THE ROLE OF DUAL SPECIFICITY PHOSPHATASE 1 IN THE ANTI-INFLAMMATORY EFFECTS OF THE GLUCOCORTICOID RECEPTOR

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"A fact in itself is nothing. It is valuable only for the idea attached to it, or for the proof which it furnishes."

Claude Bernard

Abstract

Glucocorticoids (GCs) have been used for decades in the treatment of chronic inflammatory and autoimmune diseases, thanks to their powerful anti-inflammatory properties. However, long term treatment can lead to deleterious side effects, and some patients also experience resistance to their therapeutic effects.

GCs act through the glucocorticoid receptor (GR) to regulate transcription both positively and negatively. Negative regulation of transcription involves a process known as transrepression, in which ligand-activated GR impairs transcriptional activation by nuclear factor κB (NF-κB) and other transcription factors. It is widely believed that transrepression accounts for most of the anti-inflammatory effects of GCs, whereas the activation of transcription (transactivation) is responsible for most side effects of GCs. Based on this principle, several pharmaceutical companies are trying to identify selective GR modulators (SGRMs) that preferentially induce transrepression rather than transactivation. Such compounds are predicted to retain the anti-inflammatory properties of classical GCs but cause fewer side effects.

There are several problems with a dogma that equates anti-inflammatory effects of GR with transcriptional repression. One is that GCs have long been known to destabilise many pro-inflammatory mRNAs, and this property is not explained by the transrepression model. Another issue is that GCs induce the expression of many factors with powerful anti-inflammatory effects. One of these is dual specificity phosphatase 1 (DUSP1), an enzyme that dephosphorylates and inactivates mitogen-activated protein kinases. Studies of the *Dusp1*$^{-/-}$ mouse have underlined the importance of the phosphatase in the anti-inflammatory response to GCs.

In this work, I investigated the role of DUSP1 in (1) the post-transcriptional regulation of pro-inflammatory mRNA stability by GCs and (2) the anti-inflammatory actions of SGRMs.

1- The classical dexamethasone (dex) was shown to upregulate DUSP1 in mouse macrophages, and to inhibit the expression of cyclooxygenase 2 (COX-2) in a manner that was partially dependent on DUSP1. Dex destabilised COX-2 and interleukin 1α mRNAs, and this post-transcriptional effect appeared to require DUSP1.
2- Two SGRMs were characterised and shown to preferentially mediate transrepression rather than transactivation. However, they were capable of inducing the expression of DUSP1 in several different cellular systems, and their capacity to inhibit the expression of COX-2 was correlated with DUSP1 induction. Finally, several of the anti-inflammatory effects of the SGRMs were found significantly impaired in mouse macrophages lacking DUSP1.

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This thesis is the result of my own work. Any collaboration or assistance has been appropriately acknowledged in the relevant sections. The work was carried out at the Kennedy Institute of Rheumatology Division, Imperial College London.

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Abbreviations and acronyms

ActD	actinomycin D
Amax	maximal activity
BMDM	bone marrow derived macrophage
CIA	collagen induced arthritis
COX-2	cyclooxygenase-2
Cpd1	compound 1 (RO4553059)
Cpd2	compound 2 (RO4638375)
CpdA	compound A
dex	dexamethasone
DI	dissociation index
DMSO	Dimethyl sulfoxide
DUSP1	dual specificity phosphatase 1
EC50	half maximal effective concentration
ERK	extracellular signal-regulated kinase
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRR	glucocorticoid responsive region
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	Interferon gamma-induced protein 10 (CXCL-10)
JNK	c-jun N-terminal kinase
KC	CXCL-1, Chemokine (C-X-C motif) ligand 1
KO	knock-out
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MKP-1	MAPK phosphatase 1
NF-κB	nuclear factor kappa B
pred	prednisone
RA	rheumatoid arthritis
RU	RU486, mifepristone
SB	SB 202190
SD	standard deviation
SEM	standard error to the mean
SGRM	selective glucocorticoid receptor modulator
TLR4	toll-like receptor 4
TNF	tumor necrosis factor
TTP	tristetraprolin
WT	wild type

Chapter 1

INTRODUCTION

1. INTRODUCTION

1.1. Inflammation

Inflammation is the body's reaction to pathogen invasion or injury. It establishes a physical barrier to limit the spread of infection and promote healing of any damaged tissue. Inflammation is characterised by four cardinal signs, described more than 2000 years ago by Cornelius Celsus: *rubor* (redness), *calor* (heat), *tumor* (swelling) and *dolor* (pain). A consequence of inflammation is a possible dysfunction (*functio laesa*) of the organs or tissues affected. Local inflammation is accompanied by a systemic response -acute phase response- that leads to an increased number of white blood cells and fever.

1.1.1. Overview of the immune response

Innate immunity is the first line of defence of the host from infection. It is immediate, antigen independent, non-specific and does not lead to any long lasting effect or immunological memory. It is triggered by the recognition of invariant micro-organisms markers, such as bacterial lipopolysaccharide (LPS), by the innate immune receptors. Activation leads to the production of inflammatory mediators which promote cellular migration to inflammation site and activation of macrophages and neutrophils. It can also lead to the activation of the adaptive immune response *via* antigen presentation to T cells by phagocytes.

Adaptive immunity demands time between exposure and maximal response, is antigen dependent, specific and results in immunological memory. Antigen receptors are of two types: present on T cells (TCRs) or on B cells (immunoglobulins, Ig). As these receptors are generated randomly, they cannot distinguish self from non-self. Immunological tolerance usually permits to avoid immune response against self but if there is a defect in the tolerance mechanisms, autoimmune inflammatory diseases such as rheumatoid arthritis, asthma or Crohn's disease can occur.

The final function of acute inflammation is to return damaged tissues to a functional state after the clearance of pathogens. Damaged tissue and bacterial product such as LPS trigger release and activation of histamine, prostaglandins, serotonin and leukotrienes. These chemical mediators lead to vascular dilatation, increased vascular permeability and activation of neutrophils. Activation of other chemical mediators, the complement components, leads to opsonisation of bacteria, facilitating their phagocytosis.

When an immune stimulus persists for a longer period of time than it normally takes to eliminate the antigen, chronic inflammation can occur. There are commonly three main situations leading to chronic inflammation: (i) the host immune response failed to eradicate the organism causing the infection; (ii) there is a persistent or frequent exposure to an environmental antigen; (iii) autoimmune diseases.

Macrophages are ubiquitously distributed throughout the body. Their origin is in the bone marrow, derived from pluripotent haematopoietic progenitor cells. Promonocytes undergo divisions and differentiation into monocytes which leave the bone marrow to circulate in the blood stream. Once settled in tissues, monocytes mature into resting macrophages. This differentiation can occur under the influence of granulocyte/macrophage colony stimulating factor (GM-CSF) or macrophage colony stimulating factor (M-CSF)(Naito 2008). Macrophages are the main actor of the innate immune response. They are able to recognise infectious agents thanks to their surface receptors (including Toll-like receptors, TLRs), phagocytose them and express cytotoxicity mediators, chemokines and cytokines (such as IL-1, IL-6 or tumor necrosis factor alpha, TNFα). For example, LPS promotes, *via* the TLR4/CD14 complex, intracellular signalling cascades involving kinases and transcription factors, leading to increased expression of inflammatory cytokines (Naito 2008). Macrophages also play a role in the adaptive immune response: they are part of the antigen-presenting cells (APCs) family which display a class II MHC/antigen complex to T cells.

