Pharmacology & Therapeutics xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Selective glucocorticoid receptor modulation: New directions with non-steroidal scaffolds

Nora Sundahl [a], Jolien Bridelance [a,1], Claude Libert [b,c], Karolien De Bosscher [d,*,2], Ilse M. Beck [a,2]

[a] Laboratory of Experimental Cancer Research (LECR), Department of Radiation Oncology & Experimental Cancer Research, Ghent University, Gent, Belgium
[b] Department for Molecular Biomedical Research, VIB, Gent, Belgium
[c] Department of Biomedical Molecular Biology, Ghent University, Gent, Belgium
[d] Receptor Research Laboratories, Nuclear Receptor Lab (NRL), VIB Department of Medical Protein Research, Ghent University, Gent, Belgium

ARTICLE INFO

Keywords:
Glucocorticoids
Glucocorticoid receptor
Selective glucocorticoid receptor modulator
Compound A
Side effects
Glucocorticoid resistance

ABSTRACT

Glucocorticoids remain the frontline treatment for inflammatory disorders, yet represent a double-edged sword with beneficial therapeutic actions alongside adverse effects, mainly in metabolic regulation. Considerable efforts were made to improve this balance by attempting to amplify therapeutic beneficial anti-inflammatory actions and to minimize adverse metabolic actions. Most attention has focused on the development of novel compounds favoring the transrepressing actions of the glucocorticoid receptor, assumed to be important for anti-inflammatory actions, over the transactivating actions, assumed to underpin the undesirable actions. These compounds are classified as selective glucocorticoid receptor agonists (SEGRAs) or selective glucocorticoid receptor modulators (SEGRMs). The latter class is able to modulate the activity of a GR agonist and/or may not classically bind the glucocorticoid receptor ligand-binding pocket. SEGRAs and SEGRMs are collectively denominated SEGRAMs (selective glucocorticoid receptor agonists and modulators). Although this transrepression vs transactivation concept proved to be too simplistic, the developed SEGRAMs were helpful in elucidating various molecular actions of the glucocorticoid receptor, but have also raised many novel questions. We discuss lessons learned from recent mechanistic studies of selective glucocorticoid receptor modulators. This is approached by analyzing recent experimental insights in comparison with knowledge obtained using mutant GR research, thus clarifying the current view on the SEGRAM field. These insights also contribute to our understanding of the processes controlling glucocorticoid-mediated side effects as well as glucocorticoid resistance. Our perspective on non-steroidal SEGRAs and SEGRMs considers remaining opportunities to address research gaps in order to harness the potential for more safe and effective glucocorticoid receptor therapies.

Contents

Abbreviations: AP-1, activator protein 1; CpdA, Compound A; CRH, corticotropin-releasing hormone; DBD, DNA binding domain; DUSP, dual specificity phosphatase; ENaC, epithelial Na^+ channels; GC, glucocorticoid; GILZ, glucocorticoid induced leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; HSP, heat shock protein; LBD, ligand-binding domain; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; nGRE, negative glucocorticoid responsive element; OPG, osteoprotegerin; SEGRA, selective glucocorticoid receptor agonist; SEGRM, selective glucocorticoid receptor modulator; SEGRAM, selective glucocorticoid receptor agonist and modulator.

* Corresponding author at: Receptor Research Laboratories, Nuclear Receptor Lab (NRL), VIB Department of Medical Protein Research, VIB, UGent, Albert Baertsoenkaai 3, B-9000 Gent, Belgium. Tel.: +32 92649363; fax: +32 26499490.
E-mail address: Karolien.DeBosscher@vib-ugent.be (K. De Bosscher).
[1] Moved to Molecular Signaling and Cell Death, VIB Inflammation Research center, Ghent University, Gent, Belgium.
[2] Shared senior authorship.

http://dx.doi.org/10.1016/j.pharmthera.2015.05.001
0163-7258/

1. Introduction

1.1. Glucocorticoids

Glucocorticoids (GCs) are steroid hormones produced in the adrenal cortex on a circadian rhythm. The production of these hormones is regulated by the hypothalamic-pituitary-adrenal axis. Neural, endocrine and also cytokine signals converge at the hypothalamus periventricular nucleus. These signals determine the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus into the portal system of the pituitary gland. Successively, the CRH induces secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn stimulates synthesis and secretion of the glucocorticoid cortisol (also named hydrocortisone) from the zona fasciculata of the adrenal cortex. Feedback mechanisms ensure a tight control on the cortisol production and release (Fig. 1). Approximately 10% of the secreted cortisol is free and thereby active; the other 90% is bound to systemic corticosteroid-binding globulins (Chapman et al., 2013; Nicolaides et al., 2014). GCs activate the glucocorticoid receptor (GR), a transcription factor belonging to the nuclear receptor superfamily (see Section 2.1).

1.1.1. Therapeutic use of glucocorticoids

GCs are widely used for the treatment of inflammatory, immune and allergic disorders (e.g. rheumatoid arthritis, asthma), brain edema, shock and various blood cancers (e.g. multiple myeloma); they are also used for preventing rejection after transplant, and for correcting adrenal cortical hormone insufficiency. The clinical success of exogenous GCs (e.g. dexamethasone, prednisolone…) is largely due to their anti-inflammatory characteristics. GCs suppress inflammation mainly via transrepression of inflammatory and immune genes, such as genes coding for cytokines, chemokines, inflammatory enzymes and receptors, and adhesion molecules that play a role in migration of cells towards sites of inflammation (Belvisi, 2004; Ito et al., 2006b; McMaster & Ray, 2008; Barnes, 2011). Unfortunately, the use of GCs is often not recommended, due to the wide range of side effects. These include diabetes, muscle wasting and osteoporosis (Schacke et al., 2002).

The current challenge is to minimize as many as possible of these side effects and optimize GR-associated beneficial effects. The idea of resolving all the side effects associated with glucocorticoids is, however, a utopia. It would therefore already be a great achievement to eliminate the clinically most burdening ones. Recent research has intensely focused on a class of pharmacologic compounds, selective glucocorticoid receptor agonists and modulators (SEGRAMs), that display an improved therapeutic index *in vivo* via a select skewing of the GR effector profile (Rosen & Miner, 2005). The current SEGRAMs only (or mainly) work via the transrepression pathway of glucocorticoid receptors (GRs), thereby resulting in a more specific action radius of GR (McMaster & Ray, 2008; De Bosscher, 2010; De Bosscher et al., 2010b).

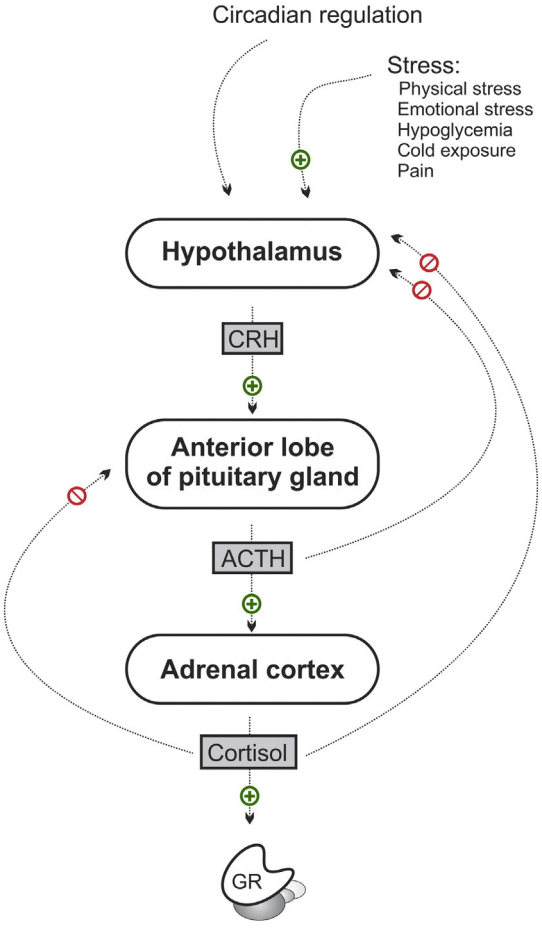

Fig. 1. Hypothalamic-pituitary-adrenal axis. The production of cortisol, the endogenous GC, has a circadian rhythm and regulation, and starts at the level of the hypothalamus. Here, neural endocrine and cytokine signals converge and instigate a secretion of CRH into the portal system of the pituitary gland. Successively, the CRH induces secretion of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary gland, which in turn stimulates the synthesis and secretion of cortisol from the adrenal cortex. Negative feedback mechanisms safeguard homeostasis of the system. ⊕ indicates positive regulation, ⊘ indicates negative regulation.

1.2. Selective glucocorticoid receptor modulators

It is assumed that the anti-inflammatory effects of GCs are largely due to GR transrepression mechanisms, while GR transactivation is accountable for the greater part of GC treatment-associated side effects. This statement has, however, turned out to be too simplistic. It has indeed been shown that some side effects are predominantly mediated via transactivation (e.g. hyperglycemia and muscle wasting), yet other side effects arise from transrepression (e.g. hypothalamic-pituitary-adrenal axis suppression), and still other side effects (e.g. osteoporosis) are mediated by both transactivation and transrepression (Schacke et al., 2002; Carballo-Jane et al., 2004). Nevertheless, examples of GR ligands exist, which can selectively induce transrepression without significant transactivation, and for which in the long run the risk of systemic side effects may be reduced, while anti-inflammatory activities are maintained.

Compounds that can activate specific GR mechanisms and thus alter GR-mediated gene expression profiles are referred to as dissociated compounds, selective glucocorticoid receptor agonists (SEGRAs) or modulators (SEGRMs) (Rosen & Miner, 2005; Beck et al., 2009) (Fig. 2). The term SEGRA was the first term used, as the compounds historically were derived from a steroidal scaffold (e.g. RU 24858) and they often still exhibited a partial agonistic effect on the transactivation mechanism of GR (Belvisi et al., 2001). The use of the term 'SEGRM' was initiated to distance the newer, non-steroidal compounds from the older ones.

