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

Halima, M.

Chapter 9

Summary and discussion

1. Novel glucocorticoid drug discovery inspired by research on ginsenosides

Glucocorticoids (GCs) are steroid drugs that are utilized to treat various inflammatory diseases [1,5]. They are highly effective and among the most prescribed drugs worldwide, but the severity of their side effects and the occurrence of resistance to these drugs limit their application [6,11]. Therefore, GC therapies with reduced side effects that do not trigger resistance are urgently needed. In this thesis, we have studied the mechanistic effects of ginsenosides, which are compounds derived from the plant Panax ginseng that has been used in traditional Chinese medicine for thousands of years [12,13]. We provide evidence that ginsenosides can bind to the receptor for GCs, the glucocorticoid receptor (GR). However, they do not act as classical GCs. We show that they can act as; 1) selective GR agonists, 2) selective GR antagonists, and 3) GC prodrugs. After studying and understanding the mechanistic effects of ginsenosides we used our novel insights to synthesize novel GCs with reduced side effects by attaching glucose group(s) to their basic steroid structure.

The zebrafish has been used as our main model organism in these studies. The zebrafish has become widely utilized as an *in vivo* model for research aimed at discovering novel drugs [14]. It is highly suitable for research on GC drugs since it has a single *gr* gene encoding a Gr, which is structurally and functionally highly similar to the human GR [15,17]. The tail fin amputation model in larval zebrafish is a well-established system for anti-inflammatory drug screening. It has been used to investigate the anti-inflammatory effect of GCs [18,22]. Upon tail fin wounding of zebrafish larvae at three days post fertilization (3 dpf), a local inflammatory response is induced, which is characterized by migration of leukocytes (neutrophils and macrophages) towards the wounded area and an increased expression of pro-inflammatory cytokines [18,22]. The tail fin will fully regenerate within several days. GCs have been shown to inhibit the migration of neutrophils and decrease the expression of pro-inflammatory genes. Thus, the tail fin wounding assay provides a convenient model to study the anti-inflammatory effects of a novel GC drug. Importantly, zebrafish have all the main organs and endocrine glands involved in the metabolic and endocrine systems and have been therefore used to study several human metabolic and endocrine disorders such as type 2 diabetes mellitus, nonalcoholic fatty liver disease, growth and regeneration, and disorders [23,25]. In this thesis, whole-body glucose, whole-body cortisol levels, larval growth and the regeneration of the tail fin have been used as readouts for the evaluation of possible GC side effects [26,28]. This research has shown that zebrafish are an appropriate model to investigate not only the anti-inflammatory effects and mechanism but also the side effects of novel GC drugs.

In **chapter 1** of this thesis, we introduced the main concepts that are investigated in this thesis and presented our research aims. In **chapter 2,** we reviewed the scientific literature on ginsenosides as promising anti-inflammatory drugs. Ginsenosides have been demonstrated to exert anti-inflammatory effects in various animal models for inflammatory disease and several neurological disorders. These effects result in most cases from the activation of GR by these compounds. Interestingly, ginsenosides function as selective GR agonists, inducing only partial activation of this receptor. As a result, ginsenosides show anti-inflammatory effects in different animal models while triggering strongly reduced side effects compared to classical GC drugs. In **chapter 3,** we investigated the anti-inflammatory

activities of the ginsenoside Rg1 in zebrafish using the tail fin wounding model. As a readout for side effects, we studied the inhibition of the ability to regenerate the wounded tissue. In **chapter 4,** we studied the mechanistic effects of steamed *Panax notoginseng (SPNE)* and its ginsenosides on modulating the immune response in the tail fin wounding model in zebrafish. In **chapter 5,** we used Rg1 as a selective GR antagonist, in combination with classical GCs as a new therapy to reduce side effects and resistance. In **chapter 6,** we investigated the mechanisms underlying the effects of ginsenosides F2 and Rb1, which act as GC prodrugs. By attaching the disaccharide gentiobiose to the classical GC drug prednisolone, we mimicked the ginsenoside structures and thereby developed a novel GC prodrug that acts locally at the site of inflammation**.** In **chapter 7,** we examined the mechanistic effects of ginsenosides F1 and Rh1. By mimicking their structures, and by attaching the monosaccharide glucose to classical GCs, we generated selective GR agonists. Finally**,** in **chapter 8**, we studied (as a side project) the suitability of tramadol and paracetamol as analgesic treatments in the collagen antibody-induced arthritis model in mice, which we used to investigate our GC prodrug concept (in **chapter 6**).

1.1. Ginsenoside Rg1 acts as a selective Glucocorticoid Receptor agonist with anti-inflammatory action without affecting tissue regeneration in zebrafish larvae

In **chapter 3**, we demonstrate, using the zebrafish tail fin wounding model, that Rg1 has anti-inflammatory effects similar to beclomethasone. Rg1 and beclomethasone inhibited wound-induced migration of neutrophils in a Gr-dependent manner. Interestingly, Rg1 shows a selective mode of action since it shows no side effects on tissue healing and regeneration, unlike synthetic GC beclomethasone. In addition, Rg1 appeared to modulate gene regulation more selectively than beclomethasone. In a previous study, we have shown that beclomethasone attenuates almost the entire transcriptional response to amputation [21]. However, whereas both beclomethasone and Rg1 were shown to inhibit the amputation-induced increases in the expression of the genes encoding Il1b, Il6, Cxcl18b, Cxcl11aa, and Mmp9, the (modest) suppression of the genes encoding Cxcl8/Il8 and Mmp13 upon beclomethasone treatment was not observed after Rg1 treatment. Moreover, the increased expression of the genes for Tlr2 and Tlr4-a, NF-κB inhibitor α-like protein A, Cxcr1, and Cxcr2 was exclusively seen after beclomethasone treatment.

The anti-inflammatory effects of Rg1 had been observed previously. It had been reported that Rg1 significantly attenuated joint swelling and injuries in adjuvant-induced arthritis (AIA) rat model. Rg1 decreased the level of TNF-α and IL-6, elevated peroxisome proliferator-activated receptor γ (PPARγ) protein expression, suppressed IκBα phosphorylation and NF-κB nuclear translocation in the inflammatory joints of AIA rats and in RAW264.7 cells stimulated by LPS [29]. Additionally, in the CAIA mice model, Rg1 was shown to have anti-inflammatory effects in CAIA mice without adverse effects on glucose levels or osteoblast proliferation and differentiation [30]. These data indicate that ginsenoside Rg1 has strong anti-inflammatory and immune-modulating capabilities.