1.1.2. Mediators of inflammation

Macrophages, and other cells involved in the inflammatory response, produce pro- (IL-1, IL-6, TNFα, COX-2) and anti-inflammatory (IL-10) mediators. Among these are pro-inflammatory enzymes, chemokines and cytokines. Cytokines are low-molecular-weight proteins produced and secreted by leukocytes. They play an important role in the development and regulation of the inflammatory response, by facilitating cell communication. Cytokines bind to specific receptors on the surface of target cells, triggering signal transduction pathways that ultimately lead to gene expression regulation. Among the many mediators involved in the inflammatory response, brief details on inflammatory mediators focused on throughout this project are given hereafter.

Interleukin-1 is a pro-inflammatory cytokine mainly produced by macrophages, monocytes and dendritic cells (Weber, Wasiliew et al. 2010). It consists of two distinct proteins, IL-1α and IL-1β, which bind to the same receptor. They are both synthesised as precursor molecules and IL-1β needs to be cleaved to be activated (Dinarello 1997). IL-1 increases the expression of adhesion molecules (cell migration to the inflammation site) and re-sets the hypothalamic thermoregulatory centre (fever) (Dinarello 1997). Inducers include LPS, TNFα, and IL-1 itself. Glucocorticoids can inhibit IL-1 synthesis at both the transcriptional and post-transcriptional level (Stellato 2004; Abraham, Lawrence et al. 2006).

Cyclooxygenase 2 (72kDa) is a pro-inflammatory enzyme which converts arachidonic acid into prostaglandins, prostacyclin and thromboxane. Its pharmacological inhibition is the method of action of non-steroidal anti-inflammatory drugs, including the famous aspirin. COX-2 is an immediate early gene, its promoter containing binding sites for NF-κB and c/EBP transcription factors. Inducers of COX-2 include LPS, IL-1 and TNFα. Its expression is regulated *via* the MAPK and NF-κB signalling pathways (Tsatsanis, Androulidaki et al. 2006). COX-2 is inhibited by glucocorticoids and this inhibition has been shown to be partly dependent on the expression of the dual-specificity phosphatase DUSP1 (Abraham, Lawrence et al. 2006). It has also been shown that COX-2 regulation by glucocorticoids was acting at the post-transcriptional level by destabilisation of its mRNA (Ristimaki, Narko et al. 1996; Newton, Seybold et al. 1998; Lasa, Brook et al. 2001).

Inducible nitric oxide synthase or iNos, is a pro-inflammatory enzyme which generates the cytotoxic nitric oxide (NO). iNos is produced by many cell types including macrophages. Its inducers include LPS and a combination of IL-1, TNFα and INFγ (Kleinert, Euchenhofer et al. 1996). iNos has been shown to be inhibited by glucocorticoids at both the transcriptional and post-transcriptional level (Kleinert, Euchenhofer et al. 1996; Korhonen, Lahti et al. 2002) TNFα was originally identified as an LPS-induced factor causing necrosis of tumours in mice (Carswell, Old et al. 1975). TNFα is a pleiotropic pro-inflammatory cytokine whose over-expression has been implicated in autoimmune diseases such as rheumatoid arthritis and Crohn's disease (Brennan and Feldmann 1996). TNFα expression has been shown to be regulated by glucocorticoids at the post-transcriptional level (Clark 2000; Smoak and Cidlowski 2006).

Interleukin 6 (IL-6) is a pro-inflammatory cytokine which, like TNF, is recognised as a major player in the pathogenesis of rheumatoid arthritis and a target of recent anti-rheumatic drugs (Buch and Emery 2011). IL-6 is induced by pro-inflammatory stimuli such as IL-1 and is a product of the p38 MAPK pathway and is activated by NF-κB (Smale 2010; Weber, Wasiliew et al. 2010). IL-6 expression has been shown to be downregulated by GCs and this is believed to be key in the therapeutic effects of corticosteroids (Tobler, Meier et al. 1992; Quante, Ng et al. 2008; Beck, Vanden Berghe et al. 2009).

Pro-inflammatory cytokine interleukin 12 (IL-12) is composed of two subunits, p35 and p40, encoded by two different genes: IL-12p35 and IL-12p40. Together, the two proteins form the biologically active dimer p70. IL-12 is secreted by most cell types implicated in inflammatory processes and function as an important transducer of inflammation. It has been shown to be strongly downregulated by GCs as well as by IL-10, as part of endogenous negative feedbacks (Haskó and Szabó 1999).

Interleukin 10 (IL-10) is generally considered as a powerful anti-inflammatory cytokine. It is mainly produced by monocytes and T cells, is tightly regulated and has a central role in inflammation, by limiting responses to pathogens. In macrophages, IL-10 is able to repress expression of TNF, IL-6, IL-8, IL-1 and GM-CSF (Lalani, Bhol et al. 1997; Saraiva and O'Garra

2010). IL-10 has been identified as a target of the p38 MAPK pathway and of the mRNA destabilising protein tristetraproline, or TTP (Stoecklin, Tenenbaum et al. 2008; Tudor, Marchese et al. 2009). IL-10 is able to upregulate DUSP1 on its own or as the result of cooperation with pro-inflammatory stimuli or dex (Hammer, Mages et al. 2005).

1.1.3. Signalling pathways involved in the regulation of inflammatory gene expression

Cell signalling is part of the complex system of communication governing and coordinating cellular activities e.g. proliferation, migration, apoptosis... It allows cells to respond to environmental changes by relaying information from the external medium to the cell in order to maintain homeostasis. Signal transduction processes can be divided into four major stages: (i) interaction of the ligand with its receptor; (ii) intracellular cascade events; (iii) transcriptional and post-transcriptional modulation and (iv) phenotypic response. Many different pathways are involved in cell signalling, all resulting in the alteration of the expression of specific proteins, including those involved in the inflammatory responses (e.g. cytokines). Signalling pathways are tightly regulated and a failure at any stage of the system can lead to pathologies such as cancer, autoimmune diseases or diabetes.

1.1.3.1. TLR4, IL-1R and TNFR signalling

Pro-inflammatory stimuli include oxidative stress, UV light but also TLR agonist such as LPS and pro-inflammatory cytokines such as TNF and IL-1. They elicit strong immune responses, consequences of the signalling events set off by specific interaction with their receptors. LPS is an endotoxin, found on the outer membrane of Gram negative bacteria, acting *via* the TLR4/CD14 receptor complex. IL-1 acts through the interleukin-1 receptor (IL-1R), while TNF regulates cellular events *via* TNF receptors (TNF-R) 1 and 2.