One of the first SEGRMs to be characterized was Compound A (CpdA) (De Bosscher et al., 2005) (Fig. 3). This non-steroidal compound showed an atypical competition binding curve in ligand-binding assays using labeled dexamethasone, a synthetic GC (De Bosscher et al., 2005; Ronacher et al., 2009). This result hinted to the fact that CpdA may use different contact points in the ligand-binding domain (LBD) of GR or may change GR's conformation in a different way. The latter hypothesis has been supported by experimental data (De Bosscher et al., 2005). As a fully detailed mechanistic characterization of many of these second-generation compounds is still anticipated, we will refer to them in this review as SEGRMs. In recent years, research on SEGRMs boomed and the amount of molecules reported explosively grew. One of the more extensively researched SEGRMs is CpdA. This molecule, widely studied *in vitro* as well as *in vivo*, has proven to favor transrepression over transactivation and therefore supports the recently challenged

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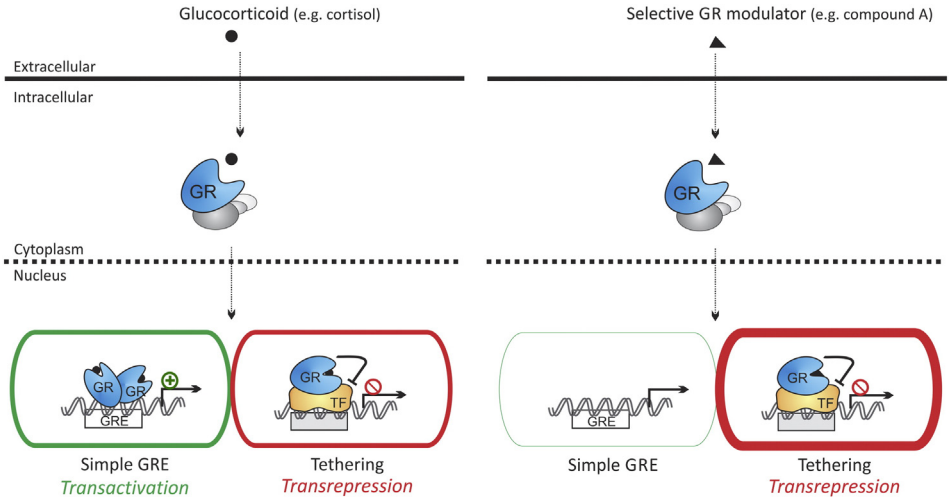

Fig. 2. Principle of a selective GR modulator (SEGRM). Glucocorticoids enter the cell and bind to the glucocorticoid receptor (GR). Successively, activated GR influences gene transcription via various mechanisms, including transactivation, i.e. stimulating the expression of certain genes via direct DNA binding, and transrepression, i.e. inhibiting the expression of certain genes via indirect DNA binding, also called a tethering mechanism. Selective GR modulators (SEGRMs) differ from GCs in the way that upon binding to GR they trigger transrepression, but do not initiate transactivation.

assumption that an anti-inflammatory therapy with less side effects remains a feasible goal (De Bosscher et al., 2005, 2010a, 2014; Zhang et al., 2009b; Reber et al., 2012; Thiele et al., 2012; Beck et al., 2013; Rauner et al., 2013; Saksida et al., 2014). However, CpdA's lability (Wust et al., 2009), in combination with a narrow therapeutic range, causes this SEGRM to be inappropriate for therapy, yet excellent as a tool compound for research purposes.

The SEGRMs discussed here have showed to exhibit anti-inflammatory effect *in vitro* as well as *in vivo* in various studies. These studies established various SEGRMs' abilities to repress inflammatory mediators *in vitro* (Supplementary Table 1). Some SEGRMs were tested in human tissues, but also in mice and rat models. With regards to the latter, the anti-inflammatory effects of the test SEGRMs were assessed using inflammatory disease models *in vivo*, such as allergic conjunctivitis (Baiula et al., 2014), (rheumatoid) arthritis (Miner et al., 2007; Dewint et al., 2008; López et al., 2008; Thiele et al., 2012; Rauner et al., 2013; Carson et al., 2014), neuro-inflammation (Zhang et al., 2009a; van Loo et al., 2010), asthma (Reber et al., 2012) and colitis (Reuter et al., 2012a,b). Yet, it needs to be said that, although it has been proven that all SEGRMs discussed here (except PF-802) can bind to GR (Coghlan et al., 2003; Schacke et al., 2004, 2009; De Bosscher et al., 2005; Chivers et al., 2006; Miner et al., 2007; Zhang et al., 2009b; van Lierop et al., 2012; Brandish et al., 2014; Carson et al., 2014), only CpdA and ZK 216346 are shown to elicit a partial or full nuclear translocation of GR (De Bosscher et al., 2005; Dewint et al., 2008; Yemelyanov et al., 2008; Robertson et al., 2010; Reuter et al., 2012b; Presman et al., 2014; Drebert et al., 2015) (also see 2.1 Structure of Glucocorticoid receptors). Therefore, it cannot be completely excluded that their observed *in vitro* and *in vivo* anti-inflammatory effect is perhaps (partially) mediated by GR-independent action mechanisms.

In the following chapters, we will discuss the paradigms of GR signaling with a critical focus on reported effects of the selective glucocorticoid receptor modulator CpdA and other SEGRMs on several aspects of GR signaling and remaining voids, in comparison with reported effects of the GR effector profile-skewing mutants. Such a GR mutant compromised in its dimerization functions is the GR*dim* mutant.

2. Review: Glucocorticoid- vs SEGRAM-mediated GR signaling

2.1. Structure of Glucocorticoid receptors

As a result of their lipophilic character, GCs can easily diffuse across the cell membrane of target cells (Smith & Cidlowski, 2010). Subsequently, GCs can bind to intracellular glucocorticoid receptors (GRs), also known as NR3C1 (nuclear receptor 3, group C, member 1), which are present in almost all human cells. These GRs are members of the steroid hormone receptor family of proteins (Rhen & Cidlowski, 2005; Kino et al., 2011).

The GR is a nuclear hormone receptor acting as a ligand-activated transcription factor and consisting of an N-terminal transactivation domain (NTD), a DNA binding domain (DBD), a hinge region and a C-terminal LBD (Fig. 4A). The gene coding for human GR contains 9 exons and is located on chromosome 5q31-32. Alternative splicing can result in different isoforms: GRα, GRβ, GRγ, GR-A and GR-P. The predominant, and most extensively researched isoform is full-length GRα. Both GRα and GRγ isoforms can bind hormone and regulate gene expression. In contrast, the GRβ isoform is incapable of binding hormone and exerts dominant-negative effects on GRα. Glucocorticoids cannot bind GR-A and GR-P isoforms as a result of their truncated LBD (Oakley & Cidlowski, 2011).

AL-438 (Ki 2.5 nM) and ORG 214007-0 (Ki 2.2 nM) show a high binding affinity for GR, comparable to prednisolone (Ki 2.4 nM) (Fig. 3) (Coghlan et al., 2003; van Lierop et al., 2012). Furthermore, also Mapracorat (alternatively known as ZK 245186 and BOL-303242X) (Schacke et al., 2009; Zhang et al., 2009a) and LGD-5552 (Ki 2.4 nM) (Miner et al., 2007) (Fig. 3) show a high affinity and selectivity towards GR (Table 1). Their direct interaction with GR was demonstrated via competitive ligand binding assays (Miner et al., 2007; Schacke et al., 2009). As for CpdA, research has proven that this SEGRM can compete with dexamethasone for binding to endogenous GR (De Bosscher et al., 2005). The binding affinity varies between cell lines, which could depend on the levels of GR in these cells (De Bosscher et al., 2005; Ronacher et al., 2009; Robertson et al., 2013b). An alternative explanation may reside in different ligand-dependent GR-associated cofactor equilibria, which were shown before to be able to modulate the properties of agonist and antagonist complexes of GR (Wang et al., 2004; Simons et al., 2014).

CpdA induces a different, currently unclarified, conformational change of GR (De Bosscher et al., 2005). Although *in silico* modeling mapped CpdA to fit the ligand-binding pocket of GR (Yemelyanov et al., 2008), other modes of binding cannot be excluded, because we still await the first elucidated crystal structure of this particular SEGRM binding to the GR-LBD. The elucidation of GR's structure, when activated by non-steroidal indazole amides, of which some show skewing towards a higher level of transrepression over transactivation, has been very informative. In this research, combined crystallography

