Ginsenosides as selective glucocorticoid drugs: agonists, antagonists, and prodrugs

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Chapter 1

Introduction

1. Inflammation

Inflammation is a critical biological process that occurs in response to a wide range of external and internal stimuli such as the presence of pathogens, toxic compounds, wounding, or disease conditions [1-3]. Inflammation involves the release of molecules that stimulate the dilation of blood vessels and guide the influx of immune cells [1-5]. This response is necessary to deal with tissue damage and to restore homeostasis as a prerequisite to tissue repair [3-5]. The classical symptoms associated with acute inflammation are well known: heat, swelling, redness, and pain [3-5]. While acute inflammation is essentially a protective response, it can become very dangerous and lead to loss of function, when it is uncontrolled or persists and becomes chronic [3,6,7]. The acute respiratory distress syndrome that is seen in critically ill coronavirus disease 2019 (COVID-19) patients is an example of an uncontrolled inflammatory response [8,10]. Persistent inflammation is associated with various chronic inflammatory or autoimmune diseases, like diabetes, cardiovascular diseases, inflammatory bowel diseases, asthma, and rheumatoid arthritis [6,11]. Anti-inflammatory medications are indispensable to relieve the symptoms of these conditions [9,12]. Considering the high prevalence and seriousness of the many diseases associated with inflammation, many research efforts go into the development of more effective and specific classes of anti-inflammatory drugs [13,15].

1.1. Regulators of inflammation

An inflammatory response can be triggered instantaneously by the release of damage signals (for example hydrogen peroxide) from injured tissue. This response is then sustained by the activation of gene expression programs leading to the production of additional inflammatory mediators and proteins in the defense response. Toll-like receptors (TLRs) are one of the main classes of receptors that recognize danger signals or molecular patterns associated with pathogens. In turn, TLRs activate the transcription factor complex nuclear factor-kappa B (NF-κB), the lynchpin of the immune response [16,17]. Together with several other immune-related transcription factors, NF-κB initiates a pro-inflammatory cascade involving the production of key cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and interleukin-6 (IL-6), as well as chemokines such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1 or CCL2)[1-5,16-20]. The spike in cytokine and chemokine production and changes in the extracellular matrix facilitates immune cell migration and further inflammatory action [1,21,22]. Innate immune cells, such as neutrophils and macrophages, are the first to migrate to the area of tissue damage [1,2,21-24]. Diapedesis, or the movement of immune cells through the capillary walls of the blood vessels into the inflamed tissue, is an essential process during this part of the inflammatory response [23,25].

The attraction of neutrophils and macrophages activates a whole array of downstream processes that directly attack the source of inflammation. The pro-inflammatory actions of neutrophils include phagocytosis, production of reactive oxygen species, and release of neutrophil extracellular traps (NETs) to capture and kill microbial invaders [23-28]. Furthermore, neutrophils produce cytokines that signal the activation and migration of macrophages [23-29]. Macrophages migrate to the site of inflammation and mature into a continuum of polarized phenotypes, of which M1 (pro-inflammatory) and M2 (anti-inflammatory) types are considered as the two extremes. The M1 type macrophages

phagocytize dead neutrophils and employ several intracellular killing mechanisms to help combat microbial threats, while the M2 type macrophages function in the wound-healing component of the immune response [11,26,30-32].

2. Glucocorticoids as anti-inflammatory drugs

Glucocorticoids (GCs) are a class of steroid hormones that are widely used to treat inflammatory diseases [33,34]. More than seventy years ago, the GC Compound E (cortisone) was used to treat rheumatoid arthritis (RA) patients, and their symptoms were found to be alleviated [35]. Since then many efforts have been made to synthesize and modify GCs for pharmaceutical purposes [36-38]. Nowadays, various synthetic GCs (e.g., prednisolone, dexamethasone, and beclomethasone) are utilized in the clinic [39]. GCs differ in their solubility, biological half-life, and affinity for the receptor that mediates their action. These drugs can be administrated via different routes, for example orally, intravenously or via nasal sprays and skin creams [12,40,41]. GCs are widely prescribed to treat various immune-related diseases, such as asthma, dermatitis, several autoimmune diseases, such as multiple sclerosis and RA, and even some cancers, such as leukemia [42,43]. They have also been applied to treat inflammatory complications of infectious diseases, such as tuberculosis [44]. Recently, dexamethasone was adopted to treat patients with COVID-19, and was found to reduce the mortality of the subset of patients with severe respiratory complications [9].

2.1. Mechanisms of action of glucocorticoids

Endogenous GCs play a crucial role in the stress response, which is conserved among all vertebrates [45-48]. Cortisol is the main stress-related GC in humans and fish, whereas in rats and mice corticosterone is the main endogenous GC [45,46]. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, causing the release of cortisol or corticosterone from the adrenal cortex [45,46]. These GCs affect virtually every molecular, cellular, and physiological system of vertebrate organisms [45,46], and they are crucial in various biological processes such as growth, reproduction, metabolism, immune and inflammatory reactions, the central nervous system, and cardiovascular functions [45-49].