Toll-like receptors (TLRs) and IL-1R are members of the Toll/IL-1 receptor superfamily which proteins are evolutionary conserved and involved in innate immunity and inflammation (Krishnan, Selvarajoo et al. 2007; Verstrepen, Bekaert et al. 2008). They are both transmembrane proteins, have common structural characteristics and their downstream

signalling pathways share similar actors including adaptor proteins, kinases-induced phosphorylation events and transcription factors (Krishnan, Selvarajoo et al. 2007). TLRs are pattern-recognition receptors (PRRs) which recognise unique pathogen-associated molecular patterns (PAMPs) –such as LPS– presented on microbial surface. There are ten members known so far in the TLR family, separated in two groups according to their localisation (plasma or endosome membrane) (Roach, Glusman et al. 2005; Chinenov and Rogatsky 2007). TLR4, localised on the plasma membrane and coupled to CD14, has been identified to be essential for LPS recognition (Hoshino, Takeuchi et al. 1999). It is mostly expressed on cells of the immune system, including macrophages and dendritic cells (Medzhitov 2001; Takeda, Kaisho et al. 2003). Two receptors for IL-1 have been identified, IL-1RI and IL-1RII (Dinarello 1997). Both their extracellular domains have an Ig(immunoglobulin)-like structure that binds to specific ligands including IL-1 and IL-18. IL-1RI is IL-1 preferred receptor and needs to be associated to the IL-1R accessory protein (IL-1RAcP) to form a functional receptor complex (Dinarello 1997; Krishnan, Selvarajoo et al. 2007). As previously mentioned, IL-1R and TLR4 share common signalling mechanisms. The receptor first recruits the adaptor protein MyD88 and then the signal is transduced *via* successive phosphorylation events involving kinases such as IRAK and IKK members as well as TRAF6 protein. The IκB kinase (IKK) phosphorylates the Inhibitor of κB (IκB) which leads to its proteosome-mediated degradation and the subsequent activation of the nuclear factor-κB (NF-κB) transcription factor complex. Signalling through IRAKs and TRAF6 also involve MAPK pathways leading to the activation of activator protein 1 (AP-1) and other transcription factors. All those events *in fine* cause the transcriptional modulation of pro-inflammatory genes expression (Dinarello 1997; Medzhitov 2001; Bhattacharyya, Diane E. Brown et al. 2007; Krishnan, Selvarajoo et al. 2007; Ringwood and Li 2008; Verstrepen, Bekaert et al. 2008).

TNF-R1 and TNF-R2 belong to the TNF receptor super-family. They also are transmembrane proteins,

1.1.3.2. Mitogen Activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases, broadly involved in the control of cellular processes, including the immune and inflammatory responses. Activation of the MAPK pathway can be induced by many stimuli including oxidative stress, UV, TLRs or IL-1R ligands. MAPKs are regulated by the upstream kinases MAPK kinase kinases (MAP3Ks) and MAPK kinases (MAP2Ks). MAP3Ks phosphorylate and activate MAP2Ks, which then phosphorylate and activate MAPKs on threonine and tyrosine residues of their activation domain. Phosphorylation by MAP3K and MAP2K causes conformational changes, leading to the activation of the targeted MAPK (Clark, Dean et al. 2003; Krishnan, Selvarajoo et al. 2007; Pimienta and Pascual 2007; Verstrepen, Bekaert et al. 2008). Activation during MAPK signalling is transient and termination is achieved by dephosphorylation of the MAPK by MAPK phosphatases (MKPs). MAPKs family include ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 MAP kinases. JNK and p38 MAPKs are activated by pro-inflammatory stimuli, ERK as well but in a smaller extent. ERK and p38 act on transcription through direct phosphorylation of transcription factors or through activation of the downstream kinase MSK, whereas JNK is responsible for the phosphorylation of c-Jun, a component of the AP-1 transcription factor (Clark, Dean et al. 2003; Clark and Lasa 2003; Wang and Liu 2007). Activated p38 also promotes mRNA stability (post-transcriptional gene regulation mechanism), by phosphorylation and subsequent activation of MAPK-activated protein kinase-2 (MK-2) (Clark, Dean et al. 2003).

1.2. Glucocorticoids

Glucocorticoids (GCs) are defined by the NIH medical dictionary as "any of a group of corticosteroids (...) involved especially in carbohydrate, protein, and fat metabolism, that tend to increase liver glycogen and blood sugar by increasing gluconeogenesis, that are anti-inflammatory and immunosuppressive, and that are used widely in medicine (as in the alleviation of the symptoms of rheumatoid arthritis)". The term glucocorticoid derives from the fusion of the words glucose, cortex and steroid. Indeed, GCs are steroid hormones, involved in the regulation of glucose metabolism and synthesised in the adrenal cortex in response to a neuro-endocrine cascade of signals along the hypothalamus – pituitary – adrenal (HPA) axis. GCs affect a large number of physiological processes and are important metabolic regulators as well as therapeutic agents, described as "miraculous" at the time of their discovery.

In this section I will review the history and physiology of these hormones, as well as the current understanding of their mechanism of action and their use in clinical medicine. Recent progresses in the search for better and safer compounds with the same therapeutic effects as GCs will also be discussed.

1.2.1. History

Cortisone, the endogenous GC, was isolated and its therapeutic potential identified during the first half of the 20th century, simultaneously by Hench, Kendall and Reichstein. This discovery will count as one of the medical milestones of the 20th century, alongside with antibiotics, aspirin and insulin. To tell its importance, some scientists of that time referred to the discovery of cortisone as a turning point in history, as "BC" (before cortisone) and "AC" (after cortisone). Here is a little chronology of the cortisone discovery, or how to be awarded the Nobel Prize of Physiology and Medicine in hardly 20 years (Hench 1950; Kendall 1950; Reichstein 1950).

1.2.1.1. On the road to Nobel Prize

Early 1930s. Extracts from the adrenal cortex are shown to control adrenalectomised animals or patients with Addison's disease for the first time (Hartman 1930; Swingle 1930). These findings, published by two groups in the same volume of the Science journal in july 1930, encouraged scientists around the globe to work with adrenal extracts, hoping to identify the compound(s) responsible for the control of adrenal deficiency. Among those scientists, two chemists: Dr Edward Kendall at the Mayo Clinic in the US and Dr Tadeus Reichstein at the ETH in Zurich, Switzerland. That same year, Dr Philip Hench, the head of the Department of Rheumatic Diseases at the Mayo Clinic in the US, starts to notice that RA patients with jaundice or who are pregnant have an improvement of their rheumatic symptoms. He suspects a common substance X to be responsible for the effects.

Mid 1930s. While Hench is still trying to understand what is happening in his pregnant and jaundice patients, Reichstein isolates 28 steroid compounds from the adrenal gland and shares his results with Kendall. The latter decides to focus on 5 of those compounds, named A to E and publishes their chemical structures in 1936 (Mason, Myers et al. 1936), simultaneously and independently with Reichstein and Pfiffner (Reichstein 1936; Wintersteiner 1936).