Initially, the mechanism underlying the selective action and the reduced side effects was unclear, but we unraveled this mechanism in the studies presented in chapters 6 and 7. In these chapters, we showed that Rg1 binds the GR but does not activate it. However, the local activity of the enzyme glucosylcermaidase beta 2 (GBA2) removes the glucose group from the C-20 position of Rg1, so it can activate the receptor in the inflamed tissue, resulting

in anti-inflammatory activity without side effects. Since the glucose group at the C-6 position is resistant to removal by GBA2, we do not observe the activity of PPT at the site of inflammation, but the more selective activity of Rh1.

Previously, selective GR activation had been shown for the aglyconic ginsenosides (20S)-Protopanaxadiol (PPD) and (20S)-Protopanaxatriol (PPT) [31], but available data for Rg1 were conflicting [29,30,32]. Our results on gene transcription generally support a transrepression-selective type of GR activation for Rg1 since the vast majority of genes downregulated by beclomethasone were also downregulated upon treatment with Rg1, whereas all studied genes of which the expression was upregulated by beclomethasone did not show this upregulation in the presence of Rg1. Since transactivation is the mechanism generally linked to the side effects of GC therapy, a lot of research is currently focused on the development of novel GR agonists that selectively induce the transrepression activity of the receptor [33]. Studies on the selectivity of Rg1 and other ginsenosides, such as PPD and PPT, and the underlying molecular mechanisms may open up new avenues in this research on novel selective GR agonists.

1.2. Steamed Panax notoginseng and its saponins inhibit the migration and induce the apoptosis of neutrophils in a zebrafish tail fin amputation model

In **chapter 4**, we show, using the tail fin wounding model in zebrafish, that steamed *Panax notoginseng* (SPNE) and its saponins (ginsenosides) modulate the immune response. SPNE significantly inhibited the migration of neutrophils toward the wounded site. Interestingly, SPNE extracts steamed at higher temperatures and for longer periods of time showed a stronger inhibitory effect. Ginsenosides Rh1, Rk3, Rh4, 20(S)-Rg3, and 20(R)-Rg3, of which the levels were increased upon longer steaming, were found to be the major active constituents contributing to the anti-inflammatory effect of SPNE (20(S)-Rg3 is a stereoisomer to 20(R)-Rg3, of which the OH-group at C-20 is closer to C-12 than the OH-group at C-12 in 20(R)-Rg1 [34]). In addition to their effect on neutrophil migration, ginsenosides Rk3, Rh4, and 20(S)-Rg3, and SPNE also promoted neutrophilic cell death, whereas the other ginsenosides did not. These data confirmed that the biological and pharmacology effects of ginsenosides can be highly specific due to minor differences in their chemical structure. For example, it has been reported that 20(S)-Rg3 has a stronger anti-cancer effect than 20(R)-Rg3 [34]. Importantly, in chapter 3, Rg1 and beclomethasone inhibited neutrophil migration without affecting the number of neutrophils in the entire tail. These data indicated that the effect of SPNE on apoptosis in neutrophils is most likely not mediated by Gr but through activation of another pathway. Thus, SPNE and its ginsenosides showed the potential to be developed as drugs with specific neutrophil-inhibiting effects for the treatment of chronic inflammatory diseases.

1.3. Co-treatment with ginsenoside Rg1 increases the therapeutic ratio of anti-inflammatory glucocorticoid treatment and prevents the downregulation of GR

In **chapter 5**, we have studied the effect of treatment with Rg1 in combination with classical GCs (beclomethasone or dexamethasone). Based on the action of Rg1 as a selective Gr agonist, which we had demonstrated in chapter 3, we hypothesized that Rg1 could also function as a selective Gr antagonist, with an additive effect on the anti-inflammatory effects but inhibiting side effects, upon co-treatment with a full Gr agonist such as

beclomethasone or dexamethasone. In line with this hypothesis, we showed that Rg1 co-treatment has an additive effect on the anti-inflammatory effect of beclomethasone or dexamethasone, but strongly reduces several side effects that can be modeled in zebrafish larvae: the decrease in tissue regeneration after wounding, the reduction in growth, the increase in glucose concentration and the decrease in cortisol level. The antagonism was competitive, since in the presence Rg1, high concentrations of beclomethasone could still induce a maximal effect on the glucose and cortisol concentrations, indicating that Rg1 binds the zebrafish Gr in a similar way as beclomethasone. Interestingly, our data confirmed that Rg1 did not induce the transactivation activity of Gr, but functioned as an antagonist, inhibiting the beclomethasone-induced upregulation of these genes upon co-treatment. The lack of transactivation activity of Gr in the presence of Rg1, while the transrepression activity is intact, thus explains the selective antagonism that we observed on phenotypical readouts such as the glucose and cortisol levels. These results were confirmed in the human HeLa cell line. Upon Rg1/beclomethasone co-treatment, an additive effect of Rg1 was observed on the transrepression of *IL1B, MMP9* and *IL8*, and the antagonistic effect of Rg1 was observed on the transactivation of *GILZ* and *SGK1*, but not *FKBP5* and *NFKBIA*.

Another phenomenon that we noticed in HeLa cells and zebrafish larvae was the reduction of GC sensitivity after long-term treatment, which appeared to be associated with a decrease in the *GR* mRNA and GR protein level. Interestingly, co-treatment with Rg1 restored these levels and thereby prevented the loss in sensitivity to GCs. Similarly, it has previously been shown that Rh1 prevented the dexamethasone-induced downregulation of GR levels and associated GC resistance in human cell cultures and in mice *in vivo* [35]. We demonstrated, using chemical inhibition of transcription and translation, that Rg1 co-treatment inhibited the beclomethasone-induced decrease in the stability of *GR* mRNA and GR protein. We therefore suggest that compounds such as Rg1 are a useful tool for further studies into the development of drugs that, either alone or as co-treatment, may alleviate the side effects of GC treatment and the acquired resistance to GC therapy that currently limit the clinical use of this class of drugs.