GCs reach their target cells through the blood stream and can diffuse through the plasma membrane and bind to an intracellular receptor, the glucocorticoid receptor (GR). The GC/GR complex can then translocate to the nucleus and modulate the expression of a plethora of target genes [49,50]. The GR consists of several domains. The N-terminal domain of the receptor contains a primary transactivation domain (the ligand-independent activation function (AF)-1), which has a significant role in interacting with transcriptional coregulators [50,51]. The DNA binding domain (DBD) contains two zinc finger motifs via which the GR binds to specific target sites, glucocorticoid-response elements (GREs), in the DNA [50,51]. GCs bind to the ligand-binding domain (LBD), which consists of 12 α-helices and four β-sheets, and contains the second (ligand-dependent) transactivation domain (AF-2), which is critical for GR activation [52].

The GR can signal through various mechanisms (Fig.1). Dimeric GRs can bind to GRE sequences to promote the transcription of responsive genes, and this process is called transactivation [53]. Additionally, GR can inhibit gene transcription when it binds to negative GREs [54]. At the same time, monomeric GRs can modulate the activity of other

transcription factors such as STAT5 and AP-1by binding to composite response elements, or by tethering to other transcription factors, such as STAT3, AP-1, and NF-kB and thereby negatively or positively regulating the transcriptional activity of these factors [55]. As a result of these actions, the GR stimulates the transcription of genes encoding anti-inflammatory proteins, whereas the transcription of pro-inflammatory genes is suppressed [55]. Besides its effects of transcription (the 'genomic' signaling pathway), GR can also trigger immediate and reversible effects through 'nongenomic' signaling pathways, through activation of cytosolic and membrane-bound GRs, thereby modulating the fluidity and composition of the membrane or interacting with membrane receptors and kinases, which results in the modulation of several intracellular signal transduction cascades [56]. As a result, through activation of GR, GCs modulate extensive functions in our body, along with the immune, metabolic,
reproductive, cardiovascular, and vital fearful devices, supporting the frame to address stress and keep homeostasis [57,58].

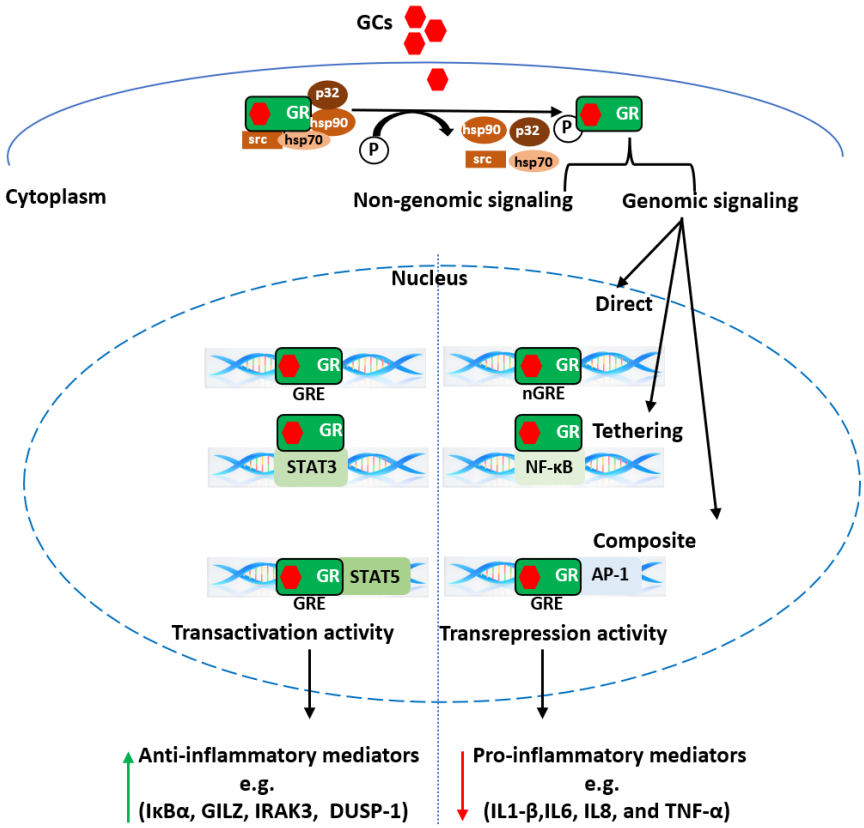


Figure 1. *GR signaling pathways. Glucocorticoids bind to GR and trigger both genomic and non-genomic signaling pathways. For its genomic action, GR translocates to the nucleus after glucocorticoid binding, where it can function in three ways. All three mechanisms can activate or repress gene expression.*