Late 1930s. Matching his own observations with studies from others, Hench begins to suspect substance X to be a bisexual hormone. At the same time he starts to collaborate with Kendall, his colleague at the Mayo Clinic, to determine the chemical nature of substance X. During this period, although the compounds isolated from the adrenal cortex received quite a lot of attention and were shown to influence the metabolism of carbohydrates and proteins (Kendall 1950), they were thought to be useful only for Addison's disease patients and therefore did not get much attention from pharmaceutical companies.

Early 1940s. Based on some 1920s observations that adrenal glands might somehow be involved in RA and on Kendall's work suggesting that compound E (17-hydroxy-11-dehydrocorticosterone) would increase the resistance to typhoid vaccine (hence have some

role in the immune system), Kendall and Hench decide in 1941 to administrate compound E to RA patients. But before they can actually do so a problem remains: they need bigger quantities of Kendall's compound E. At that time, compound E was indeed isolated from cattle adrenal glands and 1000 kg were necessary to obtain 8 g of dry extract from which to isolate the different steroids and they will therefore have to wait until synthesis of compound E is successful.

Mid 1940s. Kendall manages to synthesise compound A in 1944. A year later Merck & Co achieves the same on a large scale, with a synthetic compound A that has the same activity as the one extracted from beef adrenal gland. However, the excitement went down when no effect was shown on Addison's disease (Sprague 1948).

Late 1940s. It is finally in 1948 that Merck & Co, collaborating with Kendall, successfully synthesises a small amount of compound E, from desoxycholic acid found in cattle bile (Sarett 1948). In the summer of the same year, compound E is injected to an Addison's disease patient of the Mayo Clinic, with marked improvements in the condition (Hench 1950; Kendall 1950). Few weeks later, in September of that same year, Hench and Kendall received a small supply of compound E from Merck & Co. Seven years after their joint decision, they will finally be able to administrate some compound E to RA patients. The first patient, Mrs Gardner, a 29 year old woman with severe RA, is injected with 50 mg of compound E on 21st of September, 1948. Within few days of 100 mg/day, the results were astonishing, with marked improvements over the following months. After years in bed, she was finally able to walk, raise her arms and had much less stiffness and soreness. Between September 1948 and January 1949, due to limited supply, only four more patients were given compound E, with similar drastic ameliorations (Hench 1950; Kendall 1950; Lloyd 2002).

1949-1950. At the same time, the adrenocorticotropic hormone (ACTH, stimulates the production of cortisone) was synthesised by Armour & Co and administered in February 1949 to RA patients, with compound E-like effects. This was the beginning of the first "real" clinical study using these molecules, on RA patients. It consisted in administrating either cortisone, ACTH or a mix of both. Results were presented in front of a really enthusiastic

crowd, by Hench, in the following April, at the staff meeting of the Mayo Clinic. In this report, all 16 patients treated were reported to have a “marked” to “very marked” clinical improvement, as defined by at least 70% relief (Hench 1949; Hench 1950; Kendall 1950). Within days the news was everywhere, with the New York Times writing about the “Aid in rheumatoid arthritis is promised by new hormone” (Laurences 1949) and the magazine Life publishing, in June 1949, a photo-reportage with the headings “Arthritis. Mayo Clinic finds a treatment for man’s most crippling disease” and “Hormone stops disease” (1949). As the word of the discovery was spreading fast, and to avoid confusion with vitamin E, compound E was renamed by Hench and Kendall as cortisone and they concluded that substance X seemed to be the same (Lundberg, Grundtman et al. 2004).

In the following months, it however became evident that cortisone and ACTH did not cure RA but instead suppressed its distressing symptoms. Indeed, the hormones increased the well being of the patients but did not affect the damages caused to cartilage and bones. Moreover, the symptoms were alleviated only for the period during which the drug was given, after which relapses occurred. However, for patients desperately seeking a relief, it appeared as miraculous. During that same period, with the little hindsight they had, doctors started to notice side effects to the use of cortisone, including “reduction in carbohydrate tolerance”, “transient alteration of psyche” and “spontaneous fractures”. In those rare occurrences, it was advised to decrease the dosage or discontinue the treatment (Copeman 1950; Hench 1950). In a review published just one year after the first administration of cortisone, Carlisle, the chief manufacturer at Merck & Co lists all the diseases that are then known to be beneficially affected by the hormone: RA, psoriatic arthritis, lupus, Addison’s disease, asthma or inflammatory eye diseases. Allergies, gout, psoriasis or scleroderma were also listed as potential therapeutic targets. In that same paper he also addresses the effects of cortisone at different physiological levels, including at the immunological and metabolic levels (Carlisle 1950).

Nonetheless, in regard to their therapeutic effects, FDA approval was rapidly granted to cortisone. Simultaneously, progresses were made in the production of cortisone, as illustrated by its sale price falling to $35 per gram in November 1950, from $200 per gram in July 1949 (Kendall 1950), making the hormone available to all physicians in the US.

Barely two years after the original observations as cortisone as a potential therapeutic agent, Hench, Kendall and Reichstein were nominated for the Nobel Prize of Physiology and Medicine and were awarded in Stockholm in December 1950, “for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects" . Interestingly it was still not proven at that time that substance X and compound E were the same molecule...

1.2.1.2. 60 years on

At the dawn of the 1950s, surfing on the cortisone revolutionary wave, researchers and clinicians were however facing new challenges. How to produce more cortisone? What is its full potential, what conditions can be targeted? What are the actual mechanisms of action of cortisone? What are its limitations? The latter two questions are still today, more than 60 years after the Nobel Prize, the ground for many debates that will be detailed later in this chapter.

To deal with the limited supply of raw material available for the synthesis of cortisone (desoxycholic acid from cattle bile), two strategies were implemented, namely the search for another source of starting material and the search for a replacement drug for cortisone. The race to complete the first part of the quest was launched in 1951 when scientists from the Mexican pharmaceutical company Syntex found a cheaper way to synthesise cortisone from diosgenin, a phytosteroid contained in some local yams (Djerassi 1992). However, they were still far from the large scale production that would have satisfied the demand for cortisone. At the same time they were asked to supply a small company, Upjohn, with tons of progesterone, also derived from diosgenin. This company was about to use it as an intermediate for the production of cortisone (Ott 1956), via fermentation of progesterone with the microorganism *Rhizopus nigricans*. The conversion of progesterone to cortisone was now achieved in one single hydroxylation step! With large scale production, the prices of cortisone dropped to as low as $6 per gram in November 1952, just four years after the first use of the hormone. In the decades following, manufacturing techniques improved to continuously provide worldwide physicians and scientists with good value cortisone. The

second challenge of the "cortisone quest" was to produce synthetic cortisone analogues, with similar or even better therapeutic effects. Amongst the many synthesised, are found: hydrocortisone (cortisol) (Murray 1952), prednisone (Oliveto 1959), prednisolone (Nobile 1958), methylprednisolone (Sebek 1959), triamcinolone (Berstein 1957), betamethasone (Taub 1962), dexamethasone (Muller 1961), fluodrocortisone (Spero 1958), fluticasone (Phillips 1982) and budesonide (Brattsand 1975). Those molecules, together with the endogenous cortisone form the glucocorticoids family. They generally are reported to be much more potent than cortisone and cortisol in the treatment of autoimmune and inflammatory diseases (Becker 2001; St Clair 2004; Whitehouse 2011). Over the years glucocorticoids were found to be of use in about 200 conditions (Zetterström 2008). However, all compounds were reported with possible serious side effects.