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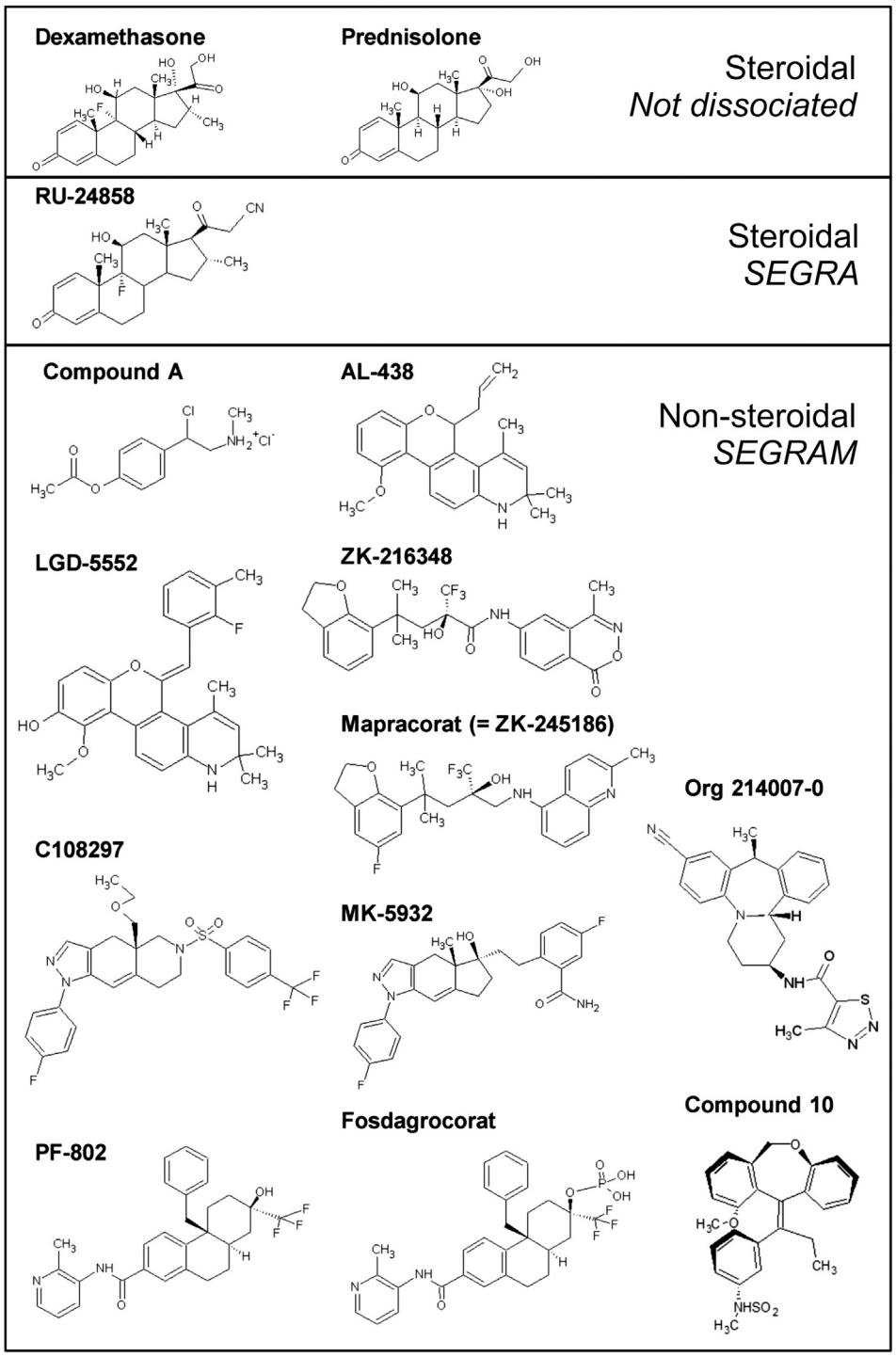

Fig. 3. Structures of selected synthetic GCs and SEGRAMs.

and modelling revealed a second binding site within the ligand-binding pocket of GR (Biggadike et al., 2009). Also the crystal structure of GR-LBD bound with compound 10, which retains full transrepression with a partial transactivation ability, shows a new binding mode, clearly different from the classic GC binding model (Carson et al., 2014). The potential binding of SEGRMs to other transcriptional isoforms besides the classical GRα remains an uncharted area.

Although the structure of most other SEGRM-bound GRs is not completely elucidated, we would assume that the ultimate conformation would differ from a classic GC-bound GR, exposing other cofactor binding surfaces and leading to an alternate cofactor-binding profile. This hypothesis is confirmed for CpdA-bound GR (Ronacher et al., 2009).

Additional GR isoforms with progressively shorter N-terminal transactivation domains are also produced due to 8 alternative translation initiation sites (e.g. GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2 and GRα-D3) with distinct gene expression profiles (Oakley & Cidlowski, 2011; Wu et al., 2013). Given this complexity of transcriptional and translational isoforms, understandably, the proportion of different GR isoforms in a cell modulates the final effects of a presented GC or SEGRM (Gronemeyer et al., 2004; Rhen & Cidlowski, 2005; De Bosscher et al., 2010a; Kino et al., 2011; Wu et al., 2013).

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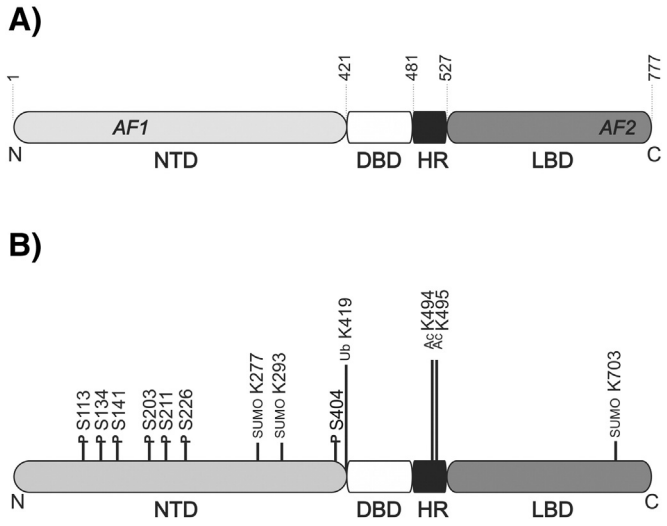

Fig. 4. GR structure. A. Structure of the human GRα-A, consisting of an N-terminal transactivation domain (NTD), a DNA binding domain (DBD), a hinge region (HR) and a C-terminal ligand binding domain (LBD). (AF, activation function). B. Post-translational modifications of human GRα-A. (Ac, acetylation; K, lysine; P, phosphorylation; S, serine; SUMO, sumoylation; Ub, ubiquitinylation).

Furthermore, it has been found that a rise in the ratio of GRβ/GRα levels appears to be a mechanism involved in the development of glucocorticoid resistance in multiple organs (Lewis-Tuffin & Cidlowski, 2006). Whether and how selective GR modulators can impact the cell-specific levels and ratios of these transcriptional and translational isoforms is not yet known.

2.2. Glucocorticoid receptor-mediated mechanisms of action

Activation of GR results both in direct gene activation and gene repression and a range of non-genomic effects indirectly influencing gene transcription, thereby all causing a decrease in inflammatory proteins and an increase in anti-inflammatory proteins.

In the absence of a ligand, a native GR resides predominantly in the cytoplasm as part of a large multiprotein complex including a heat shock protein (HSP) 90 dimer, various chaperone proteins and immunophilins. However, a continuous shuttling between the nucleus and the cytoplasm of both activated and non-activated GR takes place (Hache et al., 1999; Vandevyver et al., 2012a). After binding to its steroidal ligand, GR undergoes a conformational change, replaces the immunophilin FKBP51 with FKBP52 in its chaperoning complex and is guided by HSP90 and FKBP52 to the nucleus (Vandevyver et al., 2012a). Subsequently, activated nuclear GR can modulate the expression of GC-responsive genes either by binding to a GR-binding sequence in glucocorticoid-responsive elements (GREs) in the promoter region of specific target genes or through physical interaction with other transcription factors (Kino et al., 2011) (see Sections 2.2.1 and 2.2.2). Furthermore, also a multimodal non-genomic pathway via which GR can influence various cellular signaling cascades and events has been elucidated (Smith & Cidlowski, 2010) (see Section 2.2.4). The extent and duration of all these processes and mechanisms are co-determined by various factors, such as the identity of the ligand bound, the involved GR isoform, the available cofactors, other activated cross-talking transcription factors, other cellular protein-modifying factors and the targeted gene sequences themselves (Oakley & Cidlowski, 2011).

HSP70, one of the chaperone molecules of GR, has an anti-inflammatory effect via its capability to repress nuclear factor-κB (NF-κB) (Malhotra & Wong, 2002; Weiss et al., 2007). It has been shown that heat shock, as well as the SEGRM CpdA induce gene expression of HSP70. However, they both do this in a different manner. Heat shock induces HSP70 expression in a heat shock factor protein 1 (HSF1)-dependent and GR-independent manner, whereas CpdA induces the expression in a HSF1-independent and GR-dependent manner. Even more intriguing is the fact that following CpdA a clear HSP70 gene expression activation is observed in absence of a concomitant rise in (additional) HSP70 protein, in L929sA and A549 cell lines (Beck et al., 2013). Although the SEGRMs CpdA and ZK 216346 have shown to be able to translocate GR into the nucleus, the extent of their impact on GR's nuclear accumulation is less pronounced than achieved with classic GCs and appears to differ depending on the cell type (De Bosscher et al., 2005; Dewint et al., 2008; Yemelyanov et al., 2008; Robertson et al., 2010; Reuter et al., 2012b; Presman et al., 2014; Drebert et al., 2015). The decreased nuclear import of CpdA-bound GR was suggested to be caused by its monomeric status (Robertson et al., 2013a). Studies investigating the effect of SEGRMs on GR association with their chaperoning complex, and more in depth studies on SEGRM-mediated effects on GR's intracellular localization and GR mobility still need to be performed.

Table 1
SEGRAM-mediated binding to GR.