2.2. Anti-inflammatory effects of glucocorticoids

GCs exert anti-inflammatory effects on the immune system in several ways [34,54-56,59]. GCs inhibit the activity of transcription factors downstream of the TLR signaling pathway, such as NF-kB and AP-1, which are critical for initiating inflammation [55]. This mainly involves the tethering of GR to these transcription factors through protein-protein interactions. Moreover, GCs can enhance the expression of inhibitors of TLR signaling, such as dual-specificity protein phosphatase 1 (DUSP1) [60], IL-1 receptor-associated kinase 3 (IRAK3)[59], and NF-kB inhibitors [61]. As a result, GCs suppress the transcription of downstream genes encoding pro-inflammatory cytokines and chemokines, which are essential for the promulgation of inflammation, like IL-1, IL-6, IL-8, TNF, and CCL2/MCP-1 [62,63]. Through decreasing the levels of these pro-inflammatory mediators, GCs also reduce the extravasation of leukocytes and their migration towards inflammatory foci [64][65]. Moreover, GCs suppress antigen presentation by dendritic cells (DCs), T-cell stimulation, and immunoglobulin production by B-cells. GCs also inhibit vascular permeability and expansion by suppressing the production of lipid mediators such as eicosanoids and prostaglandins [63]. Finally, GCs mediate the resolution phase of inflammation via increasing the expression of Annexin-1, directing neutrophil apoptosis [66], and promoting the differentiation of macrophages to an anti-inflammatory (M2) phenotype with high expression of scavenger receptors, which is fundamental for the clearance of apoptotic cells and debris [67-69]. These various effects of GCs promote the clearance of inflammation and restoration of homeostasis [67-69].

2.3. Roles of GCs beyond immune suppression

Despite GCs being renowned for their repressive effects on the immune system, it has become clear that their effects are much more complex. Under specific biological conditions, GCs have been shown, in specific cell types, to play pro-inflammatory roles rather than their classical anti-inflammatory roles [70,71]. For instance, GCs can increase the level of TLR2, leading to increased secretion of critical cytokines in HeLa cells (IL-13, IL-8, TNF-α) [72,73]. Induction of the expression of a member of the NOD-like receptor family (NLRP3) was also reported by GCs in macrophages, triggering the secretion of IL-1B, IL–6, and TNF-α [74]. GCs have also been shown to increase the expression of the purinergic receptor P2Y2R in a microvascular endothelial cell line, causing an increase in IL-6 secretion [75]. Besides the effects of cell type-specificity, the timing and treatment conditions of GC treatment also play an essential role in determining the effects. For example, whereas prior exposure to GCs triggers the pro-inflammatory response to a lipopolysaccharide (LPS) challenge, post-exposure to GCs inhibits this response [75]. The dose of GCs can also influence their effects, since low dose GC treatment was found to promote inflammation, while a high dose of GCs resulted in suppression of inflammation in macrophages [76]. This GC-triggered induction of inflammatory signals may represent a sensitization of cells to inflammatory mediators to enhance the propagation of the inflammatory reaction [76]. Taken together, the physiological and therapeutic outcomes of GCs result from the complex signaling mechanisms and the treatment conditions.

2.4. Side effects of GCs

While GCs are highly effective anti-inflammatory drugs, their clinical use is limited by their side effects [77]. GCs suppress the HPA axis during prolonged systemic exposure and subsequently prevent cortisol production [34,78]. GR transactivation activity enhances the expression of genes encoding proteins such as tyrosine aminotransferase (TAT), phosphoenolpyruvate carboxykinase 1 (PCK1), and FK506 binding protein 5 (FKBP5)[79,80]. This transactivation activity triggers several severe side effects like type II diabetes, hypertension, and muscle and skin atrophy [77,81-86]. However, GR's transrepression activity has been shown to contribute to decreased cortisol levels and osteoporosis [77-79,83].The therapeutic immunosuppressive effect of GCs, affecting the function and production of immune cells, can also induce side effects by enhancing infectious complications [44,78]. To improve the benefit/risk ratio of GC therapy, different methods for delivering GCs have been adopted to decrease the systemic effects. For instance, inhaled GCs to treat asthma [87], intra-articular injection to treat RA [88], topical creams applied on the skin for dermatological problems [89], and ocular drops for eye conditions [90] induce local effects. However, due to the systemic distribution of the GCs upon absorption into the bloodstream, side effects are not fully abolished [38,41,77]. To overcome these problems regarding GC therapy, developing a GC prodrug, acting specifically at the site of inflammation rather than having a systemic effect, would be beneficial for treating inflammatory diseases.

2.5. Resistance to GCs

Besides the side effects of GC therapies, another issue that restricts the usage of GCs is the occurrence of resistance [91-94]. GC resistance is described by a decreased sensitivity and a reduction of the maximal response to GCs, which may be triggered in patients with different diseases, such as chronic obstructive pulmonary disease (COPD), asthma and RA [91-94]. Multiple molecular mechanisms have been investigated to account for this GC resistance [98]. In GC-resistant asthma patients, defective GR ligand binding and nuclear translocation were observed, triggered by GR phosphorylation by mitogen-activated protein kinase (MAPK) or GR nitrosylation. These types or resistance could therefore be targeted by utilizing kinase inhibitors or nitric oxide (NO) synthase inhibitors [99,100]. Another mechanism of GC resistance is related to an increased expression of an alternative splice variant of GR, GRβ, which functions as a dominant-negative inhibitor of the canonical GR α-isoform [101]. Increased activation or expression of some pro-inflammatory transcription factors, including AP-1, NF-kB, and STATs, have also been reported to play a role in GC resistance [102,103]. Other possible mechanisms underlying GC resistance include reduced histone deacetylase 2 (HDAC2) activity [104], which influences the suppression of inflammatory gene expression by GCs, and elevated expression of the efflux pump P-glycoprotein, which transports foreign substances out of the cell [91,95-97]. The downregulation of GR expression upon GR activation is a well-established phenomenon that is known to contribute to GC resistance in patients receiving chronic GC treatment [105]. Although the exact mechanisms underlying GC-induced GR downregulation have not been unraveled yet, it has recently been shown that the dimerization status of the receptor plays a key role. Receptor dimerization is suggested to be required for binding to a negative GRE in the *GR* gene resulting in transcriptional repression, for transactivating the gene encoding