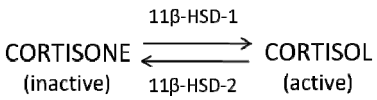
From the early observations made by Hench and his contemporaries to more recent studies, the unwanted effects of the glucocorticoids have been a growing concern amongst the scientific and medical communities. In 1953, Hench was already writing "any truth is everybody's business", referring to the bulky deleterious effects of cortisone (Hench 1953). Recently reviewed conditions targeted by glucocorticoids and unwanted effects are developed further down in this chapter.

What can be said is that, in spite of their side effects, GCs are still one of the most important and most often drugs used in medical practice; the annual worldwide market reached in the early 2000s, a staggering $10 billion and about 10 million new prescriptions are issued in the US alone every year (Schacke, Docke et al. 2002). With such an extensive market, it is easy to understand why the search for better and safer glucocorticoid is the modern version of the race for the cortisone synthesis.

1.2.2. Product of the HPA axis

As previously mentioned, GCs are steroid hormones, end product of the HPA axis in response to many psychogenic, physical and immune challenges. GCs have an important role in glucose metabolism and catabolism of fat and proteins. They also play a pivotal role in the immune system, thanks to their anti-inflammatory ans immunosuppressive actions

(Brook 1998; Becker 2001; Nussey 2001; Chrousos and Kino 2009; Marques, Silverman et al. 2009). To maintain normal homeostasis, the parvocellular neuroendocrine cells (PNCs) in the paraventricular nucleus of the hypothalamus respond to stressful environmental changes by an increased activity and the synthesis of corticotrophin-releasing hormone (CRH). CRH will in turn induce the secretion of the adrenocorticotropic hormone (ACTH) by the anterior lobe of the pituitary into the general circulation, leading to the final activation of glucocorticoids synthesis in the zona fasciculata of the adrenal cortex. Cortisol, the major active glucocorticoid in man (corticosterone in rodents) is synthesised from hydroxylation of cholesterol, as all other steroids. Each step of the reaction is catabolised by different hydroxylases. The final two steps consist in the conversion of 17α-hydroxy-progesterone by CYP-21 into 11-deoxycortisol. 11-deoxycortisol is in turn hydroxylased by CYP-11β1 into the active cortisol. The majority of cortisol being converted into inactive cortisone by the kidney, an active synthesis is required when necessary. Once released in the blood, bioavailability of GCs is therefore tightly regulated, to produce the best possible response. 90% of circulating glucocorticoids is bound to corticosteroid binding globulin (CBG) (Brook 1998). Regulation of CBG concentration and binding ability appears as one of the main regulation of GCs availability. The other main regulatory mechanism involves 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2. These enzymes regulate GCs bioavailability by converting inactive cortisone into the active cortisol and *vice-versa*. Regulation of 11β-HSDs expression is therefore an indirect way to regulate GCs bioavailability.



It is believed that GCs exert their therapeutic and undesired effects mainly *via* the glucocorticoid receptor (GR), a nuclear receptor expressed in most tissues, including in the different components of the HPA axis and immune cells. This ubiquitous expression explains the negative regulatory feedback loop that GCs exert on their own synthesis, to maintain their endogenous levels. Disturbance of this balance is the background for specific pathologies. Cushing syndrome is due to an exaggerated production of cortisol, resulting

from tumors overstimulating the HPA axis and, more commonly, from iatrogenic intake of corticosteroids. Symptoms of GCs hypersensitivity include upper body obesity, hypertension, diabetes mellitus and depression. On the other hand, hypocortisolism (Addison's disease) is characterised by hypotension, hyperpygmentation, fatigue, weight loss, mood changes and autoimmunity/inflammation. In a majority of cases Addison's disease is caused by autoimmune adrenalitis. More rarely it can be the result of impaired steroidogenesis or adrenal dysgenesis, a rare genetic condition involving mutations of the ACTH receptor (Ten, New et al. 2001). Treatment of Addison's disease consists in restoring functionality to the HPA axis by administrating controlled doses of GCs. It is also worth mentioning here the strong relationship that exists between the HPA axis and the immune system. Indeed, the different components of the HPA axis are sensitive to circulating cytokines, present during inflammation and immune diseases. Stimulation of the HPA axis leading to the synthesis of GCs, and GCs having some anti-inflammatory function, this mechanism is regarded as a negative control, once again to maintain endogenous homeostasis, endangered by ongoing inflammation.

1.2.3. Glucocorticoid receptor

The endogenous GC cortisol exerts its effects *via* both the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), having similar affinity for the latter than aldosterone. However, circulating GCs are metabolised into keto-derivatives with low affinity for MR, contributing to aldosterone selectivity. Dex binds to GR with greater affinity than cortisol or aldosterone and displays significant effects *via* MR only at very high doses (Hellal-Levy, Couette et al. 1999). Other synthetic GCs however, have few or very few MR activity (Nussey 2001). For the purpose of this report, I decided to focus on the description of the structure and mechanisms of action of GR only.

1.2.3.1. Structure

GR, a nuclear receptor expressed in most tissues, is encoded by the *NR3C1* gene, located on the chromosome 5q31-q32 (Turner, Alt et al. 2010; Oakley and Cidlowski 2011), was identified in 1968 and cloned for the first time in 1985 (Munck and Brinck-Johnsen 1968;

Hollenberg, Weinberger et al. 1985). Its ubiquitous expression explains the wide spectrum of action of the glucocorticoids, which interact with GR to regulate gene expression. GR is composed of four domains (Nicolaides, Galata et al. 2010). (1) A C-terminal ligand binding domain (LBD) made of eleven α-helices and four β-sheets, forms a hydrophobic pocket for binding of lipophilic GCs (Bledsoe, Montana et al. 2002). (2) A hinge region (HR) that facilitates the flexibility of the protein. (3) A DNA binding domain (DBD) comprising of two zing finger motifs which recognise glucocorticoid-responsive elements (GREs) on targeted DNA. DBD also shares dimerisation and nuclear localisation interfaces with LBD. (4) A N-terminal transactivation domain (NTD), with activation function (AF) 1, serving the recruitment of basal co-regulators and transcriptional machinery. On the other hand AF-2, overlapping LBD, is considered as the element facilitating the recruitment of ligand specific co-regulators (Heitzer, Wolf et al. 2007; Beck, Vanden Berghe et al. 2009; Nicolaides, Galata et al. 2010; Oakley and Cidlowski 2011).