1.4. Glycosylated glucocorticoid prodrugs show strongly reduced side effects due to specific Glucosylceramidase Beta 2–mediated activation in inflamed tissue

In **chapter 6, we** demonstrate that glycosylated ginsenosides such as F2 and Rb1 elicit their anti-inflammatory effects by activation of GR, but without inducing side effects. Interestingly, the anti-inflammatory activity of F2 and Rb1, which consist of a steroid structure to which saccharide groups are conjugated, required the activity of the enzyme Glucosylceramidase beta 2 (Gba2), of which the activity was strongly induced locally in inflamed tissue. We concluded that the inflammation-induced Gba2 activity led to cleavage of the saccharide groups from the ginsenosides only in inflamed tissues, resulting in GR activation restricted to these sites. In line with this idea, we showed that conjugation of the disaccharide gentiobiose to prednisolone preserved the anti-inflammatory effect of this drug in zebrafish larvae, but made this effect dependent on the activity of Gba2. Therefore, gentiobiose-prednisolone (GbPdn) showed strongly reduced side effects compared to prednisolone.

Additionally, gentiobiosylation strongly reduced the binding affinity of prednisolone to the human GR *in vitro* and abolished its ability to induce nuclear translocation and

transcriptional activity of GR in human (HeLa) cell cultures. However, upon treatment of these cells with the pro-inflammatory cytokine TNF-α, GBA2 was induced. As a result, under these conditions GbPdn treatment did result in nuclear translocation and transcriptional activation of GR. Finally, we demonstrated in a mouse model for arthritis that GbPdn significantly reduced inflammation without triggering side effects (Fig.1).

The localized GBA2-dependent conversion of the prodrugs is probably also responsible for the selective action of glycosylated GCs observed in two earlier studies using rat models of inflammation [36,37]. A strong reduction of side effects was reported upon glycosylation in these studies, but the mechanism of action remained elusive. Our discovery that GBA2 is the enzyme responsible for the conversion of glycosylated glucocorticoids in inflamed tissue has revealed the mechanism underlying this specific action of glycosylated glucocorticoids. Thus, we have established a promising approach for developing novel glucocorticoid prodrugs with an improved therapeutic ratio. Since glucocorticoid drugs are frequently prescribed against a range of immune-related disorders, the possible clinical applications of this approach are manifold and may alleviate the severe and often debilitating side effects associated with this treatment in numerous patients.

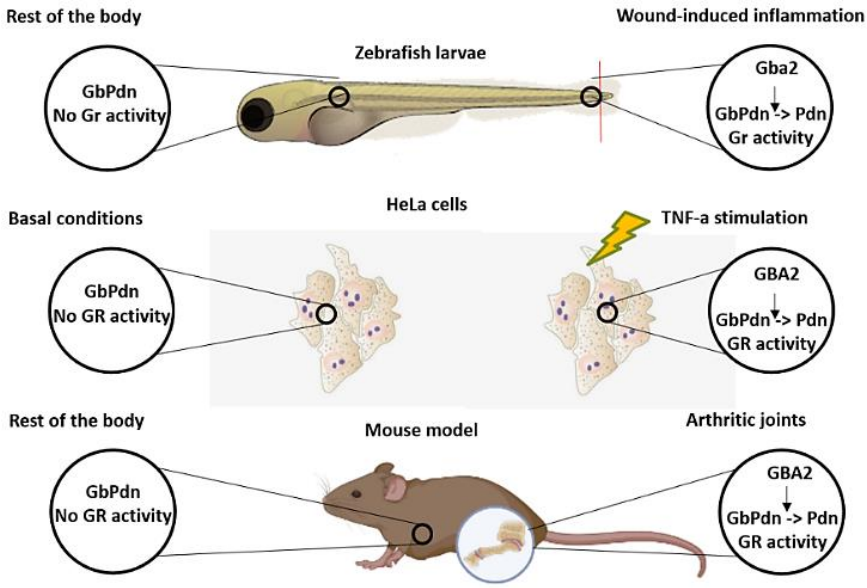

***Figure 1**. Gentiobiose-prednisolone (GbPdn) selectively activates GR under inflammatory conditions, as a result of local conversion into prednisolone (Pdn) by the enzyme glucocylceramidase 2 (GBA2).*

1.5. Monoglycosylation of glucocorticoids converts them into selective glucocorticoid receptor modulators

In **Chapter 7**, we have explored the potential of PPT, Rh1, and F1, besides Rg1, as selective GR agonists. We have used the tail fin wounding model in zebrafish larvae and the HeLa cell line to determine the mechanistic effects of the monoglycosylated ginsenosides F1 and Rh1. We showed that these ginsenosides have anti-inflammatory effects, and that this effect is independent of the activity of GBA2. Apparently, the presence of a single glucose group does not harm the anti-inflammatory effect of ginsenosides. Interestingly, side effects such as effects on glucose and cortisol levels, the ability to regenerate the wounded tissue and the growth, were strongly reduced compared to the effects of classical GCs, indicating that single glucose groups do inhibit the side effects of ginsenosides. We showed that the single glucose group makes the compound transrepression-selective, which explains the ability of these compounds to inhibit the inflammatory gene expression.

Based on these results, we decided to mimic the structure of Rh1 and F1 by attaching a single glucose group to prednisolone and a glucuronide group to dexamethasone, resulting in glucose-prednisolone (GPdn) and glucuronide-dexamethasone (GDex). In zebrafish, we found anti-inflammatory effects of these compounds, independent of GBA2, with reduced side effects, similar to the effects found for F1 and Rh1. In zebrafish and in HeLa cells, the monoglycosylated compounds induced the transrepression activity of GR, but not its transactivation activity. Interestingly, the transactivation activity of these glycosylated GCs in HeLa cells was not even induced upon TNF-α treatment, which increased the GBA2 activity. In chapter 6, we showed that this increase in GBA2 activity was high enough to activate GbPdn. Apparently, for activation of monoglycosylated GCs higher GBA2 activity levels are required, because when we overexpressed GBA2 in the HeLa cells, administration of the compounds did induce GR activation. We hypothesize that the monglycosylated compounds GDex and GPdn can antagonize Pdn and Dex, because of their higher affinity for GR compared to GbPdn, so a higher conversion rate of GDex and GPdn is required to achieve GR activation.