miR-29a which binds the *GR* mRNA resulting in destabilization and degradation of the RNA molecule, and for ubiquitination which targets the receptor for proteasomal degradation [106]. Thus, the problem of GC resistance, as well as the serious GC side effects, underly the need for the development of novel anti-inflammatory therapies.

2.6. Development of novel glucocorticoids

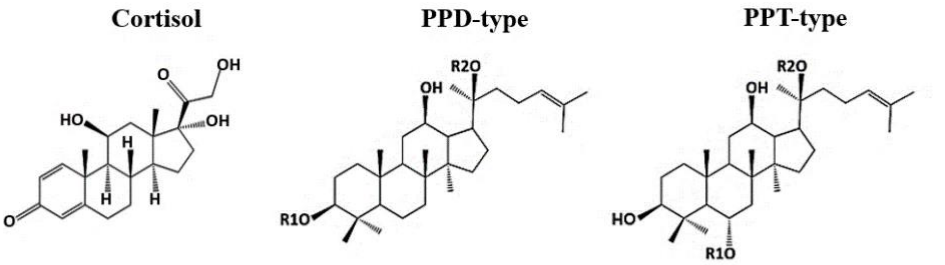
The transrepression activities of GR have been considered the primary mechanism that mediates the anti-inflammatory actions of GCs. In addition, the classical view is that most side effects of GCs result from the transactivation activities of GR [15,107]. Therefore, significant effort has been put into developing novel GR agonists that induce the transrepression activity of GR but not its transactivation activity to improve the therapeutic ratio of GC drugs [108]. However, although several promising compounds have been developed [109-111], success has been limited, most likely because the immune suppression by GCs also appears to depend on the transactivation of anti-inflammatory genes and because some side effects also result from the transrepression activity of GR [39,112]. Therefore, it will be interesting to attempt novel approaches to develop GC drugs with reduced side effects, such as modifying the structure of existing GCs. For example, an anti-CD163-dexamethasone conjugate designed to target activated macrophages, showed an improved therapeutic ratio in rats [13], and the conjugation of hydrolyzable polyethylene glycol (PEG) to prednisolone increased the retention time in the lungs of rats, thereby not causing any systemic side effect [113]. Furthermore, adding γ-lactones and cyclic carbonates can make GCs more easily activatable by specific enzymes once they enter the bloodstream [114]. Another strategy is the development of prodrugs, which are activated locally. Glucuronide-dexamethasone (GDex) is a dexamethasone with a glucose group conjugated at the C-21 position, and has been proposed as a potential prodrug for colonic delivery of dexamethasone [115-117]. When orally administered to rats, GDex is converted in the gut by the beta-glucosidase activity of the microbiota. Therefore, GDex exerted a promising therapeutic effect in a colonic inflammatory bowel disease model. Significantly, GDex accelerated the healing of colitis in rats without adrenal suppression. GDex also showed a selective advantage for the delivery of dexamethasone in cecal tissues in the guinea pig and mice [117].

3. Ginsenosides as potent anti-inflammatory agents

Plants and other natural sources contain wide variety of compounds that may be harnessed for medical applications, including the treatment of inflammatory diseases. Radix Ginseng (also called ginseng) is one of the world's most widely used herbal remedies, derived from the root of the plant *Panax ginseng* C.A. Mayer. It has a lengthy historical medical application for many different purposes, such as wound healing, anti-aging, anti-inflammatory, anti-cancer, vaso-relaxation, and anti-oxidation. Scientific studies indicate that ginseng has various effects on the immune response [118-121]. Ginseng has a diverse multi-chemical composition of polysaccharides, ginsenosides, peptides, polyacetylene alcohols, fatty acids, phenolic compounds, which may all contribute to its pharmaceutical activities. A class of compounds called ginsenosides are proven to be the principal and most active constituents in ginseng [118-121].

Each ginsenoside presents different biological and pharmacological effects [122]. The differences in aglyconic backbone structures, nature of acyl substituents, and radical glucose groups at the carbon positions 3, 6, and 20 are the distinguishing factors underlying their differential activities [122,123].

Ginsenosides are known as saponins (or sapogenin glycosides), which consist of a steroid or triterpene sapogenin backbone structure consisting of 17 carbons in a 4-ring structure, to which one or more acyl and/or glucose groups are attached. Thus, ginsenosides resemble the structure of cortisol or synthetic GCs [118-121] (Fig.2).