SEGRAM	GR origin	GR binding	Ref
RU24858	A549, h	$K_i = 110.0 \pm 24.0$ nM	[a]
Org214007-0	Recombinant hGR	$K_i = 2.2 \pm 1.3$ nM	[b]
AL-438	SF-9 moth cells infected with recombinant baculovirus expressing hGR	$K_i = 2.5$ nM	[c]
	COS-1, s, TT hGR	$IC_{50} = 61 \pm 13$ nM	[d]
Compound A	L929sA, m	$IC_{50} = 6.4$ nM (CI 1.9-20.5 nM)	[e]
	BWTG3, m	$K_d = 81.8$ nM	[f]
	COS-1, s, TT hGR	$IC_{50} = 0.003 \pm 0.004$ nM	[d]
Compound 10	HEK293, h, TT hGR	$K_i = 0.268 \pm 0.026$ nM	[g]
LGD-5552	SF-9 moth cells infected with recombinant baculovirus expressing hGR	$K_i = 2.4 \pm 0.6$ nM	[h]
		$K_d = 2$ nM	[i]
MK-5932	insect cell-expressed hGR	$K_i = 3.7$ nM	[j]
C108297	Recombinant hGR	$K_i = 0.9$ nM	[k]
ZK 216348	Recombinant hGR	$IC_{50} = 20.3 \pm 2.6$nM	[l]
Mapracorat	SF-9 moth cells infected with recombinant baculovirus expressing hGR	$CF = 1.9 \pm 0.5$	[m]
PF-802		Not published	

Abbreviations:
CF, Competition factor CF; defined as IC_{50} of test compound/IC_{50} of reference compound DEX.
CI, confidence interval; h, human; m, murine; s, simian; TT, transiently transfected.
IC_{50}, concentration at which compound inhibited 50% of specific binding of labeled dexamethasone.
Ref, *References*.
[a] (Chivers et al., 2006), [b] (van Lierop et al., 2012), [c] (Coghlan et al., 2003), [d] (Ronacher et al., 2009), [e] (De Bosscher et al., 2005), [f] (Robertson et al., 2010), [g] (Carson et al., 2014), [h] (Miner et al., 2007), [i] (López et al., 2008), [j] (Brandish et al., 2014), [k] (R. D. Clark et al., 2008), [l] (Schacke et al., 2004), [m] (Schacke et al., 2009).

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2.2.1. Stimulation of gene transcription

Gene expression can be stimulated via three main different mechanisms (Fig. 5): (1) the GR forms a dimer and binds to an imperfect palindromic GRE, thus activating the promoter (this is called transactivation), (2) the (monomeric) GR binds to DNA together with a transcription factor, in this way cooperatively enhancing gene expression (this is called composite transactivation), and (3) the GR can also interact with transcription factors without interacting with DNA itself (this is called tethering). These three mechanisms stimulate, among others, the expression of anti-inflammatory proteins and also of metabolic gene products, which can give rise to side effects associated with GC therapy, such as diabetes, glaucoma, hypertension, and muscle wasting (Beck et al., 2009).

Since transactivation is the mechanism of action generally linked to the side effects associated with GC-therapy, research mainly focused on the development of compounds not exerting enhanced expression of GRE-regulated genes (Barnes, 2011).

Unlike GCs, CpdA does not cause GR dimerization, and does not allow the binding of GR to a classic GRE. Hence, CpdA does not support a transactivation mechanism (De Bosscher et al., 2005; Dewint et al., 2008; Robertson et al., 2010, 2013b; Presman et al., 2014). As increasing GR concentrations allow a ligand-independent GR dimerization, it is striking that CpdA-bound GR, even under these conditions, maintains its predominant monomeric state (Robertson et al., 2013b). Also several other SEGRMs, including AL-438 (Coghlan et al., 2003), Mapracorat (Schacke et al., 2009), PF-802 (Hu et al., 2011), ZK 216348 (Schacke et al., 2004, 2007), LGD-5552 (Miner et al., 2007; López et al., 2008), and Org 214007-0 (van Lierop et al., 2012), have shown a reduced transactivating potential in comparison to classic GCs (Supplementary Table 1), however, without studying if and how these SEGRMs may affect GR dimer formation. Yet, it should be noted that the simple idea of ligand-induced GR dimerization translating into GR transactivation is challenged (see 2.2.3 GR*dim* action mechanism). Further, the effect of CpdA or other SEGRMs on gene expression enhancement via the tethering and composite GRE mechanisms still needs to be investigated.

2.2.2. Inhibition of gene transcription

On the other hand, GCs also repress gene transcription via a number of different mechanisms (Fig. 5): (1) GR can interact with a transcription factor, thereby inhibiting the transcription factor and repressing transcription. This is the predominant transrepression mechanism and is called tethering; it is the mechanism frequently used to inhibit the pro-inflammatory transcription factors NF-κB (De Bosscher et al., 2006; Beck et al., 2009) and activator protein 1 (AP-1). Sometimes this mechanism can also lead to sequestration of the transcription factor. The "Number and Brightness" technology, a moment-based analysis of the average number of moving fluorescent molecules and their brightness at every pixel, shows that GC-activated GR also remains in a dimeric state around GR:NF-κB interactions, arguing against an exclusive role for monomeric GR in GR transrepression (Presman et al., 2014). Alternatively, (2) another transcription factor-responsive element overlaps with the GRE, and the subsequent GR binding to the GRE blocks the transcription factor from binding to the response element of the respective transcription factor. This is called a competitive GRE. (3) When a GR binds to its GRE and cross-talks with the transcription factor bound to its designate transcription factor-responsive element we consider this a composite GRE, and finally (4) there is evidence of GR binding to a negative GRE (nGRE), resulting in transcriptional repression. These mechanisms typically inhibit the transcription of pro-inflammatory proteins (e.g. cytokines, enzymes and adhesion molecules) (Stahn & Buttgereit, 2008; Beck et al., 2009). Hence, the transrepression mechanism has since long been linked to the

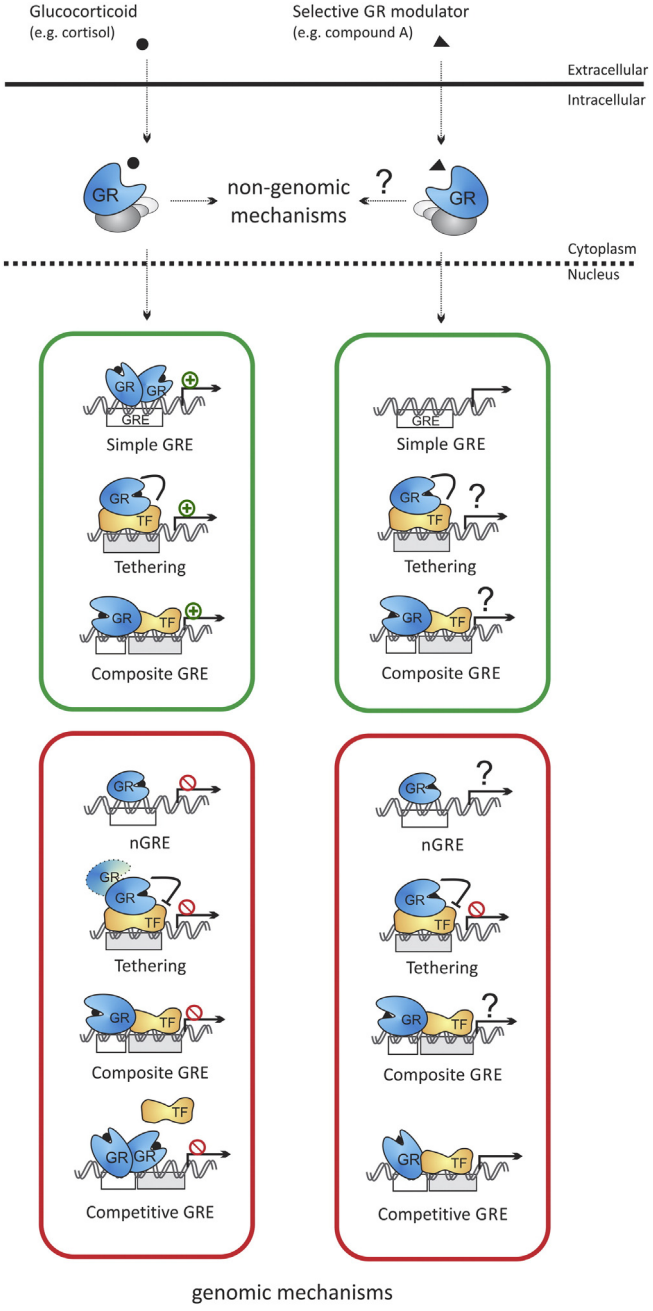

Fig. 5 GR mechanisms.Upon binding to GR, GCs induce non-genomic mechanisms and genomic mechanisms. The genomic mechanisms can be divided into two groups, namely stimulation of gene transcription (indicated in green) and inhibition of gene transcription (indicated in red). Gene expression can be stimulated by three different mechanisms: (1) the GR can interact with transcription factors without interacting with DNA itself (i.e. tethering); (2) the GR forms a dimer and binds to a GRE (white box), thus activating the promoter (i.e. transactivation); (3) the monomeric GR binds to DNA together with another transcription factor, in this way cooperatively enhancing gene expression (i.e. composite transactivation). Gene transcription can be inhibited by a number of different mechanisms, including: (1) GR binding to a negative GRE (nGRE), resulting in transcription repression; (2) in the tethering mechanism, GR can interact with a DNA-binding transcription factor, thereby inhibiting the transcription factor and repressing transcription; (3) a GR bound to its GRE can cross-talk with another transcription factor bound to its respective transcription factor-responsive element (TFRE) (grey box) and form a composite GRE; (4) a transcription factor-responsive element (TFRE) can overlap with the GRE, and the subsequent GR binding to the GRE blocks the transcription factor from binding to the response element of the respective transcription factor, forming a competitive GRE. SEGRMs, specifically CpdA, differ from classic GCs in the way that they do induce the transrepression mechanism, yet do not induce the transactivation mechanism. Other SEGRMs have a compromised transactivation capacity. Although further studies are still required on other competitive GRE-regulated genes, the SEGRM-mediated osteocalcin gene expression regulation suggests a preliminary model of a SEGRM-bound monomeric GR disengaging the competitive GRE and allowing the binding of the driving transcription factor (Coghlan et al., 2003; Hu et al., 2011; Rauch et al., 2011). The effect of SEGRMs on the four other mechanisms mentioned above has not yet been elucidated in detail.