1.6. Tramadol/paracetamol treatment attenuates the development of collagen antibody-induced arthritis and interferes with prednisolone treatment in mice

In chapter 8, we have studied whether analgesics can be used to reduce animal discomfort when testing novel GCs in the collagen antibody-induced arthritis (CAIA) model in mice [38]. For this purpose, we studied the effects of tramadol in combination with paracetamol as an analgesic (TP) treatment in the CAIA model, in which we investigate the anti-inflammatory effect of prednisolone. Our data showed that prednisolone-treated mice showed rapidly decreasing arthritis scores after the start of the treatment. However, mice receiving TP analgesia showed a narrower window between prednisolone and vehicle treatments. Moreover, TP treatment also impacted prednisolone effects on several metabolic parameters and gene expression levels. Interestingly, TP induced the upregulation of *Cyp3a11*, a homolog to human *CYP3A4*, encoding an enzyme involved in prednisolone metabolism [39]. This finding offers an explanation for the modulation of prednisolone-induced effects by TP. Based on these results, we did not apply TP during our GC prodrugs study in chapter 6. We conclude that using TP as an analgesic is incompatible with the CAIA

model since it suppresses the induction of arthritis Moreover, when testing glucocorticoids such as prednisolone, TP administration may interfere with the activity of these compounds.

2. Ginsenosides can act as selective GR agonists, selective GR antagonists and as glucocorticoid prodrugs

Taken together, the work described in this thesis, resulted in several new insights into the mechanistic effects of PPT-type (Rg1, F1, Rh1, Rh4, Rk3, and PPT) and PPD- type (Rb1, F2, 20(S)-Rg3, 20(R)-Rg3 and PPD) ginsenosides. All ginsenosides were shown to have anti-inflammatory effects in zebrafish larvae dependent on Gr action, and showed reduced side effects compared to classical GCs such as beclomethasone, prednisolone and dexamethasone. However, there are differences in the severity of the side effects induced by different ginsenosides. Below we discuss how these differences may relate to selective agonistic or antagonistic activities, and to prodrug properties of the ginsenosides.

The aglyconic ginsenosides PPT and PPD showed reduced side effects compared to classical GCs. In particular, PPD and PPT did not affect glucose levels, whereas it shows similar (although sometimes more minor) effects as beclomethasone on all other readouts (tissue regeneration, cortisol levels and larval length). It is well established that the GC-induced increase in glucose concentration solely depends on the transactivation activity of the GR [6], so we hypothesized that PPD and PPT binding to Gr does not induce this transactivation activity. Our data presented that PPD and PPT and their glycosylation forms did not trigger gr transactivation activities in *Tg(9xGCRE-HSV.UI23:EGFP^{ia20})* reporter zebrafish line and in endogenous *gr* target genes *fkbp5*, *pck1*, confirming that PPT and PPD do not induce the transactivation activity of GR. In line with our results, it has been published that these ginsenosides can trigger GR translocation and mediate GR transrepression activities without triggering GR transactivation activities [13, ,30,32,40].

Glycosylation of PPD and PPT adds an extra layer of selectivity compared to their aglyconic forms. Interestingly, our data demonstrate that the number of glucose groups conjugated to the steroid backbone of ginsenosides is an essential factor underlying the differential mechanistic pathways of ginsenosides. Ginsenosides F1 and Rh1 (both containing one glucose group), did not affect the cortisol level or the length of the larvae under basal condition. Upon tail fin wounding, F1 affected the regeneration capacity and the cortisol level, in a Gba2-dependent way, whereas Rh1 did not affect these parameters at all. Thus, these data indicate that Gba2 can cleave off the glucose group at C-20 position (in F1) but not at C-6 (in Rh1). Therefore, in inflamed tissue Gba2 converts F1 to PPT, while it does not convert Rh1 to PPT. In line with the results of our study, it has been reported that the presence of glucose in positions C-20 or C-3, rather than at C-6, could be more favorable for the binding of the ginsenoside to their possible receptors and hence enhance their biological effects [41,42]. We conclude that monoglycosylation of ginsenosides turns them into selective GR agonists, with a higher level of selectivity compared to the aglyconic PPT and PPD.

Other ginsenosides contain more than one glucose group. For example, Rb1 (4 glucose groups), Rg1 and F2 (2 glucose groups) show anti-inflammatory effects which are dependent on the Gba2 activity in the inflamed tissue. Additionally, our data presented that the position at which the glucose is bound to the aglycone backbone of ginsenosides, C- 6

and/or -20 for PPT-type and C -3 and/or -20 for PPD-type, is also an essential factor underlying the differential pharmacology effects. Ginsenosides F2 and Rb1 (C-3 and C-20) and ginsenoside Rg1 (C-6 and C-20) mediated their anti-inflammatory effects dependent on Gba2. However, F2 and Rb1 show slight side effects on cortisol levels and regeneration of wounded tissue capacity. These minor effects on both readouts were abolished in the presence of a Gba2 inhibitor(MZ31). In contrast, Rg1 mediated anti-inflammatory effects without triggering side effects in the presence or the absence of MZ31. This data confirm that Gba2 may cleave off glucose groups at C-3 and C-20 (in F2 and Rb1), but not at C-6 (in Rg1). Thus, we conclude that polyglycosylated ginsenosides have anti-inflammatory effects, and that these effects are dependent on Gba2 activity. Therefore, polyglycosylated ginsenosides act as GC prodrugs which are not active outside the inflamed tissue.

Due to the selective agonistic effects of ginsenosides, we hypothesized that ginsenosides might also act as selective antagonists. This would imply that using ginsenosides as co-treatment may competitively antagonize specific GC-induced side effects. We showed that Rg1 had an additive effect on the anti-inflammatory effects of beclomethasone. However, Rg1 acted as a selective GR antagonist as well, which inhibited the transactivation activity of GR and therefore the side effects. Significantly, Rg1 also restored the expression and activity of GR in the HeLa cell line, which is reduced upon chronic treatment with GCs. Our data was supported by previous work showing that ginsenoside Rh1 improves the anti-inflammatory effects of dexamethasone in animal models for chronic inflammatory disease by reversing the dexamethasone-induced downregulation of GR expression and the resulting GC resistance [35]. Collectively, our data suggest that ginsenoside co-treatment may improve the therapeutic ratio of classical GCs such as beclomethasone and prevent resistance to GC treatment (Table 1, Fig.2).