PPD-type	R1	R2	PPT-type	R1	R2
Rb1	Glc-Glc	Glc-Glc	Re	Glc-Rha	Glc
Rb2	Glc-Glc	Glc-Arap	Rf	Glc-Glc	H
Rc	Glc-Glc	Glc-Araf	Rg1	Glc	Glc
Rd	Glc-Glc	Glc	Rg2	Glc-Rha	H
F2	Glc	Glc	F1	H	Glc
Rg3	Glc-Glc	H	Rh1	Glc	H
Rh2	Glc	H	PPT	H	H
Compound K	H	Glc			
PPD	H	H			

Figure 2. *An overview of the dammarane-type class of ginsenosides, which are the main subject of this thesis. The structures of PPT-type and PPD-type ginsenosides resemble the structure of glucocorticoids such as cortisol. Functional groups are indicated as follows. Glc: b-D-glucopyransosyl; Arap: α-L-arabinopyranosyl, Rha:α-L-rhamnopranosyl.*

Depending on the structure of the aglycone backbone, ginsenosides can be categorized into three different types: a) dammarane types, including protopanaxadiols (PPDs) such as PPD, Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, F2 and compound K, as well as protopanaxatriols (PPTs) such as PPT, Rg1, Re, Rf, Rg2, F1 and Rh1; b) Oleanane (oleanolic acid) types, such as Ro and polyacetylene-ginsenoside Ro; and c) Ocotillol types, such as majonosides R1, majonosides R2, vina-R1, vina-R2, vina-R6, vina-R14,24- pseudoginsenoside RT4 and pseudoginsenoside F11 [118-121]. The first type of ginsenosides, the dammarane, are the main subject of this thesis. The glycosylated forms of these ginsenosides can be involved in several metabolic processes *in vivo* and *in vitro*, and they can be transformed from glycosylated (e.g., Rg1, Rb1) into partially de-glycosylated (e.g., F1, F2) and aglycone forms (e.g., PPT, PPD) [121]. The key enzymes mediating these transformations belong to β-glucosidases from the microbiome in the intestine. Also, liver enzymes mediate the transformation of ginsenosides via different metabolic pathways, including isomerization, oxidation, oxygenation, and hydrolysis [122]. Due to the differences in chemical structures between ginsenosides, each ginsenoside presents different biological and pharmacological effects [122]. The differences in aglyconic backbone structures, nature of acyl substituents, and radical glucose groups at the carbon positions 3, 6, and 20 are the distinguishing factors underlying their differential activities [122,123].

Ginsenosides can regulate inflammatory responses on early upstream targets by downregulating TLR4 [132]. However, most ginsenosides regulate inflammatory responses mainly by blocking the expression of downstream targets of the NF-κB signaling pathway [122,123,133-135]. Therefore, ginsenosides inhibit the production of pro-inflammatory cytokines like TNF-α, IL-1b, and IL-6. In addition, ginsenosides suppress the production of inflammatory enzymes such as iNOS and COX-2 [122,123,133-135]. Therefore it is not surprising that pharmacological and biological studies, both *in vitro* and *in vivo*, have shown different bioactivities of ginsenosides related to immune response modulation. Ginsenosides and their metabolites exhibit anti-inflammatory, anti-oxidative properties and present potent pharmacological effects in humans' metabolic, neurological, and endocrine systems. This highlights the adaptogenic and anti-stress properties of ginsenosides [122,123,133-135]. To properly exploit ginsenosides' potential as anti-inflammatory drugs, it is fundamental to understand the precise details of their mechanism of action.

Structural similarity to GCs may enable ginsenosides to interact with cellular membranes or sub-cellular organelles and exert effects on extra- and/ or intracellular receptors similarly to GCs. Importantly, it has been shown that ginsenosides are functional ligands of the GR [122,124,125]. In addition, ginsenosides are thought to regulate the function of various other receptors, for instance receptor tyrosine kinases (RTK), serotonin (5-HT) receptors, N-methyl-D-aspartate (NMDA) receptors and nicotinic acetylcholine receptors (AChRs) [122]. However, direct interaction of ginsenosides with receptor ligand-binding sites has only been shown for the GR and other steroid receptors, including the estrogen receptor [122][126], androgen receptor and progesterone receptor [127]. For example, ginsenosides Rg1 and its metabolites Rh1, Re, PPT, and PPD are all functional ligands of the GR [122,124,125,128]. Recently, many reports demonstrated that ginsenosides are not as potent as synthetic GCs such as dexamethasone, because they bind to the human GR with a 10-100-fold lower affinity [122,124,125,129,130,131]. Despite their lower affinity for the GR, ginsenosides

have been shown to alter both transcriptional and non-transcriptional actions of this receptor in cultured cells [130,131].