Please cite this article as: Sundahl, N., et al., Selective glucocorticoid receptor modulation: New directions with non-steroidal scaffolds, *Pharmacology & Therapeutics* (2015), http://dx.doi.org/10.1016/j.pharmthera.2015.05.001

anti-inflammatory effects associated with GCs, and thus, in general, the therapeutic effect. The combination of a transrepressive activity with less or no transactivating activity is thought to lead to a compound with an improved therapeutic index, meaning more on target, wanted therapeutic effects and less side effects (van Lierop et al., 2012).

SEGRMs still exhibiting this transrepressive capability were therefore sought and found. CpdA (De Bosscher et al., 2005), Mapracorat (Schacke et al., 2009; Cavet et al., 2013), PF-802 (Hu et al., 2011), AL-438 (Coghlan et al., 2003), ZK 216348 (Schacke et al., 2004, 2007, 2009; Reuter et al., 2012b), LGD-5552 (Miner et al., 2007; López et al., 2008), MK-5932 (Bungard et al., 2011) and Org 214007-0 (van Lierop et al., 2012) and many others (Carson et al., 2014; Edman et al., 2014; Razavi et al., 2014) have all proven to transrepress the expression of inflammatory genes (Supplementary Table 1). The effect of these compounds on the transcriptional inhibition via nGREs and composite GREs is unresolved. For competitive GREs some initial work has been done on the osteocalcin gene expression regulation (see Section 2.4), but research with a wider view on this matter is still warranted. Interestingly, although Mapracorat inhibits the expression of certain pro-inflammatory genes via inhibition of the classical NF-κB pathway, it also upregulates certain anti-inflammatory genes (such as RelB) via the alternative NF-κB pathway (Spinelli et al., 2014).

While CpdA can initiate the tethering GR transrepression mechanism, the compound, however, works transcription factor-specifically in certain cell types. CpdA-bound GR, namely, represses NF-κB regulated gene transcription, yet sustains AP-1 regulated gene transcription, in contrast to GC-bound GR, which represses both (De Bosscher et al., 2014). Also AL-438 demonstrated a similar transcription factor specificity in which NF-κB-driven reporter genes were found to be more efficiently repressed than AP-1-driven reporter genes (Ronacher et al., 2009).

Although the SEGRM CpdA suppresses inflammation, *in vivo* studies indicate that stability issues, an alkylating potential, and hence a narrow therapeutic range (causing high doses to be toxic) has as result that for the same dose its anti-inflammatory effect is less efficient compared to the classic GC dexamethasone, and that therefore its clinical potential is severely limited (Rauner et al., 2013). Not all SEGRMs are unstable, however, a study where Mapracorat was topically administered to guinea pigs with induced allergic conjunctivitis, showed that Mapracorat caused more eosinophil apoptosis than dexamethasone. This would indicate that not all non-steroidal ligands suffer from reduced activity, as this SEGRM has an even larger therapeutic effect than the classical GCs (Baiula et al., 2014). Mapracorat has also been the study product in a dose finding phase II clinical trial as an ointment for atopic dermatitis (USNIH, 2014d,g,j). Also phase I, II and III clinical trials have been performed, investigating the topical use of Mapracorat in an ophthalmic suspension for the treatment of allergic conjunctivitis and for the treatment of inflammation and pain following cataract surgery (USNIH, 2014b,h,i,o,p,q). Moreover, a first clinical trial phase II for systemic use of the SEGRM Fosdagrocorat (also known as PF-04171327) in rheumatoid arthritis, has just been completed (USNIH, 2014l). At the moment, no study results of these trials are available.

2.2.3. GRdim action mechanism

Over the past decade, the view that side effects are merely resulting from transactivation and desired-GC effects can solely be attributed to transrepression has turned out to be oversimplified. Furthermore, recent studies even challenge the initial work from which the general transrepression/transactivation hypothesis arose. This GR mechanism hypothesis stems mostly from research performed with a mutant GR, called 'GR*dim*', harbouring the GR A458T mutation in the DBD (Heck et al., 1994; Reichardt et al., 1998). However, it was later shown that the GR dimerizes not only via this DBD, but also via an LBD interface (Bledsoe et al., 2002). Initial *in vitro* tests with this GR*dim* variant indicated that this mutant receptor was incapable of forming dimers and that it was impaired in its GR transactivation mechanism, by using GRE model systems such as MMTV, yet still exhibited a clear transrepression capability (Heck et al., 1994; Reichardt et al., 1998). Subsequently, the hypothesis was formed that a dimerized GR can only translate to the transactivation mechanism and that monomer GR is restricted to the transrepression mechanism. Recent research argues that there is no watertight relation between the monomer or dimer state of the GR in solution and its capability to transrepress or transactivate specific gene promoters, but that GR's ability to induce gene expression is co-dictated by the GR-binding sequence and its context and cofactors (Meijsing et al., 2009; Jewell et al., 2012; Watson et al., 2013; Presman et al., 2014). As expected, severely impaired GR transactivation mechanisms were observed in GR*dim* cells, compared to wild type GR cells. However, using immunoprecipitation or the "Number and Brightness" technology, these GR*dim* variants were recently shown to still support GR dimer formation in solution, albeit slightly - yet significantly - less pronounced than wild type GR (Jewell et al., 2012; Presman et al., 2014). Nevertheless, the GR*dim* mutant does show a sequence-specific decreased GRE binding (Presman et al., 2014), which corresponds with a gene expression profiling study comparing livers of prednisolone-treated wild type versus GR*dim* mice, in which the level of prednisolone-induced gene expression was significantly reduced for GR*dim*, as compared to wild type (Frijters et al., 2010). The observation of a small amount of residual gene induction by prednisolone in GR*dim* mice tempts speculations on the existence of alternate GR dimers involving NTD-LBD contacts, conventional yet more unstable GR dimers, GR:MR (mineralocorticoid receptor) heterodimers, GR multimeric complexes or combinations hereof (Nixon et al., 2013). Interestingly, an attenuating effect of the GR*dim* mutation was also retrieved for some genes that were downregulated by classic GCs (Surjit et al., 2011). This observation feeds the hypothesis that genes, failing to be repressed by GCs in GR*dim* mice, may either be regulated through GR binding to negative GRE elements (nGREs) or may be subject to indirect regulation via other GR target genes. However, further research into these suggested mechanisms is still required. Taken together, the propensity of GRs to dimerize appears to be of significance, but not sufficient for GR transactivation to take place. Nevertheless, the earlier finding that the SEGRM CpdA actively supports GR monomer formation (Dewint et al., 2008; Robertson et al., 2010, 2013b), still stands, also in an analysis with the more recent "Number and Brightness" technology (Presman et al., 2014).

Combined with the knowledge that GR-mediated promoter activation extends further than the classic GRE-mediated transactivation, these findings indicate that dimerization is not the sole key player in the activation mechanism. GR is more likely to be regulated in a more complex manner involving co-factors and the cellular environment (Presman et al., 2014). These findings also indicate that more research is needed to conclude whether or not the GR-dimerization-inducing capability of SEGRMs can be extrapolated to their transactivation capability. Importantly, it is now clear that results generated with GR*dim* should be interpreted with caution and thus, one cannot extrapolate GR*dim* experimental interpretations to mechanistic conclusions on wild type GR. SEGRM-based research and their exerted effects on wild type GR could help to pick apart the action mechanisms of wild type GR. In that perspective, the work with the current transrepressing-favoring selective GR modulators should ideally be complemented with research into their counterparts, i.e. transactivation-favoring selective GR modulators or agonists.

2.2.4. Non-genomic pathway

Besides above described GR-mediated genomic mechanisms, several rapid non-genomic pathways have also been reported. These pathways can result in the induction of downstream signaling cascades, changes in cytoplasmic calcium, sodium or potassium concentrations, an increase in mitochondrial production of reactive oxygen species, ceramide and hydrogen peroxide, and the lysosomal release of cathepsin B (Smith & Cidlowski, 2010). For this non-genomic pathway, several mechanisms

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have been postulated: (1) membrane-associated GRs, (2) direct membrane effects of GCs, (3) classic GRs that target signaling proteins associated or not with the plasma membrane and (4) classic GRs that translocate into the mitochondria (Norman et al., 2004; Rhen & Cidlowski, 2005; Stahn & Buttgereit, 2008; Kino et al., 2011).

The influence of most SEGRMs on this non-genomic pathway has not yet been investigated. For the SEGRM CpdA it has been reported that CpdA blocks all mitogen-activated protein kinases (MAPKs) in human rheumatoid arthritis fibroblast-like synoviocytes, albeit in a GR-independent manner (Gossye et al., 2009). Furthermore, CpdA enhances the phosphorylation of c-Jun N-terminal kinase JNK and thus its kinase activity in L929sA cells, also in a GR-independent manner. This enhanced phosphorylation occurs in absence of a CpdA-mediated upregulation of the expression of the MAPK phosphatase DUSP1, which is in contrast to a classical GC. Yet, the enhancement of AP-1-driven gene expression by CpdA is GR-dependent, and tied to a lack of GR recruitment to these promoters. Finally, in the same cells, CpdA blocks the phosphorylation of extracellular regulated signaling kinase ERK but not of p38 MAPK, illustrating not only a remarkably cell-type dependency concerning MAPK regulation (De Bosscher et al., 2014), but also that it is wise not to regard non-genomic and genomic pathways as separate entities.

2.3. Post-translational modifications of GR

The GR protein is modified by various processes. Several mechanisms have been elucidated, such as phosphorylation, acetylation, nitrosylation, sumoylation, and ubiquitination (Fig. 4B) (Gronemeyer et al., 2004; Lu & Cidlowski, 2004; Beck et al., 2009; Vandevyver et al., 2014).