1.2.3.2. Mode of action

When inactive, GR is sequestrated in the cytosolic compartment, as a dynamic multi protein complex. GR was shown to interact with numerous chaperones such as heat shock proteins (HSPs) and immunophilins. HSP40 and HSP70 would be involved in folding of GR when HSP90 would bind the folded protein and help for its activation. Immunophilins, such as FKBP51 and 52, serve ligand binding (Beato, Chalepakis et al. 1989; Heitzer, Wolf et al. 2007). By binding GR, glucocorticoids cause a conformational change, dissociation from the accessory protein complex and subsequent exposure of nuclear localisation signal. GR in turn translocates to the nucleus where it functions as a transcription factor to regulate targeted gene expression (Beck, Vanden Berghe et al. 2009; Nicolaides, Galata et al. 2010; Oakley and Cidlowski 2011). Moreover, it is worth mentioning that ligand-activated GR does not function alone to regulate gene expression; it requires coactivators (e.g. CBP/p300, p160 proteins) and corepressors (e.g. TIF2/GRIP1) to help modify and remodel chromatin structure. These co-regulatorss can have histone acetylase (coactivators of transcription) or deacetylase (corepressors of transcription) activities (Chinenov and Rogatsky 2007; Kassel and Herrlich 2007).

1.2.4. Classical model of regulation of gene expression by glucocorticoids

Glucocorticoids interact with numerous components of signaling pathways to regulate the expression of target genes by increasing or reducing their level of expression. Regulation of pro- and anti-inflammatory mediators expression is thus responsible for the anti-inflammatory properties of glucocorticoids, whereas regulation of metabolic genes would explain their undesired effects. This regulation takes effect at the transcriptional (known for years) as well as at the post-transcriptional (more recently studied) levels (Figure 1.1).

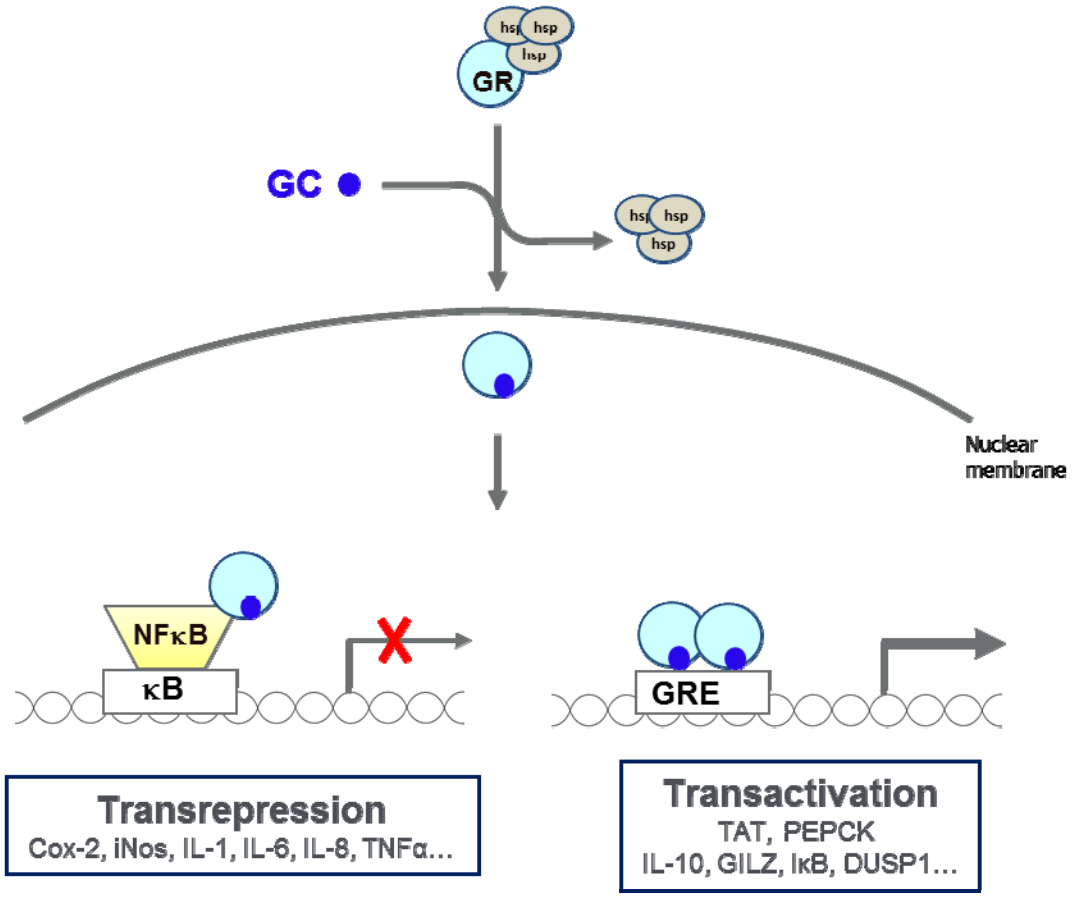


Figure 1.1. Schematic model of classical regulation of gene expression by glucocorticoids.

1.2.4.1. Transactivation

Transactivation (transcriptional activation) of gene expression has been associated for many years with binding of a GR-GR homodimer to a DNA consensus sequence consisting of two inverted and imperfect repeats of the hexanucleotide TGTTCT separated by an element of any 3 nuleotides (5'-GGTACAnnnTGTTCT-3'). This element, located on the promoter of target genes, was defined as glucocorticoid responsive element or GRE (Beato, Chalepakis et al. 1989). Recently, extensive variation of this sequence was reported across different GREs of a given organism, while considerable conservation was observed for an individual GRE, across four mammalian species (So, Chaivorapol et al. 2007). The same group determined the consensus sequence of those GR binding regions (GBRs) as containing only five invariant residues: nGnACAnnnnGnnnn. According to the consensus matrix weight they obtained compiling GBR sequences; one hexanucleotide is more conserved than the other which can influence binding of the GR (So, Chaivorapol et al. 2007). Indeed, GR binds as homodimer to the imperfect palindromic sequence (GRE) and to single hexanucleotides as a monomer (half-GRE) (Segard-Maurel, Rajkowski et al. 1996; Lefstin and Yamamoto 1998). Interaction of the GR with GBRs has been reported as a dynamic model, or "hit and run", in which GR transiently binds to DNA, promote chromatin remodelling (to make it more accessible to other transcription factors and transcriptional machinery) and then is released from the GBR (Nagaich, Rayasam et al. 2004; Stavreva, Muller et al. 2004).