4. β - glucosylceramide 2 (GBA2)

β- glucosylceramide-1 (GBA1) and -2 (GBA2) are both glucosylceramide (GlcCer)-degrading enzymes, cleaving the beta-glycosidic bond to release the glycoside and ceramide [136]. They are structurally unrelated enzymes, present at different cellular locations, and act on different cellular pools of GlcCer [137]. GBA1 localizes to the lysosome [138], plays a central role in the degradation of complex lipids and the turnover of cellular membranes, and participates in cholesterol metabolism [139]. GBA2 is reported to be a single-pass transmembrane protein, localized at the plasma membrane. GBA2 is involved in glycosphingolipid homeostasis, particularly in brain tissue where it plays a role in neural development. Loss of GBA2 causes accumulation of glucosylceramide and has been associated with locomotor dysfunction [140] and altered cytoskeletal dynamics [141]. A recent study showed that the administration of the potent GBA2 inhibitor miglustat reduced the inflammation in *P. aeruginosa* infection in primary bronchial epithelial cells from cystic fibrosis patients [142], and it was demonstrated that inhibiting β-glucosidases in LPS-stimulated mice reduced the number of neutrophils recruited to the airways [143]. Furthermore, miglustat-mediated inhibition of GBA2 caused anti-inflammatory effects in mice [144]. Together, these reports indicate that GBA2 could play an important role in inflammation.

5. The zebrafish as an animal model for studying inflammation and GC action

Over the last several decades, the zebrafish has become widely utilized as a model organism in biomedical research due to its suitability at the embryonic and larval stages for genetic and chemical screening, as well as microscopic imaging of cellular behavior during development and disease [145,146]. The immune system is well conserved between fish and humans, both in terms of immune cell functions and in terms of the molecular signaling pathways that mediate inflammatory responses and the immune defense [147,148]. The major cell types of the innate branch of the immune system, macrophages and neutrophils, are already present at the embryonic and larval stages, and these cells can mount inflammatory responses to different cues such as wounding or infections [147,148]. These characteristics make the zebrafish a suitable model for research into inflammatory diseases and for anti-inflammatory drug screening [148,149]. Zebrafish also have all the main organs and endocrine glands involved in the metabolic and endocrine systems. Zebrafish have been therefore been used to study several human metabolic and endocrine disorders associated with inflammation, for example type 2 diabetes mellitus and nonalcoholic fatty liver disease [148-153].

Like in humans and all other teleost fish species, endogenous GC productions in the zebrafish is regulated by stress and in a circadian rhythm [154-156]. This process is organized by the hypothalamus-pituitary-interrenal (HPI) axis, the fish equivalent of the human HPA axis [154-157]. The main GC in the zebrafish is cortisol, which functions through Gr, the zebrafish orthologue of GR [145-148]. Most teleost fish contain two genes encoding a Gr due to a genome duplication during their evolution [157-161]. However, only a single Gr-encoding gene is found in zebrafish, which is structurally and functionally highly similar

to the human GR [158-161]. Furthermore, both the human and zebrafish gene encode two splice variants, the α-isoform, and the β–isoform [160]. Like their human equivalents, the zebrafish Grα performs the canonical GR functions, while the zebrafish Grβ acts as a dominant-negative inhibitor of Grα in cultured cells and is expressed at a significantly lower level compared to Grα [160,162]. In various recent studies, the similarities with the human GR and practical advantages of the model system, have been exploited to investigate GCs and GR function in zebrafish [150-153].

sRecently, genetic studies on GCs and GR have been performed on zebrafish to advance our knowledge of their mode of action [148]. For this purpose, gene knockdown techniques have been conducted in those studies [163]. Gene knockdown approaches have been used by injecting morpholino (MO) antisense oligomers at the 1-cell stage, suppressing translation or mRNA splicing [163]. Using a splice-blocking MO, it was found that the inhibitory effect of GCs on zebrafish caudal fin regeneration is dependent on Gr activation and that the suppression of the *cripto-1* gene by Gr is implicated in this process [164]. Transcriptome analysis showed that the knockdown of the *gr* gene by a splice-blocking MO changed the expression of a distinct cluster of genes compared to the genes affected by treatment with the synthetic GC dexamethasone, indicating that Gr modulates different sets of genes under basal conditions than upon increased activation, e.g. after stress [165]. Another study, using a translation-blocking MO, revealed multiple developmental defects, illustrating the crucial role of maternal *gr* transcripts [166,167]. These results are in line with the programming function of cortisol in the development of multiple organs such as muscle, heart, bone, and the nervous system [168,169].

In addition to knockdown studies, different *gr* mutant lines have been generated [170,171]. The first *gr* mutant zebrafish line, gr^{s357}, was identified in a forward genetic screen based on behavioral assays [172]. This gr^{s357} mutant contains a point mutation in the DBD of Gr, resulting in defective GRE binding activity and high cortisol levels [173,174]. It has been utilized to investigate HPI hyperactivation related to depressive behavior, and may provide a model to screen for potential anti-depressant drugs [174]. Moreover, analysis of the gr^{s357} line revealed that *gr* signaling elevates embryonic hematopoietic stem and progenitor cell production [174], triggers the expression of white skeletal muscle genes [175], modulates the visual function of the retina [176], and elevates anxiety-related behavior in adults [177]. These studies on the gr^{s357} line confirm the essential role of GC/Gr signaling during development. More recently, a null mutant of the zebrafish *gr* was generated by CRISPR/Cas9-mediated gene editing [170]. This mutant has a 5-nucleotide insertion in the *gr* gene, causing a frameshift that causes a premature stop codon, truncating the receptor upstream of its DBD. Larvae of this mutant line show unresponsiveness to GCs and have high cortisol levels, similar to the observations in the gr^{s357} line [170]. Interestingly, the *gr* null mutant larvae did not display an inflammatory response upon treatment with dextran sodium sulfate (DSS), whereas gr^{s357}did show this response, indicating that partial Gr function is retained in the latter mutant which may be required for eliciting certain inflammatory responses [170].