It has been reported that ginsenosides bind to the human GR with a 10-100-fold lower affinity than dexamethasone, which means that high concentrations of ginsenosides are needed to activate the receptor [30,32,40]. Our data showed that the relative binding affinity of ginsenoside PPT for the human GR was ~1600 fold lower than that of dexamethasone. The relative affinity binding of the glycosylated ginsenoside Rh1 was around ~2600 folds lower than that of dexamethasone and for Rg1 ~4000 fold lower. However, in the zebrafish model 50 μM of Rg1 countered the effects of 10 μM beclomethasone. We suggest that the kinetics of beclomethasone (e.g. absorption or excretion) in zebrafish are such that higher doses are required for activation of the Gr compared to the doses that are necessary for GR activation in human cell cultures. Notably, the low-affinity binding of ginsenosides makes ginsenosides not suitable for utilization in the clinic. The interesting properties of these compounds described in this thesis, i.e. their actions as selective agonists, selective antagonists and prodrugs, highlight them very interesting tools for research into the development of novel GC therapies with reduced side effects.

Table 1. The effects of PPT- and PPD-type ginsenosides in zebrafish.

Treatment	Anti-inflammatory biomarkers	Cortisol	Glucose	Length	Regeneration	Transrepression	Transactivation
PPT	+	+	-	+[2]	+	+	-
F1	+	+[1]	-	-	+[1]	+	-
Rh1	+	-	-	-	-	+	-
Rg1	+	-	-	-	-	+	-
PPD	+	+	-	+[2]	+	+	-
F2	+	+[1]	-	-	+[1]	+	-
Rb1	+	+[1]	-	-	+[1]	+	-

- No effect; + Gba2-independent effect; + Gba2-dependent effect; [1] Minor effect, only observed after wounding; [2] Minor effect.

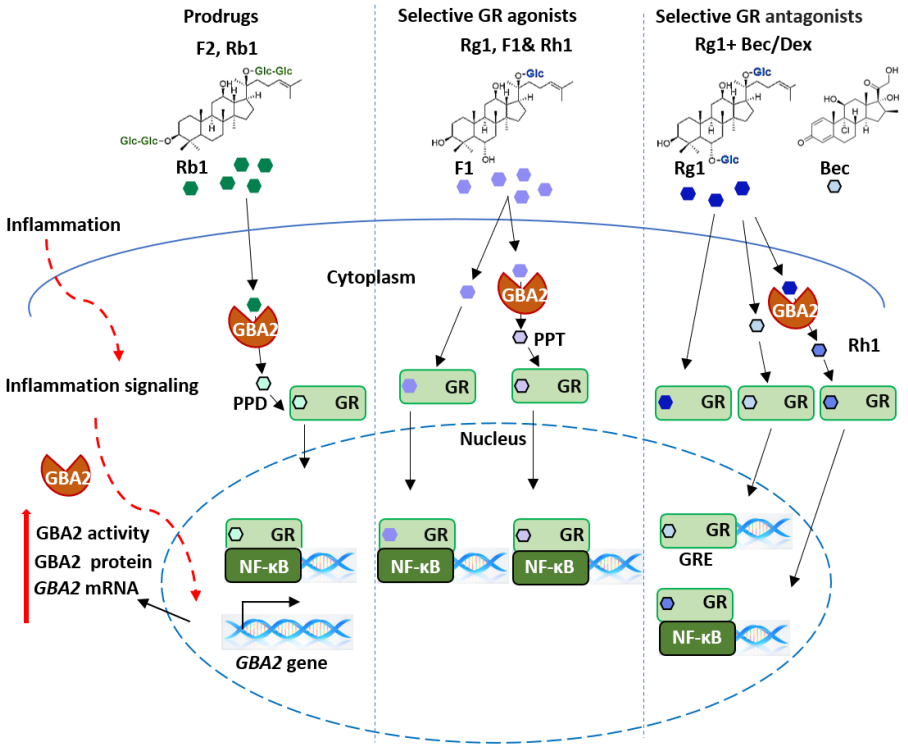

Figure 2. *The mechanisms underlying the effects of ginsenosides, which can act as selective GR agonists, antagonists and as prodrugs.*

3. Insights from ginsenoside research inspired the development of novel glucocorticoid drugs

After unraveling the molecular mechanisms underlying the effects of ginsenosides, we hypothesized in chapters 6 and 7 that adding glucose groups to classical GCs may result in novel GC drugs having potent anti-inflammatory effects but reduced side effects. Interestingly, our data showed that monoglycosylated glucocorticoids (GDex and GPdn) exerted a potent anti-inflammatory effect mediated via Gr. There were no significant differences in anti-inflammatory drugs between GDex and GPdn and their classical aglyconic forms (at the same dose of 25 μM) in the zebrafish model. Interestingly, GDex and GPdn showed significantly reduced side effects compared to their classical aglyconic forms. Our results on the *Tg(9xGCRE-HSV.UI23:EGFP^{ia20})*reporter zebrafish line, the expression of immune-related genes, and endogenous GR target genes in zebrafish and in HeLa cells indicate that GDex and GPdn exhibit their selectivity via inducing mainly transrepression activity of GR. We conclude that adding one glucose group to classical GCs reduces their side effects and makes them promising selective GR agonists.

When we attached the disaccharide gentiobiose to prednisolone, our data showed that the resulting compound, GbPdn, had preserved the anti-inflammatory effect of prednisolone in zebrafish larvae and in a mouse model for arthritis, but this effect was dependent on Gba2 activity. In the zebrafish model, GbPdn showed strongly reduced side effects on glucose and cortisol levels, growth, and tissue regeneration. Additionally, GbPdn did not trigger Gr transactivation activities in *Tg(9xGCRE-HSV.UI23:EGFP^{ia20})* reporter zebrafish line and endogenous Gr target genes. However, upon Gba2 overexpression in the zebrafish larvae, GbPdn did trigger the transactivation activity of Gr, and induced side effect, similarly to prednisolone. Additionally, GbPdn strongly reduced the binding affinity of prednisolone to the human GR *in vitro* and abolished its ability to induce nuclear translocation and transcriptional activity of GR in human (HeLa) cell cultures. However, upon treatment of these cells with the pro-inflammatory cytokine TNF-α, GbPdn treatment did result in nuclear translocation and transcriptional activity of GR. Finally, we demonstrated that these effects of GbPdn were dependent on an increase in GBA2 expression that was induced by the TNF-α treatment. In mouse model for arthritis, GbPdn did not affect corticosterone or insulin levels and endogenous Gr target genes in the liver and muscles. However, GbPdn triggered GR activity locally in inflamed joints due to the local induction of Gba2. We concluded that GbPdn acts as a GC prodrug, which provides a novel approach towards further development of anti-inflammatory GC drugs with an improved therapeutic ratio (Table 2, Fig.3).

