

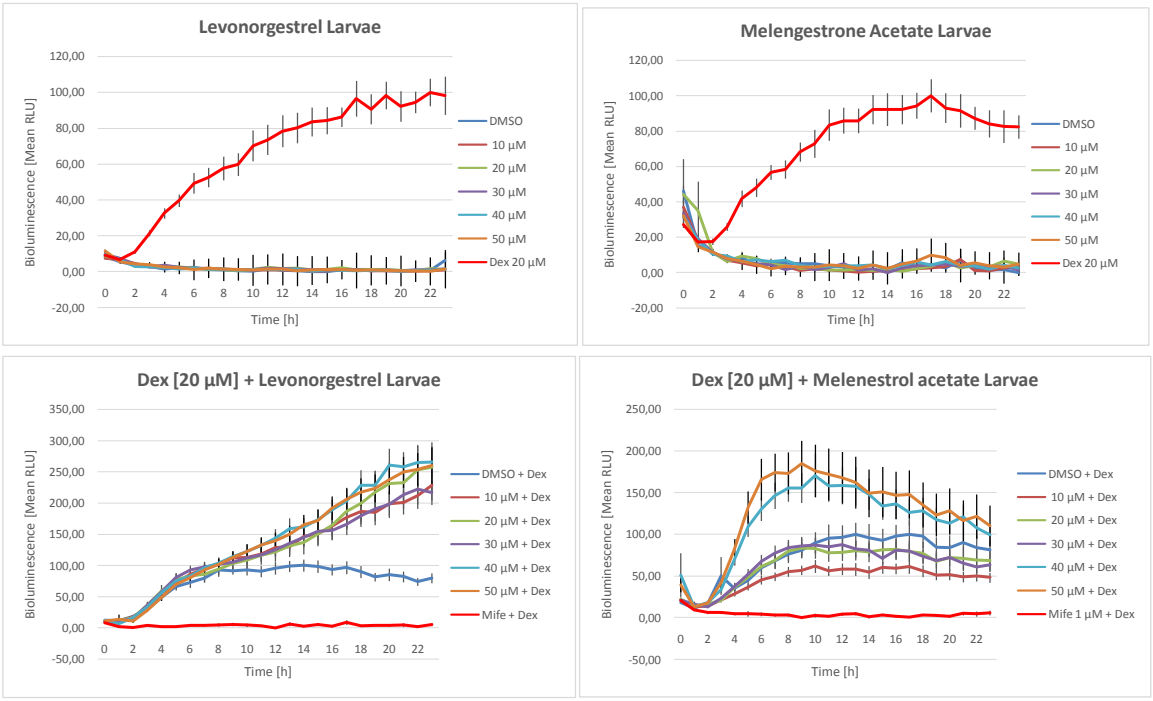

Figure IV: Bioluminescent traces of Levonorgestrel when exposed alone to larvae (**a**) or in co-treatment with Dex (**b**) and of Melengestrol acetate alone (**c**) and with Dex (**d**).

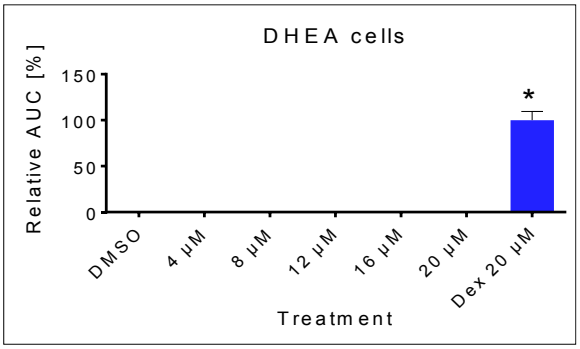

Figure V: No signal activation by DHEA via receptor binding to the 4 x GRE sequence in transgenic AB.9-*GRE:Luc* cells.

Tabelle A Optimized ESI-MSMS parameters and MRM transitions between precursor ions and product ions for each compound

Analyte	RT [a] [min]	Precursor ion [m/z]	DP [b] [V]	Product ions [c] [m/z]	CE [d] [V]	CXP [e]
Cortisol	0.57	363.1	86	120.9/105.0	35/63	10/10
Corticosterone	0.76	347.1	91	329.3/120.9	23/33	20/10
Dexamethasone	0.71	393.1	61	373.3/355.2	13/19	22/20
Progesterone	1.17	315.2	86	97.0/109.1	35/33	8/10
Pregnenolone	1.14	299.3	96	281.3/159.2	18/32	10/15
Pregnenolone-D4	1.14	303.1	96	284.9	31	10

[a] Retention time
[b] Declustering potential
[c] Quantifier ion/ qualifier ion
[d] Collision energy
[e] Cell exit potential