A convenient tool for *in vivo* visualization and monitoring of the transcriptional activity of the Gr is the reporter zebrafish line *Tg(9xGCRE-HSV.UI23:EGFP^{ia20})* [178,179]. In this line, the enhanced green fluorescent protein (EGFP) gene is driven by a GRE-containing promoter

so that the effect of Gr transactivation by GC treatment can be measured systemically in larvae. By 2–3 days post fertilization (dpf), the fluorescence is noticeable in GC target organs and tissues, such as the liver, pancreas, intestine, and optic vesicles. The EGFP signal is increased significantly in the presence of exogenous GCs [169,170]. Due to the sensitivity and spatial resolution of the *Tg(9xGCRE-HSV.UI23:EGFP^{ia20})* line, it is considered a useful readout model for investigating the physiological functions of GC signaling *in vivo* during zebrafish development or in response to different stressors [178,179]. This line has successfully been used to identify compounds able to activate Gr in pharmacological and toxicological experiments [178,179].

To study Gr function and the immune-suppressive effects of GCs during inflammation, a variety of assays in embryonic and/or larval zebrafish have been employed such as; LPS-induced inflammation [148,180,181], $CuSO_4$-promoted inflammation [148,182], and dextran sodium sulfate (DSS)-stimulated enterocolitis [148,183]. Among these, wounding-induced inflammation [69,148,179,184,186], induced using the tail fin amputation assay, is one of the most frequently used systems for anti-inflammatory drug screening, showing prominent anti-inflammatory effects of GCs [69,184] (Fig.3). Upon tail fin amputation of 3 dpf larvae of the double transgenic line *Tg(mpx:GFP^{i114}/mpeg1:mCherry-F^{umsF001})*, in which neutrophils are labeled with GFP and macrophages with mCherry, [185], neutrophils, and macrophages can be observed migrating towards the wounded area, and increased expression of pro-inflammatory mediators can be detected [184]. The tail fin will fully regenerate within several days [179,186]. GCs have been shown to inhibit the migration of neutrophils, dependent on Gr, without affecting macrophage migration [69,179,187]. In addition, GCs reduce the abundance and activity of pro-inflammatory mediators and increase the concentration and activity of anti-inflammatory factors [69,179,187]. However, GCs also show effects in the zebrafish model that can be used as readouts for GC side effects. For example, GCs suppress the regeneration capacity of the wounded tail, the velocity of the embryonic growth, and disrupt their endocrine and metabolic systems, which manifests itself as an increased whole body glucose concentration and suppressed whole body cortisol level [141,142,186,188,189]. Therefore, the tail fin amputation model in zebrafish larvae provides a convenient system to screen for the anti-inflammatory and side effects of novel GC drugs [148].

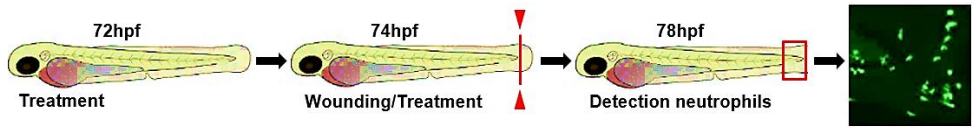


***Figure 3**. Schematic overview of the experimental design of the tail wounding assay. At 72 hpf, zebrafish larvae of the Tg(mpx:GFP^{i114}/mpeg1:mCherry-F^{umsF001}) line were treated with compounds for 6 h (2 h pre-wounding and 4 h post-wounding). At 74 hpf, tail fin wounding was performed at the indicated site (red line). At 78 hpf, the larvae were imaged by fluorescence microscopy, and the area for quantification of the fluorescently labeled neutrophils and macrophages (i.e., the area posterior to the tail vein) is indicated (red box).*

6. Outline of the thesis

The aim of the research described in this thesis was to unravel the molecular mechanisms underlying the anti-inflammatory action of ginsenosides, using the zebrafish as a main model organism. We first showed that ginsenosides, elicit these immune-suppressive effects by activating the glucocorticoid receptor (GR). Interestingly, we also demonstrated that the side effects of these compounds are strongly reduced compared to classical GCs such as beclomethasone. After studying the effects of ginsenosides in detail, we found that, depending on the structure of the ginsenosides, they can act as selective GR agonists, selective GR antagonists and as GC prodrugs. Based on our understanding of the mechanistic effects of ginsenosides, we have developed a novel way of modifying the structure of GC drugs which may strongly reduce their side effects.