The GR is subject to intense phosphoregulation, which affects GR ligand- and DNA-binding affinity, subcellular localization, GR interactions and half-life, culminating in altered transactivation and transrepression capabilities of GR. Its basal phosphorylation is low, but upon addition of an agonist, GR becomes hyperphosphorylated. It is proposed that phosphorylation of the S211 residue is a hallmark in the transactivation potential of GR (Blind & Garabedian, 2008; Chen et al., 2008). However, also other phosphorylation sites have a profound impact on GR function (Galliher-Beckley et al., 2011). It was observed that the phosphorylation of residues S211 and S226 was ligand-specific (Avenant et al., 2010). In contrast to classic GCs, CpdA does not cause an increase in S211 and S226 phosphorylation (De Bosscher et al., 2005; Avenant et al., 2010). This could correspond to a different allosteric conformation upon binding of CpdA to GR (De Bosscher et al., 2005). AL-438 led to less phosphorylation of S211 and S226, compared to classical GCs. In general, a correlation between the efficacy and potency of transactivation and ligand-induced phosphorylation status of S211 and S226 was observed. This correlation showed, on the one hand, that phosphorylation of S226 inhibited maximal efficacy for transactivation and, on the other hand, that phosphorylation of S211 is required for maximal efficacy for transactivation. Furthermore, also a correlation was seen with respect to the transrepressive capacity, since phosphorylation of residues S211 and S226 slightly inhibited the maximal efficacy for GC-activated GR-dependent transrepression on an AP-1 and NF-κB promoter (Avenant et al., 2010). The ligand-specific phosphorylation profiles could therefore play a role in determining the transrepression *vs*. transactivation potential of GR (De Bosscher et al., 2005; Avenant et al., 2010).

Also acetylation has an effect on the activity of GR. It has been suggested that acetylation limits the capability of GR to inhibit the transcription factor NF-κB. Furthermore, the acetylation of GR by the transcription factor Clock is shown to limit both the transactivation and transrepression capabilities of GR, causing GC-insensitivity in certain tissues (Ito et al., 2006b; Nader et al., 2009; Charmandari et al., 2011; Oakley & Cidlowski, 2013). How SEGRMs influence the acetylation of GR is currently unknown. Facing the lack of in depth knowledge on SEGRM effects on GR phosphorylation and acetylation, it is no wonder that potential SEGRM effects on other posttranslational modifications of GR, such as SUMOylation, are still completely uncharted.

Potentially linked to differences in GR ubiquitination, SEGRMs appear to differ to classic GCs with regard to their impact on GR protein degradation. Although GCs evoke a clear, but timing-related cell type-dependent, homologous downregulation of the GR via proteosomal degradation subsequent to GR K419 ubiquitination (Deroo et al., 2002), the SEGRM CpdA does not at all induce GR degradation in various cell types (Avenant et al., 2010; Gossye et al., 2010; Drebert et al., 2015). Unfortunately, GR exposure to CpdA is not sufficient to fend off GC-evoked GR downregulation (Drebert et al., 2015). A GR mutant with at least 3 Ser phosphorylation sites mutated to Ala (mGR S212, S220, S234, the murine equivalents for the hGR S203, S211 and S226) is not subject to GC-dependent downregulation (Webster et al., 1997). Hence, ligand-induced hyperphosphorylation appears to be key to the onset of the ubiquitination-mediated proteasomal degradation of GR. This hypothesis further fits with the observation that CpdA does not invoke GR S211 and S226 phosphorylation in different cell types (De Bosscher et al., 2005; Avenant et al., 2010) and is also supported by the observed inverse correlation between ligand-dependent GR S211 phosphorylation and the GR half-life (Avenant et al., 2010). However, further research is necessary to consolidate the mechanistic basis for this compound-specific presence or absence of GR degradation.

2.4. Side effects

Major issues with the therapeutic use of GCs are the side effects associated with long-term and/or high dose usage and the occurrence of GC resistance (Beck et al., 2009; Van Bogaert et al., 2010; Dejager et al., 2014). GC therapy has been associated with various side effects, including skin and muscle atrophy, disturbed wound healing, growth inhibition in children, osteoporosis, cataract, glaucoma, disturbances of affect and behavior, hyperglycemia leading to diabetes mellitus, adrenal insufficiency, peptic ulcers and gastrointestinal bleeding, hypertension, and increased risk of infections (Ito et al., 2006a; McDonough et al., 2008; Reichardt et al., 2014). The incidence and severity of these side effects depends on the time, amount, dosing regimen, the specific GC that is used and its mode of application (Ito et al., 2006a; McDonough et al., 2008). Overall, prolonged use is a high-risk factor (Schacke et al., 2002). It should be noted that these GC-induced "side effects" are actually on-target GR-mediated effects and that therefore the term 'side-effect' is misleading (Beck et al., 2009; Clark & Belvisi, 2012). Namely, many or all unwanted effects of synthetic GCs can be seen as versions of normal physiological effects of the endogenous cortisol, inappropriately intensified, prolonged or at the wrong point in time of the circadian cycle initiated and continued (Clark & Belvisi, 2012).

In general, these side effects were believed to result from mainly GR transactivation. Yet again, note that this is a simplified version of reality. In fact, the onset and maintenance of side effects is a more complex matter. Whereas a lot of side effects result predominantly from GR transactivation (e.g. glaucoma, hypertension, diabetes …), there are also side effects resulting from GR transrepression (e.g. repression of the hypothalamic-pituitary-adrenocortical axis, susceptibility to infections). Moreover, the mechanisms of some side effects have been attributed to both transactivation and transrepression (e.g. osteoporosis) or else have not been fully elucidated (Beck et al., 2009; Baschant et al., 2012).

Studies on the SEGRMs CpdA (De Bosscher et al., 2005), Mapracorat (Schacke et al., 2009), PF-802 (Hu et al., 2011), AL-438 (Coghlan et al., 2003), LGD-5552 (Miner et al., 2007), ZK 216348 (Schacke et al., 2004) and ORG 214007-0 (van Lierop et al., 2012) all reported on *in vivo* anti-inflammatory activities with an improved therapeutic index, meaning more anti-inflammatory effects and less pronounced side effects, which will be discussed in further detail below.

The molecular mechanisms mediating the GC-induced side effects are well-known for osteoporosis, hyperglycemia and diabetes, hypertension, and skin and muscle atrophy (Schacke et al., 2002).

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Osteoporosis results from a culminated decrease in osteoblast proliferation and activity, and an increase in osteoclast activity. Osteoclast activity is indirectly increased due to the GC-induced decrease in gastrointestinal Ca^{2+} absorption and the increase in urinary Ca^{2+} excretion. The drop in serum Ca^{2+} level is counteracted by an increase in parathyroid hormone levels, resulting in increased osteoclastic bone resorption. GCs can also stimulate osteoclast activity via transactivation of osteoprotegerin-ligand (OPG-L, also known as receptor activator of NF-κB ligand, RANKL), which enhances osteoclast differentiation and activity, and inhibits osteoclast apoptosis. Besides this, GCs also transrepress osteoprotegerin (OPG), which binds to OPG-L and prevents its activities. As such, this enhancement in the OPG-L/OPG ratio favors bone resorption. A decrease in osteoblast activity, and as such a decrease in bone formation, can result from different mechanisms, including a decrease in adrenal steroidal hormones caused by a GC-induced suppression of the adrenals, GC-induced osteoblast and osteocyte apoptosis, and GC-mediated suppression of growth hormone, insulin-like growth factor-1 and transforming growth factor-β, which are bone homeostasis mediators. Next to the increase in osteoclast activity and the decrease in osteoblast activity, reduced synthesis of bone-forming extracellular matrix proteins (e.g. osteocalcin and collagen type I) also contributes to osteoporosis upon long-term GC-therapy (Schacke et al., 2002).

Studies on mice models with arthritis comparing CpdA to prednisolone or dexamethasone showed that CpdA, on the one hand, was less potent in suppressing inflammation compared to the GCs, yet, on the other hand, was able to maintain bone mineral density (Thiele et al., 2012; Rauner et al., 2013) and did not inhibit osteoblast differentiation (Rauch et al., 2011), in contrast to prednisolone. These findings indicate that CpdA has a bone sparing effect compared to classical GCs (Rauch et al., 2011; Thiele et al., 2012; Rauner et al., 2013). Of special interest, the ability of CpdA to preserve osteoblast differentiation (Rauch et al., 2011) is most probably linked to CpdA's maintenance of AP-1 activity (De Bosscher et al., 2014), a crucial regulatory factor for IL-11, in turn indispensable for proper bone metabolism (Rauch et al., 2010).

Also the SEGRM AL-438 has shown promising *in vivo* results in rat models with arthritis, namely no inhibition of osteoblast activity in cancellous bone and less inhibition of bone formation in cortical bone (Coghlan et al., 2003). Furthermore, AL-438 did not cause a reduction in cell proliferation or proteoglycane synthesis in chondrocytes, in contrast to dexamethasone and prednisolone, indicating that it has a reduced side effect profile on chondrocytes compared to GCs (Owen et al., 2007).

Treatment with LGD-5552 resulted in a smaller decrease in bone formation compared to prednisolone in mice models (Miner et al., 2007). As for ZK 216348, *in vitro* results indicate an inhibition of OPG, albeit less pronounced than for dexamethasone or prednisolone (Humphrey et al., 2006). No *in vivo* experiments have been reported regarding the effect of ZK 216348 on bone metabolism.