Transactivation is not achieved by GR alone, but by the interaction of GR with other transcription factors and co-regulators. For this reason, GBRs are usually within composite regulatory regions of DNA, designated as GCs responsive regions (GRRs). These regions contain binding sequences for GR and other transcription factors involved in the responses to GCs, such as C/EBP and Octamer-binding transcription factor-1 (Oct-1), activating-protein 1 (AP-1) or STAT5 (Rogatsky and Ivashkiv 2006). This can lead GR to interact directly with transcription factors: formation of heterodimers or even tethering mechanisms at some promoters. Indirect interactions were also reported, with necessity to have concomitant binding of GR and other transcription factors at GRRs (Lefstin and Yamamoto 1998; Newton and Holden 2007; So, Chaivorapol et al. 2007; Beck, Vanden Berghe et al. 2009).

Other components influencing GR function are nuclear co-regulators. Comprising co-activators and co-repressors of GR transcriptional activity, they are cell- and gene- specific and will, *in fine*, influence chromatin remodelling. Chromatin "opening" by co-activators is essential to transcriptional activation and involves ATP-dependent chromatin remodelling complex (e.g. SWI/SNF), histone acetyltransferases (e.g. p300/CBP and p160/CAF), histone methyltransferases (e.g. CARM1) and regulators of the interaction with RNA polymerase II machinery (e.g. components of the Mediator complex) (Perissi and Rosenfeld 2005; Nicolaides, Galata et al. 2010). Once the latter finally steps in, transcription of the target GC-regulated gene is promoted.

1.2.4.2. Transrepression

Transrepression is a mechanism by which GR prevents transcriptional activation. It has been shown to be independent of GR dimerisation and DNA binding (Reichardt, Kaestner et al. 1998). Although some cases of "negative" or "competitive" GREs have been documented (Newton and Holden 2007; Beck, Vanden Berghe et al. 2009), in most cases GR does not bind directly to DNA but instead is recruited to DNA via direct or indirect interactions with transcription factors, notably members of the AP-1 and NF-κB families. Typically GR would "tether" to AP-1 or NF-κB proteins and alter the assembly of the co-activator complex thereby reducing chromatine remodelling, histone acetylation and RNA polymerase II actions. This accounts for blocking initiation of transcription and subsequent gene expression (Kassel and Herrlich 2007; Beck, Vanden Berghe et al. 2009; Glass and Saijo 2010).

Direct interaction between the p65 subunit of the NF-κB complex and GR has been reported as of major importance for the repressive effect of the GCs. A proposed mechanism is that by tethering to p65, GR would prevent the association of the co-activators interferon regulatory factor 3 (IRF3) and positive transcription elongation factor b (PTEFb) which have both been shown to be essential for transcription of NF-κB target genes (De Bosscher, Schmitz et al. 1997; Luecke and Yamamoto 2005; Glass and Saijo 2010). Another factor important for GR repression activity is GR-interaction protein 1 (GRIP1), a member of the

p160 family of nuclear co-activators. Its interaction with GR was originally reported to enhance GC-mediated transrepression of AP-1, but also NF-κB, target genes and therefore act as a co-repressor. Recruitement of GRIP1 by GR, tethered to a transcription factor, would activate GRIP1-specific repression domain and lead to inhibition of transcription initiation (Rogatsky, Luecke et al. 2002). A more recent two-hybrid screen study demonstrated an interaction between GRIP1 and IRF3 in mammalian cells. Moreover, a competition mechanism between GR and IRF3 for GRIP1 was identified. As IRF3 is a downstream actor of TLR3/4 pathways, this interaction GRIP1-IRF3 was suggested as a novel target for GC-mediated immunosuppression (Reily, Pantoja et al. 2006). Again, another element cooperating with GR is the thyroid receptor-interacting protein 6 (Trip6), precisely its nuclear isoform nTrip6 (Kassel, Schneider et al. 2004). This co-activator of AP-1 dependent transcription interacts only with Fos family members of AP-1 and is an essential component of GR-mediated transrepression, as it links GR to c-Fos:c-Jun driven promoters (Diefenbacher, Sekula et al. 2008).

These molecular mechanisms of transrepression therefore appear as diverse as those of transcriptional activation. They are cell type and gene specific and contribute to the fine tuning of gene expression regulation by GCs (Kassel and Herrlich 2007; Beck, Vanden Berghe et al. 2009; Glass and Saijo 2010).

1.2.4.3. Post-transcriptional regulation

Other studies showed that glucocorticoids could also regulate certain gene expression at a post-transcriptional level, modulating the rates of translation or degradation of mRNA (Stellato 2004; Clark 2007; Newton and Holden 2007) (Figure 1.2). This mechanism can not be explained by the transrepression function of GR, but there is no reason why glucocorticoids should not control genes by both transrepression and post-transcriptional mechanisms. However, active transcription of inflammatory genes may occur for a relatively short period, and mechanisms that target transcription can only work during this window. In contrast, a mechanism that influences mRNA stability may be effective for a longer period. For example, a transcriptional inhibitor could block the expression of COX-2 only if it was added very soon after the inducing stimulus. Dex could block COX-2 expression even if it was

added long after the stimulus, so it must act at a post-transcriptional level (Newton, Seybold et al. 1998)

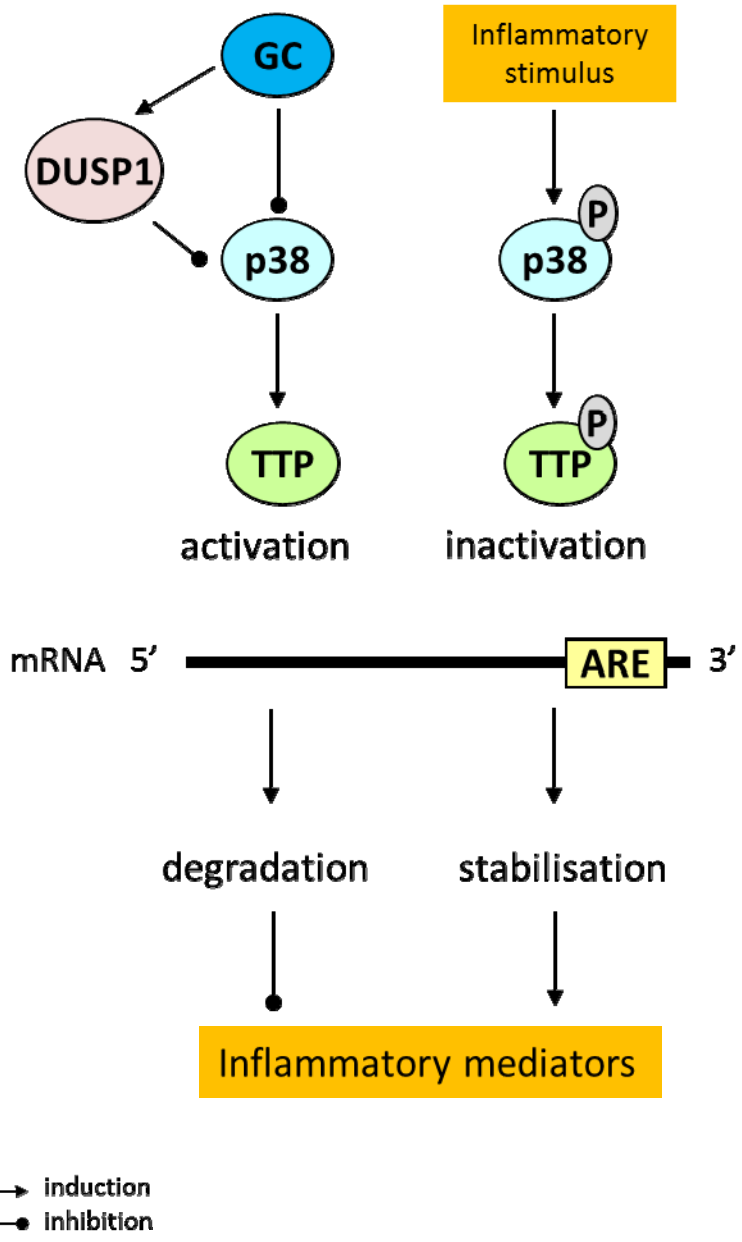


Figure 1.2. Schematic overview of post-transcriptional mechanisms of regulation of gene expression.