The present introductory chapter, **Chapter 1,** provides background information on the cellular and molecular mechanisms of inflammation. Subsequently, we present the mechanistic effects of GCs as anti-inflammatory agents which also induce GR resistance and side effects. After that, we introduce the mechanistic effects of ginsenosides as anti-inflammatory agents, which are mediated by GR.

Chapter 2, a literature review, presents evidence that ginsenosides are potential candidates as selective GR agonists. Ginsenosides present anti-inflammatory effects dependent on GR and selectively activate this receptor. Interestingly, ginsenosides have been shown to exert their anti-inflammatory effects while triggering strongly reduced side effects compared to GC drugs. Ginsenosides have been shown to alleviate metabolic, neuronal, and endocrine disorders in various disease models. We conclude that understanding the unique properties of ginsenosides opens the door to develop novel selective GC drugs which will be the new generation of anti-inflammatory drugs.

Chapter 3 presents evidence that the ginsenoside Rg1 acts as a selective GR agonist in the zebrafish model, with potent anti-inflammatory effects but without affecting tissue regeneration, opening up a novel avenue towards the development of improved anti-inflammatory GCs. Furthermore, in this chapter we show that the zebrafish may serve as a translational model to unravel the mechanisms underlying the selectivity of Rg1 and related ginsenosides and for screening compounds to evaluate their potential for clinical use.

Chapter 4 demonstrates that steamed *Panax notoginseng* (SPNE) and its ginsenosides modulate the immune response in the tail-fin wounding model in zebrafish larvae. SPNE significantly inhibited the neutrophil migration towards the wounded area. SPNE extracts steamed at higher temperatures and for longer time periods showed a stronger inhibitory effect. Ginsenosides Rh1, Rk3, Rh4, 20(S)-Rg3, and 20(R)-Rg3, of which the levels were increased along with the duration of steaming, were found to be the major active constituents contributing to the neutrophil-inhibiting effect of SPNE. Rh1 and 20(R)-Rg3 inhibited the migration of neutrophils, while ginsenosides Rk3, Rh4, 20(S)-Rg3, and SPNE inhibited the migration of neutrophils and also promoted neutrophilic cell death.

Chapter 5 shows that Rg1 has an additive anti-inflammatory effect when it is used in combination with GCs, as shown by its effect on neutrophil migration and pro-inflammatory gene expression. However, Rg1 reduced the side effects induced by the GC treatment, because it acts as a selective GR antagonist. Data collected from HeLa cells confirmed the

additive anti-inflammatory effects. In addition, Rg1 reverses the beclomethasone-induced downregulation of GR. Collectively, our data indicated that adding Rg1 to classical GC treatment may improve the therapeutic ratio and reduce acquired GC resistance.

Chapter 6 demonstrates that glycosylation of GCs offers an approach to reduce their side effects because it converts them into inactive prodrugs, which are specifically activated in inflamed tissue by the enzyme Glucosylceramidase beta 2 (GBA2). This approach is based on our observation that ginsenosides act as anti-inflammatory GCs in the zebrafish model, but hardly induce any side effects. This selective activity was demonstrated to be a result of localized Gba2 activity. By conjugating the disaccharide gentiobiose to the GC prednisolone, we generated a prodrug with similarly reduced side effects. In human HeLa cell cultures, gentiobiose-prednisolone (GbPdn) activated the GR when cells were stimulated with the pro-inflammatory cytokine TNF-α, which induced the GBA2 activity required for prodrug conversion. Moreover, in this chapter we have demonstrate that gentiobiose-prednisolone effectively alleviates the inflammatory response in a mouse model for arthritis without inducing side effects.

Chapter 7 illustrates that monoglycosylated ginsenosides, such as F1 and Rh1, show anti-inflammatory effects independent of Gba2 activity, while inducing strongly reduced side effects. We show that F1 and Rh1 selectively induce the transrepression activity of the GR in the zebrafish model. Based on these data, we investigated the mechanistic actions of monoglycosylated GCs glucuronide-dexamethasone (GDex) and glucose-prednisolone (GPdn) in the zebrafish model and in HeLa cells. We found that GDex and GPdn exerted their anti-inflammatory effects independent of GBA2, with fewer side effects than their non-glycosylated forms. Importantly, both compounds induced selective transrepression activity of the GR without promoting its transactivation activity. We conclude that monoglycosylated GCs are selective GR agonists.

Chapter 8 explores the possible use of tramadol and paracetamol (TP) analgesia in the collagen antibody-induced arthritis (CAIA) model in mice. Our results showed that in the TP-treated groups, the arthritic response was significantly decreased, and thereby the therapeutic window between prednisolone-treated mice and vehicle-treated mice was diminished. TP treatment abolished prednisolone-induced amelioration of CAIA and several other prednisolone-induced effects, such as a reduction in thymus weight or an increase in the insulin level. We conclude that TP analgesia is unsuitable for the murine CAIA model since it affects the arthritic response, and because it interferes with the effects of prednisolone, most likely by influencing the hepatic metabolism of this drug in mice.

Chapter 9 summarizes the results from the research chapters and discusses the findings in the context of the current scientific literature.

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