In vitro effects of PF-802 (the pro-drug of Fosdagrocorat) on osteocalcin, a component of the bone matrix typically inhibited by GCs, indicated that this SEGRM did not suppress osteocalcin expression to the same extent as prednisolone, concomitant with unaffected levels of secreted osteocalcin protein (Hu et al., 2011). These results are in line with the findings that also CpdA and AL-438 did not inhibit osteocalcin production (Coghlan et al., 2003; Rauch et al., 2011). This can be explained by the fact that classic GCs suppress the expression of osteocalcin via GR binding to a GRE which overlaps with a TATA-box, thus forming a competitive GRE, and not through direct interaction with a transcription factor (Meyer et al., 1997). The absence of SEGRM-mediated regulation of the competitive GRE of osteocalcin suggests that SEGRM-bound GR is not able to inhibit binding of the key fueling transcription factor in this case. Additional research into other competitive GRE-regulated genes and the SEGRM-bound GR-mediated mechanisms in this constellation are still required.

Furthermore, a clinical trial investigating Fosdagrocorat in healthy human subjects, also showed that the compound had less impact on osteocalcin (Stock et al., 2009). Research concerning the potential effects of Org 214007-0 and Mapracorat on bone metabolism is yet to be performed.

Furthermore, it has been shown that leptin plays a detrimental role in the pathogenesis of *osteoarthritis*. And, although it is unclear what the effect of classic GCs is on the progression of osteoarthritis, it has been proven that they cause an increase in leptin and its receptor (Ob-R) (Relic et al., 2009). Recent research, however, demonstrated that CpdA, in contrast to glucocorticoids, does not cause an increase in the expression of leptin or its receptor, thereby potentially indicating an improved risk:benefit ratio (Malaise et al., 2014).

Long-term GC treatment can also cause muscle *atrophy*, via the stimulation of protein degradation and inhibition of protein synthesis. Stimulation of protein degradation can result from GR-mediated transactivation of genes encoding components of the ubiquitin-proteasome pathway. For example, MuRF1 and atrogin-1 and members of the forkhead box superfamily of transcription factors (e.g. FOXO3) are believed to be important for the catabolic effect of GCs in muscle (Hasselgren et al., 2010). Yet, GCs rather activate FOXO3 indirectly, i.e. via the inhibition of PI3K/AKT signaling (Zheng et al., 2010). Also, the induction of myostatin gene expression could possibly be a mechanism via which GCs are able to evoke muscle atrophy (Ma et al., 2003). Skin atrophy results from a GC-mediated reduction in keratinocyte and dermal fibroblast proliferation and a decreased protein synthesis by dermal fibroblasts. Collagen type I synthesis is decreased by GCs through protein-protein interaction between GR and the transcription factor Smad3, which is required for transcription of the COL1A2 gene, coding for collagen type I. The regulation of other extracellular matrix proteins is also involved in the development of skin atrophy. GCs e.g. also decrease tenascin C gene expression (Schacke et al., 2002).

Mapracorat (Schacke et al., 2009) and ZK 216348 (Schacke et al., 2004) have shown reduced skin atrophy compared to classical GCs after long-term topical treatment, yet the pathways or exact targets involved were not documented. Related, ZK 216348 and CpdA also do not inhibit intestinal epithelial cell restitution *in vitro* (Reuter et al., 2012b). *In vivo* studies of CpdA on mice indicated that CpdA did not stimulate protein degradation and had an ameliorative effect on intermediate markers of muscle dystrophy, compared to GCs (Huynh et al., 2013). The effect of CpdA was also examined on a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced model of skin inflammation and hyperplasia in which it was shown that CpdA's ability to reverse this induction was less explicit compared to a classical GC. However, an increase in epidermal thickness and keratinocyte proliferation was observed after CpdA-treatment in a dose-dependent manner (Kowalczyk et al., 2013). It still needs to be investigated what the effect is of other SEGRMs, such as AL-438, PF-802, Org-214007-0 and LGD-5552 on muscle and skin metabolism.

Hyperglycemia and the concomitant increased *risk of diabetes* can also be caused by long-term GC treatment. Both insulin resistance and a decrease in β-cell insulin production are an unwanted effect of GC excess. GCs increase glucose synthesis mainly via a GR transactivation-stimulated expression of enzymes involved in the gluconeogenesis pathway. The increased glucose synthesis is followed by increased glycogen storage in the liver due to a GC-mediated activation of glycogen synthase (Schacke et al., 2002).

SEGRMs that cannot induce GRE-regulated gene expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase, such as CpdA, were expected not to induce hyperglycemia or hyperinsulinemia (De Bosscher et al., 2005). This was confirmed by *in vivo* studies in mice and rats where blood glucose levels -which reflect the risk of induction of diabetes- were increased after treatment with GCs, but were not increased after CpdA treatment (Dewint et al., 2008; Zhang et al., 2009b). Neither the collagen-induced arthritis model, nor an experimental autoimmune encephalomyelitis model showed CpdA-evoked hyperinsulinemia (Dewint et al., 2008; van Loo et al., 2010). Moreover, recent studies even indicate that CpdA could

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protect against the development of immune-inflammatory diabetes in mice (Saksida et al., 2014).

Also PF-802 showed a reduced induction of phosphoenolpyruvate carboxykinase and tyrosine aminotransferase gene expression in primary human hepatocytes, compared to prednisolone. The fact that it still induces the expression of these genes, to some extent, indicates that the compound would exhibit a reduced side effect profile concerning the risk of diabetes, yet not a complete lack of effects on glucose metabolism (Hu et al., 2011). Furthermore, Pfizer just completed a phase I clinical trial investigating the effect of the PF-802-related compound Fosdagrocorat, when compared to prednisone on glucose metabolism (USNIH, 2014f). Results of this trial have not yet been posted.

In vivo studies in rats using AL-438, ZK 216348 or MK-5932 and in mice using Org-214007-0 also supported that these compounds cannot cause an increase in plasma glucose, in contrast to prednisolone (Coghlan et al., 2003; Schacke et al., 2004; Bungard et al., 2011; van Lierop et al., 2012; Brandish et al., 2014). Also contrary to prednisolone therapy, Org-214007-0 did not shift the liver glucose/glycogen balance (van Lierop et al., 2012). AL-438 was even able to prevent GC-induced hyperglycemia (Coghlan et al., 2003). Research on the effect of LGD-5552 on glucose housekeeping still needs to be performed (Reeves et al., 2012).

GCs can induce *hypertension* by causing Na^+ retention. Synthetic GCs that bind to the mineralocorticoid receptor can increase the activity of the epithelial Na^+ channels (ENaCs) via this receptor. Besides this, GCs can elevate ENaC gene expression via GR transactivation (Boyd & Naray-Fejes-Toth, 2007) and increase ENaC activity through enhanced transcription of the serum- and GC-regulated kinase SGK, which phosphoregulates ENaC (Schacke et al., 2002). Only the effect of LGD-5552 on blood pressure has been previously investigated. Low doses of LGD-5552 (1-3 mg/kg) did not induce a raise in arterial blood pressure in rats, in contrast to treatment with prednisolone which did induce a raise even at low dosages (López et al., 2008).

Although select researchers have investigated the effects of particular SEGRMs on hypertension (López et al., 2008), and fertility (Louw & Swart, 1999), these particular domains and also research into effects of SEGRMs on depression and memory in the long run, still warrant additional research. Indicative, a recent report on C108297, a partial GR agonist and antagonist, indicates the possibility to selectively target and allow GR signaling in the brain (Zalachoras et al., 2013). Also, two phase II clinical trials investigating the potential suppression of the hypothalamic-pituitary-adrenal axis by a Mapracorat ointment in adults with atopic dermatitis were performed, yet no results have been posted (USNIH, 2014m,n).

Taken together, the currently developed SEGRMs have indeed a better side effect profile *in vitro* and *in vivo*. However, a full side effect profile of one particular SEGRM is lacking, even for the more intensely examined CpdA.

2.5. Glucocorticoid resistance

A second major pitfall in the use of GC-therapy, besides the side effects, is GC resistance. Such GC resistance can be innate, disease-dependent, or can be acquired due to a prolonged GC treatment (Beck et al., 2009; Van Bogaert et al., 2010; Dejager et al., 2014; Vandevyver et al., 2014). Innate GC resistance is predominantly, but not necessarily, caused by a mutation of the GR itself, leading to abnormal GR concentrations, ligand-binding affinity, GR stability, GC-induced nuclear translocation, and/or interactions between GR and its cofactors (van Rossum & Lamberts, 2006; Charmandari et al., 2008; Yang et al., 2012; Hakim et al., 2013; Quax et al., 2013). Also several polymorphisms are known to be associated with a genetic predisposition to GC resistance, such as in the interleukin 4 promoter causing increased gene transcription (Ito et al., 2006a) or in genes of enzymes regulating the bioavailability of GR (Quax et al., 2013).

The mechanisms involved in the acquisition of GC resistance are divergent and cell-type specific. GC resistance has been associated with an altered level of expression of GR isoforms (e.g. increased expression of the 'dominant-negative' GRβ isoform), a lack of GR auto-induction, homologous down-regulation of GR, a decreased GR ligand-binding affinity, impaired GC-induced GR nuclear translocation, reduced ability of GR to bind DNA, altered cofactor activity and expression, alterations in GR phosphorylation, aberrant expression of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein, failure to induce Bim expression and interactions between various kinase pathways and GR signaling (Ito et al., 2006a; Beck et al., 2009; Smith & Cidlowski, 2010; Mercado et al., 2012; Rossios et al., 2012; Hakim et al., 2013; Papi et al., 2013; Quax et al., 2013). Reduction in GC responsiveness is also associated with an increase in the levels of FK506 binding protein 51 (FKBP51), an element of the GR chaperone complex, and of inflammation-associated transcription factors (e.g. AP-1), the latter of which could compete with GR for DNA binding at specific gene promoters. In addition, a multidrug resistance membrane transporter can limit GC activity by extruding GCs out of the cell (Ito et al., 2006a; Beck et al., 2009; Quax et al., 2013). Interestingly, an *in vitro* and *in vivo* reported cholesterol-induced selective GC resistance, in which only GR transactivation and not GR transrepression events are impaired, occurs via a JNK-dependent mechanism (Papi et al., 2013; Yang et al., 2014).