Table 2. The effects of classical glucocorticoids (Bec, Dex, Pdn) and their glycosylated forms (GDex, GPdn, GbPdn) in zebrafish.

Treatment	Anti-inflammatory biomarkers	Cortisol	Glucose	Length	Regeneration	Transrepression	Transactivation
Bec/Dex/Pdn	+	+	+	+	+	+	+
GDex	+	+[1]	+[1]	-	+	+	-
GPdn	+	+[1]	+[1]	-	+	+	-
GbPdn	+	+[2]	+[2]	-	+[2]	+[3]	+[3]

- No effect; + Gba2-independent effect; + Gba2-dependent effect; [1] Minor effect under basal conditions, larger effect after wounding/inflammation (although still smaller than effect of Dex/Pdn); [2] Very minor effect, only observed after wounding.

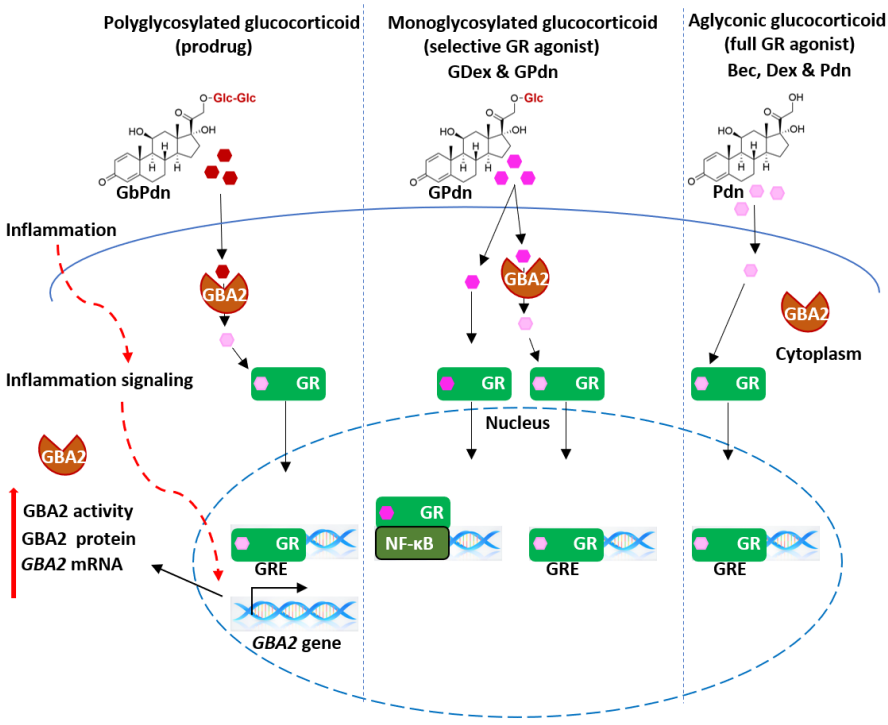

Figure 3. *The mechanisms underlying the effects of polyglycosyalted glucocorticoids (acting as prodrugs), monoglycosylated glucocorticoids (acting as selective GR agonists), and aglyconic glucocorticoids (acting as full GR agonists).* wounding/inflammation; [3] local effect.

In summary, we have presented ginsenosides as valuable tools for further studies into the development of novel GC drugs. Ginsenosides alone or as co-treatment induce anti-inflammatory effects, alleviate the side effects of GC treatment, and may provide a solution to the acquired resistance to GC therapy that currently limits the clinical use of this class of

drugs. However, their low binding affinity to GR makes them unlikely candidates for application in the clinic. In addition to our studies on ginsenosides, we investigated the effect of glycosylation of GC drugs. We showed that monoglycosylated GCs such as GDex and GPdn function as selective GR agonists, and that both compounds have anti-inflammatory effects without triggering side effects. However, monoglycosylated GCs are less suitable for clinical use than polyglycosylated drugs, because they bind the GR relatively well and selectively induce the transrepression activity of the receptor. As a result, they will induce side effects that are a result of this transrepression activity. Furthermore, when the rate of deglycosylation by GBA2 in inflamed tissue is not high enough, and relatively high levels of monoglycosylated GCs are present in this tissue, these monoglycosylated GCs will act as antagonists for the transactivation activity of GR inside the inflamed tissue. Thereby they will inhibit the induction of anti-inflammatory genes such as *NFKBIA* and *GILZ* and limiting the efficacy of the drug [1,3]. Strikingly, the polyglycosylated GC gentiobiose-prednisolone (GbPdn) mediates its anti-inflammatory actions without inducing side effects. GbPdn triggers full GR activities (transrepression and transactivation), locally at the site of inflammation, depending on degylcosylation by GBA2. Therefore, we conclude that polyglycosylation of GCs makes them favorable candidates for treating chronic inflammations and could be an appropriate replacement for currently used GCs in the clinic.

4. Conclusion

Glucocorticoids are potent anti-inflammatory drugs widely used clinically to treat various inflammatory and immune conditions [1,2]. However, two main clinical problems limit their use. GCs trigger severe side effects and they induce acquired glucocorticoid resistance, especially during chronic systemic treatment [6-11]. Therefore, developing novel strategies to improve the treatment inflammatory and immune conditions becomes urgent. Here, we present evidence that ginsenosides act as selective GR agonists, antagonists, and prodrugs. Moreover, our data illustrate that the number and positions of glucose groups bound to the steroid backbone of ginsenosides and different chemical structures are essential factors underlying the differential mechanistic effects of ginsenosides. Understanding the molecular mechanisms and the effects of natural compound (ginsenosides) opens a novel road towards developing improved anti-inflammatory GCs. Attaching one glucose group to classical GCs produced selective Gr agonists, such as GDex and GPdn, which exert anti-inflammatory effects without triggering side effects due to the absence of GR transactivation activities. Attaching two glucose groups to classical GCs resulted in the creation of GC prodrugs, such as GbPdn, which mediates its action locally at the site of inflammation, dependent on GBA2 activity, without triggering side effects.