As mentioned above (see 2.3 Post-translational modifications of GR), studies in cell lines and an *in vivo* study in mice showed that CpdA did not cause a homologous down-regulation of the GR-protein in contrast to dexamethasone in a multitude of cells (Avenant et al., 2010; Gossye et al., 2010; Drebert et al., 2015). In line with these observations, CpdA is known to preserve its anti-inflammatory potential even in long-term treatment (Gossye et al., 2010). The molecular basis for this finding has not yet been elucidated, but several mechanisms have been postulated. First of all, it could result from a different CpdA-induced conformational change of GR, causing it not to be marked for ubiquitination. Secondly, the lack of GR phosphorylation CpdA failed to induce, as discussed above, could circumvent homologous down-regulation as phosphorylation is known to mark the GR for degradation. This would mean a regulation of GR on a post-translational level. However, the down-regulation of GR following dexamethasone treatment is already apparent on an mRNA level, implying that post-translational regulation cannot be the only level of regulation (Gossye et al., 2010). A third mechanism by which CpdA could possibly maintain GR protein levels is via its possible inability to form an NCoR1 repression complex. This idea unfolded from the observation that agonist-bound GR binds to an nGRE in exon 6 of the GR gene, followed by the formation of the NCoR1 repression complex at the transcription initiation site of the GR gene, leading to reduced transcription of GR (Ramamoorthy & Cidlowski, 2013). Perhaps CpdA-bound GR cannot bind to this nGRE in exon 6, and thus does not instigate this mechanism. The latter hypothesis is supported by the observed lack of interaction between CpdA-bound GR and NCoR in a mammalian two-hybrid assay (Ronacher et al., 2009).

In vitro experiments showed that ZK 216348 also did not induce a homologous downregulation of GR (Reuter et al., 2012a). Research on the effect of the other SEGRMs on GR half-life and acquired glucocorticoid resistance still needs to be performed.

2.6. Critical perspectives

Although CpdA and the other SEGRMs, including the newest one in clinical trial, i.e. Fosdagrocorat, provide new insights in the physiology of GR, they can never be the holy grail with regard to all GR-related side effects. As stated before, the situation is not just black and white; the therapeutic effects are not all due to the transrepressive mechanism of action and the side effects are not all due to the transactivating mechanism of action (Beck et al., 2009; De Bosscher, 2010; De Bosscher et al.,

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2010b). For instance, research shows that an important part of the anti-inflammatory effect of GR is mediated by an increased expression of dual specificity phosphatase 1 (DUSP) and glucocorticoid induced leucine zipper (GILZ) (Newton & Holden, 2007; Ayroldi & Riccardi, 2009; Beck et al., 2009; Ayroldi et al., 2014; Newton, 2014). For DUSP1, it was recently shown that this phosphatase plays a transient and partial role in the glucocorticoid-regulated transrepression (Shah et al., 2014). Subsequently, an anti-inflammatory effect is induced via inactivation of MAPKs by both proteins (Ayroldi & Riccardi, 2009; Beck et al., 2009; Newton, 2014), suppression of Cox-2 by DUSP1 (Joanny et al., 2012), inhibition of NF-κB by GILZ and a negative effect on the function of macrophages, T-cells and dendritic cells by GILZ (Ayroldi & Riccardi, 2009; Beck et al., 2009; Newton, 2014).

Of certain SEGRMs it is known how they affect the expression of these genes. Despite being defective at classic GRE-dependent transactivation, the SEGRA RU24858 induced the expression of GILZ. This puts forth the possibility of inducing GILZ expression, by another mechanism than the classical GRE or could more likely, be explained by an incomplete dissociating potential, depending on the target gene and tissue (Chivers et al., 2006). Furthermore, also ORG 214007-0 behaves as a partial agonist, by inducing the expression of GILZ and DUSP1 (van Lierop et al., 2012). Additionally, experiments investigating two SEGRMs, the ZK 218348-related compound 1 and the LGD-5552-related compound 2, showed that their anti-inflammatory effect was directly proportional to their capability of inducing DUSP1 (Joanny et al., 2012), indicating again a potentially incomplete dissociation profile. Of note, also the dexamethasone-stimulated GR*dim* mutant is capable of inducing the expression of DUSP1 in COS-7 cells and murine bone marrow macrophages (Abraham et al., 2006; Tchen et al., 2010), albeit not in prednisolone-treated GR*dim* murine livers (Frijters et al., 2010) . In contrast to the aforementioned SEGRMs, CpdA is unable to induce DUSP1 (Reber et al., 2012; De Bosscher et al., 2014) or GILZ (Drebert et al., 2015; Malaise et al., 2015) expression in various cell types. The effect of Mapracorat, PF-802, AL-348, LGD-5552 and ZK 216348 on DUSP and GILZ expression has not yet been investigated. Considering the potential relevance of GILZ and DUSP1 steady state levels, no knockdown or knockout experiments have been performed to analyze the importance of pre-existing GILZ or DUSP1 to the anti-inflammatory mechanism of completely dissociated SEGRM-bound GRs.

These findings all together indicate that the simple, convenient idea that transactivation is solely mediated by the classical GRE-based mechanism is outdated. Rather, a more complex and diverse system of GR-mediated gene expression is probably the reality. However, this line of experiments also brought to light that, like for the GR*dim* mutant, many of the SEGRMs display only a decrease in GR transactivation events and so far only CpdA (De Bosscher et al., 2005) and PF-802 (Hu et al., 2011) can still uphold the most strongly dissociated profile.

As a result of the increasing evidence for a role for GILZ and DUSP1, the benefit of therapeutic strategies relying on the transrepression hypothesis has recently come under pressure (Clark & Belvisi, 2012). Another reason for this is that certain inflammatory mouse models were found to depend on GR's full activity, including transactivation, to resolve the inflammation, e.g. contact allergy (Tuckermann et al., 2007) and systemic TNF-induced lethal shock (Vandevyver et al., 2012b). Moreover, and as mentioned earlier, the GR*dim* mouse model in which the GR should be hampered in its dimerization and subsequent DNA binding ability, appears still able to support some dimerization and DNA binding (Jewell et al., 2012; Presman et al., 2014). Although this observation casts a shadow over predictions and interpretations of the past, the use and validity of findings with true dissociating ligands and their impressive effects in inflammatory models are nice examples that the baby needs not to be thrown out with the bathwater. Indeed, based on the transrepression hypothesis, the pharmaceutical company Pfizer performed a phase I clinical trial evaluating the safety of systemic use of the dissociated GR modulator Fosdagrocorat, a phosphate ester pro-drug of PF-802, which concluded that it had less impact on plasma osteocalcin, a biomarker of adverse effects on bone, and similar effect on biomarkers of GC activity (Stock et al., 2009). Moreover, Pfizer just completed further phase I (USNIH, 2014a,e,f,k) clinical trials and phase II (USNIH, 2014c,l) clinical trials with the same compound. These phase II clinical trials investigated Fosdagrocorat on a methotrexate background, destined for usage in rheumatoid arthritis. The results of all these trials have not yet been posted.

Even though the to date observed side effects using SEGRMs are fewer and/or less pronounced than those observed using GCs, problems that may arise because of systemic administration are currently not being tackled in the SEGRM research field. A number of research lines have focused on topical skin and eye preparations (Schacke et al., 2009; Zhang et al., 2009; Kowalczyk et al., 2013; Baiula et al., 2014; Spinelli et al., 2014; USNIH, 2014d,g,h,j,o,p). Nevertheless, additional research into tissue-specific delivery systems for these pharmacological compounds would aid in reducing the remaining side effects even more.

Although the anti-inflammatory potential of certain SEGRMs seems to turn out less powerful than anticipated, the selective nature of its pharmacological profile does entail a reduced count of side effects. When more is known on how GR exactly works and how ligands influence the effects of GR, perhaps ligands with another kind of selectivity and a more specific target, for example solely causing transactivation of DUSP, can be developed for research purposes and/or to eventually compile a select compendium of beneficial GR-mediated events.

3. Conclusions

The activated glucocorticoid receptor displays an intricately layered mechanism of action, controlled by ligands, cofactors, post-translational modifications and promoter-specific events. Picking these mechanisms apart using selective GR modulators and agonists has yielded new insights in GR biology. However, the flawed assumption that genetic models using GR mutants should lead to similar conclusions as approaches using small molecules targeting GR and the hitherto comparison of completely and incompletely dissociated SEGRAM effector profiles, has created a great deal of controversy over the past years. Furthermore, although the pharmacological endeavors of the past decade have yielded a surge of new and more selective GR modulators, their mechanism at heart still remains largely unresolved and warrants further research.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Role of the funding source

The funding agency had no role in design, analyses, decision to publish, or preparation of the manuscript.

Acknowledgments

IMB is a postdoctoral fellow of the Research Foundation-Flanders (FWO).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pharmthera.2015.05.001.

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Please cite this article as: Sundahl, N., et al., Selective glucocorticoid receptor modulation: New directions with non-steroidal scaffolds, *Pharmacology & Therapeutics* (2015), http://dx.doi.org/10.1016/j.pharmthera.2015.05.001

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Please cite this article as: Sundahl, N., et al., Selective glucocorticoid receptor modulation: New directions with non-steroidal scaffolds, *Pharmacology & Therapeutics* (2015), http://dx.doi.org/10.1016/j.pharmthera.2015.05.001

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Please cite this article as: Sundahl, N., et al., Selective glucocorticoid receptor modulation: New directions with non-steroidal scaffolds, *Pharmacology & Therapeutics* (2015), http://dx.doi.org/10.1016/j.pharmthera.2015.05.001