References

1. Oppong, E.; Cato, A.C.B. Effects of Glucocorticoids in the Immune System BT - Glucocorticoid Signaling: From Molecules to Mice to Man. In; Wang, J.-C., Harris, C., Eds.; Springer New York: New York, NY, 2015; pp. 217–233 ISBN 978-1-4939-2895-8.
2. Cain, D.W.; Cidlowski, J.A. Immune regulation by glucocorticoids. *Nat. Rev. Immunol.* **2017**, *17*, 233–247, doi:10.1038/nri.2017.1.
3. Coutinho, A.E.; Chapman, K.E. The anti-inflammatory and immunosuppressive effects of

glucocorticoids, recent developments and mechanistic insights. *Mol. Cell. Endocrinol.* **2011**, *335*, 2–13, doi:10.1016/j.mce.2010.04.005.
4. Franchimont, D. Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. *Ann. N. Y. Acad. Sci.* **2004**, *1024*, 124–137.
5. Perretti, M.; D'Acquisto, F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat. Rev. Immunol.* **2009**, *9*, 62–70, doi:10.1038/nri2470.
6. Schäcke, H.; Döcke, W.D.; Asadullah, K. Mechanisms involved in the side effects of glucocorticoids. *Pharmacol. Ther.* **2002**, *96*, 23–43, doi:10.1016/s0163-7258(02)00297-8.
7. Joseph, R.M.; Hunter, A.L.; Ray, D.W.; Dixon, W.G. Systemic glucocorticoid therapy and adrenal insufficiency in adults: A systematic review. *Semin. Arthritis Rheum.* **2016**, *46*, 133–141, doi:10.1016/j.semarthrit.2016.03.001.
8. Straub, R.H.; Cutolo, M. Glucocorticoids and chronic inflammation. *Rheumatology (Oxford).* **2016**, *55*, ii6–ii14, doi:10.1093/rheumatology/kew348.
9. Lee, M.-J.; Pramyothin, P.; Karastergiou, K.; Fried, S.K. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. *Biochim. Biophys. Acta* **2014**, *1842*, 473–481, doi:10.1016/j.bbadis.2013.05.029.
10. Pasieka, A.M.; Rafacho, A. Impact of Glucocorticoid Excess on Glucose Tolerance : Clinical and Preclinical Evidence. **2016**, doi:10.3390/metabo6030024.
11. Sato, A.Y.; Richardson, D.; Cregor, M.; Davis, H.M.; Au, E.D.; McAndrews, K.; Zimmers, T.A.; Organ, J.M.; Peacock, M.; Plotkin, L.I.; et al. Glucocorticoids Induce Bone and Muscle Atrophy by Tissue-Specific Mechanisms Upstream of E3 Ubiquitin Ligases. *Endocrinology* **2017**, *158*, 664–677, doi:10.1210/en.2016-1779.
12. He, M.; Huang, X.; Liu, S.; Guo, C.; Xie, Y.; Meijer, A.H.; Wang, M. The Difference between White and Red Ginseng: Variations in Ginsenosides and Immunomodulation. *Planta Med.* **2018**, *84*, 845–854, doi:10.1055/a-0641-6240.
13. Leung, K.W.; Wong, A.S.-T. Pharmacology of ginsenosides: a literature review. *Chin. Med.* **2010**, *5*, 20, doi:10.1186/1749-8546-5-20.
14. MacRae, C.A.; Peterson, R.T. Zebrafish as tools for drug discovery. *Nat. Rev. Drug Discov.* **2015**, *14*, 721–731, doi:10.1038/nrd4627.
15. Schaaf, M.J.M.; Champagne, D.; van Laanen, I.H.C.; van Wijk, D.C.W.A.; Meijer, A.H.; Meijer, O.C.; Spaink, H.P.; Richardson, M.K. Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. *Endocrinology* **2008**, *149*, 1591–1599, doi:10.1210/en.2007-1364.
16. Schaaf, M.J., A. Chatzopoulou, and H.P.S. The zebrafish as a model system for glucocorticoid receptor research. *Comp Biochem Physiol A Mol Integr Physiol 2009. 153(*.
17. Schoonheim, P.J.; Chatzopoulou, A.; Schaaf, M.J.M. The zebrafish as an in vivo model system for glucocorticoid resistance. *Steroids* **2010**, *75*, 918–925, doi:10.1016/j.steroids.2010.05.010.
18. Renshaw, S.A.; Loynes, C.A.; Trushell, D.M.I.; Elworthy, S.; Ingham, P.W.; Whyte, M.K.B. A transgenic zebrafish model of neutrophilic inflammation. *Blood* **2006**, *108*, 3976–3978, doi:10.1182/blood-2006-05-024075.
19. Chatzopoulou, A.; Heijmans, J.P.M.; Burgerhout, E.; Oskam, N.; Spaink, H.P.; Meijer, A.H.; Schaaf, M.J.M. Glucocorticoid-Induced Attenuation of the Inflammatory Response in Zebrafish. *Endocrinology* **2016**, *157*, 2772–2784.
20. Xie, Y.; Tolmeijer, S.; Oskam, J.M.; Tonkens, T.; Meijer, A.H.; Schaaf, M.J.M. Glucocorticoids inhibit macrophage differentiation towards a pro-inflammatory phenotype upon wounding without affecting their migration. **2019**, doi:10.1242/dmm.037887.
21. Chatzopoulou, A.; Heijmans, J.P.M.; Burgerhout, E.; Oskam, N.; Spaink, H.P.; Meijer, A.H.; Schaaf, M.J.M. Glucocorticoid-Induced Attenuation of the Inflammatory Response in Zebrafish. *Endocrinology* **2016**, *157*, 2772–2784, doi:10.1210/en.2015-2050.
22. Xie, Y.; Papadopoulou, P.; de Wit, B.; d'Engelbronner, J.C.; van Hage, P.; Kros, A.; Schaaf,

M.J.M. Two Types of Liposomal Formulations Improve the Therapeutic Ratio of Prednisolone Phosphate in a Zebrafish Model for Inflammation. *Cells* **2022**, *11*, doi:10.3390/cells11040671.

23. Ghaddar, B.; Diotel, N. Zebrafish: A New Promise to Study the Impact of Metabolic Disorders on the Brain. *Int. J. Mol. Sci.* **2022**, *23*, doi:10.3390/ijms23105372.
24. Löhr, H.; Hammerschmidt, M. Zebrafish in endocrine systems: recent advances and implications for human disease. *Annu. Rev. Physiol.* **2011**, *73*, 183–211, doi:10.1146/annurev-physiol-012110-142320.
25. Mathew, L.K.; Sengupta, S.; Kawakami, A.; Andreasen, E.A.; Löhr, C. V; Loynes, C.A.; Renshaw, S.A.; Peterson, R.T.; Tanguay, R.L. Unraveling Tissue Regeneration Pathways Using Chemical Genetics. *J. Biol. Chem.* **2007**, *282*, 35202–35210, doi:https://doi.org/10.1074/jbc.M706640200.
26. Nesan, D.; Vijayan, M.M. Role of glucocorticoid in developmental programming: evidence from zebrafish. *Gen. Comp. Endocrinol.* **2013**, *181*, 35–44, doi:10.1016/j.ygcen.2012.10.006.
27. Willi, R.A.; Faltermann, S.; Hettich, T.; Fent, K. Active Glucocorticoids Have a Range of Important Adverse Developmental and Physiological Effects on Developing Zebrafish Embryos. *Environ. Sci. Technol.* **2018**, *52*, 877–885, doi:10.1021/acs.est.7b06057.
28. Chatzopoulou, A.; Roy, U.; Meijer, A.H.; Alia, A.; Spaink, H.P.; Schaaf, M.J.M. Transcriptional and metabolic effects of glucocorticoid receptor α and β signaling in zebrafish. *Endocrinology* **2015**, *156*, 1757–1769, doi:10.1210/en.2014-1941.
29. Zhang, L.; Zhu, M.; Li, M.; Du, Y.; Duan, S.; Huang, Y.; Lu, Y.; Zhang, J.; Wang, T.; Fu, F. Ginsenoside Rg1 attenuates adjuvant-induced arthritis in rats via modulation of PPAR-γ/NF-κB signal pathway. *Oncotarget* **2017**, *8*, 55384–55393, doi:10.18632/oncotarget.19526.
30. Du, J.; Cheng, B.; Zhu, X.; Ling, C.; Alerts, E. Ginsenoside Rg1, a Novel Glucocorticoid Receptor Agonist of Plant Origin, Maintains Glucocorticoid Efficacy with Reduced Side Effects. **2019**, doi:10.4049/jimmunol.1002579.
31. Karra, A.G.; Konstantinou, M.; Tzortziou, M.; Tsialtas, I.; Kalousi, F.D.; Garagounis, C.; Hayes, J.M.; Psarra, A.-M.G. Potential Dissociative Glucocorticoid Receptor Activity for Protopanaxadiol and Protopanaxatriol. *Int. J. Mol. Sci.* **2018**, *20*, 94, doi:10.3390/ijms20010094.
32. Lee, Y.J.; Chung, E.; Lee, K.Y.; Lee, Y.H.; Huh, B.; Lee, S.K. Ginsenoside-Rg1, one of the major active molecules from Panax ginseng, is a functional ligand of glucocorticoid receptor. *Mol. Cell. Endocrinol.* **1997**, *133*, 135–140, doi:10.1016/s0303-7207(97)00160-3.
33. Sundahl, N.; Bridelance, J.; Libert, C.; De Bosscher, K.; Beck, I.M. Selective glucocorticoid receptor modulation: New directions with non-steroidal scaffolds. *Pharmacol. Ther.* **2015**, *152*, 28–41, doi:10.1016/j.pharmthera.2015.05.001.
34. Wang, W.; Rayburn, E.R.; Zhao, Y.; Wang, H.; Zhang, R. Novel ginsenosides 25-OH-PPD and 25-OCH3-PPD as experimental therapy for pancreatic cancer: anticancer activity and mechanisms of action. *Cancer Lett.* **2009**, *278*, 241–248, doi:10.1016/j.canlet.2009.01.005.
35. Li, J.; Du, J.; Liu, D.; Cheng, B.; Fang, F.; Weng, L.; Wang, C.; Ling, C. Ginsenoside Rh1 potentiates dexamethasone's anti-inflammatory effects for chronic inflammatory disease by reversing dexamethasone-induced resistance. *Arthritis Res. Ther.* **2014**, *16*, R106, doi:10.1186/ar4556.
36. Ishii, T.; Kibushi, N.; Nakajima, T.; Kakuta, T.; Tanaka, N.; Sato, C.; Sugai, K.; Kijima-Suda, I.; Kai, H.; Miyata, T. Local Anti-inflammatory Activity and Systemic Side Effects of NM-135, a New Prodrug Glucocorticoid, in an Experimental Inflammatory Rat Model. *Jpn. J. Pharmacol.* **1998**, *78*, 505–509, doi:10.1254/jjp.78.505.
37. Hirschmann, R.; Strachan, R.G.; Buchschacher, P.; Sarett, L.H.; Steelman, S.L.; Silber, R. An Approach to an Improved Antiinflammatory Steroid. The Synthesis of 11β,17-Dihydroxy-3,20-dione-1,4-pregnadien-21-yl 2-Acetamido-2-deoxy-β-D-glucopyranoside. *J. Am. Chem. Soc.* **1964**, *86*, 3903–3904, doi:10.1021/ja01072a086.

38. Khachigian, L.M. Collagen antibody-induced arthritis. *Nat. Protoc.* **2006**, *1*, 2512–2516, doi:10.1038/nprot.2006.393.
39. Skauby, R.H.; Bergan, S.; Andersen, A.M.; Vethe, N.T.; Christensen, H. In vitro assessments predict that CYP3A4 contributes to a greater extent than CYP3A5 to prednisolone clearance. *Basic Clin. Pharmacol. Toxicol.* **2021**, *129*, 427–436, doi:https://doi.org/10.1111/bcpt.13645.
40. Hu, C.; Lau, A.J.; Wang, R.; Chang, T.K.H. Comparative analysis of ginsenosides in human glucocorticoid receptor binding, transactivation, and transrepression. *Eur. J. Pharmacol.* **2017**, *815*, 501–511, doi:10.1016/j.ejphar.2017.10.019.
41. Chen, R.J.Y.; Chung, T.; Li, F.; Lin, N.; Tzen, J.T.C. Effect of sugar positions in ginsenosides and their inhibitory potency on Na+/K+-ATPase activity. *Acta Pharmacol. Sin.* **2009**, *30*, 61–69, doi:10.1038/aps.2008.6.
42. Nag, S.; Qin, J.; Wang, W.; Wang, M.-H.; Wang, H.; Zhang, R. Ginsenosides as anticancer agents: in vitro and in vivo activities, structure-activity relationships, and molecular mechanisms of action . *Front. Pharmacol.* 2012, *3*, 25.