1.2.4.3.1. Role of TTP

It has been shown that regulation of mRNA stability occurs mainly through adenosine- and uridine- rich elements (AREs) located in the 3' UTR region of mRNAs (Garneau, Wilusz et al. 2007). AREs are thought to control mRNA stability through interaction with AREBPs (ARE binding proteins). One of these proteins, tristetraprolin (TTP), causes mRNA destabilisation and degradation *via* recruitment of exonucleases (Carrick, Lai et al. 2004). Many of the mRNAs that are destabilised by glucocorticoids contain AREs, and several are known to be targets of TTP. It is possible that glucocorticoids downregulate gene expression by a mechanism that involves TTP. In fact glucococorticoids have been shown to increase the expression of TTP, at the mRNA and protein levels in A549 cells, pulmonary bronchial epithelial cells and MEFs (Smoak and Cidlowski 2006; Clark 2007; Ishmael, Fang et al. 2008). The same groups (Smoak and Cidlowski 2006; Ishmael, Fang et al. 2008) also showed that TTP was a key mediator in the post-transcriptional effects of the glucocorticoids on the TNFα mRNA expression and that the lack of TTP ($TTP^{-/-}$ cells) was affecting a large proportion of glucocorticoid-regulated gene expression, including cytokines and chemokines.

1.2.4.3.2. Role of p38 MAPK

The p38 MAPK pathway plays an important role in the post-transcriptional regulation of inflammatory gene expression (Clark, Dean et al. 2003; Dean, Sully et al. 2004). p38 MAPK activates a downstream kinase, MK-2, which then phosphorylates and inactivates TTP, preventing the recruitment of exonucleases and the degradation of the mRNA (Clark, Dean et al. 2003; Dean, Sully et al. 2004; Stoecklin, Stubbs et al. 2004). Prolonged activation of p38 is necessary to maintain stability of COX-2 and other pro-inflammatory mRNAs. Inhibiting p38 at a late time point after the inflammatory stimulus causes rapid mRNA degradation. In macrophages from TTP knockout mice, pro-inflammatory mRNAs are extremely stable and cannot be destabilised by p38 inhibitors (Tudor, Marchese et al. 2009). This suggests that p38 MAPK controls mRNA stability exclusively through TTP (at least in mouse macrophages).

1.2.5. Clinical outcomes of GCs treatments

1.2.5.1. Anti-inflammatory effects

Thanks to their anti-inflammatory properties, GCs have been used in the clinic for more than 60 years and remain a cornerstone therapy to fight chronic inflammatory and autoimmune diseases. They are also being used to treat cardiovascular diseases and cancers, both types of pathologies being linked to inflammation. Based on an analysis of the THIN (The Health Improvement Network) database, a very recent study (Fardet, Petersen et al. 2011; Fardet, Petersen et al. 2011) report that at any time t, about 1% of the UK population (e.g. ~ 600 000) are treated with oral GCs and that this prevalence has been increasing over the past 20 years. Moreover, according to Asthma UK, about 5.4 million people are currently being treated for this condition (> 300 million worldwide, representing a global $15 billion market), almost half of them with inhaled GCs. Fardet and colleagues also describes the main indications for long-term (> 3 months) GCs prescriptions: asthma (18.7%), COPD (13.4%), RA (4.1%), dermatitis (7%) and cancer (6.1%); amongst more than 200 conditions targeted with GCs.

Anti-inflammatory properties of GCs are the result of their ability to regulate inflammatory gene expression by the transcriptional and post-transcriptional mechanisms described previously. GCs have long been known to down-regulate pro-inflammatory mediators expression, such as IL-1, IL-6, IL-8, TNFα, COX-2 or iNOS (Goulding 2004; Hayashi, Wada et al. 2004). More recently, growing evidence has also demonstrated up-regulation of the anti-inflammatory mediators GILZ, Annexin-1, IL-10, IκB and DUSP1 expression in response to GC treatment(Lasa, Abraham et al. 2002; Varga, Ehrchen et al. 2008) (Berrebi, Bruscoli et al. 2003). Because of their unfavourable risk/benefit ratio (detailed in the next section), researchers and clinicians have developed different strategies to optimise GCs treatments since their early use: (i) lowering the dose, (ii) shortening the length of treatment (e.g. several short periods instead of longer ones and (iii) using GCs in combination with other treatments. As (i) and (ii) were proven problematic options regarding the maintenance of optimal anti-inflammatory effects, (iii) has been widely considered over the past 15 years.

Historically proven very effective in RA, GCs are nowadays mostly used as bridging therapy or in intra-articular injections. They also are considered for use in conjunction with disease-modifying antirheumatic drugs (DMARDs). On top of rapid relief of RA symptoms, low to medium doses of GCs have also been shown to have long-lasting beneficial effects in early RA, via an inhibition of radiographic damages after 1-2 years treatment. Several trials reviewed in a recent article (Hoes, Jacobs et al. 2010) have studied the outcome of combined treatment versus DMARD alone, demonstrating co-therapy as better in term of symptomatic relief but also long-term reduction of joint destruction. A recent study also showed beneficial impact of a combination treatment, using subtherapeutic doses of prednisolone and the antithrombotic drug dipyridamole (Zimmermann, Avery et al. 2009). Zimmermann and colleagues reported synergistic immunosuppressive effects in a rat model of collagen induced arthritis and no typical GCs-mediated adverse effects.

In asthma, GCs are being used as a first-line treatment, in their inhalable form. Inhaled GCs, such as fluticasone or budesonide, improve lung function, control exacerbations and they may prevent irreversible airway changes. Their use is recommended to treat patients with mild asthma. In persistent asthma, were GCs doses taken daily can make systemic adverse effects become a problem, patients are now treated with combination drugs. Such drugs consist of a GC coupled to a long-acting β_2-adrenoceptor agonist (LABA) and were proven to increase bronchodilatation and anti-inflammatory effects with minimal adverse effects, compare to GCs or LABAs alone. Mechanistically GCs have been shown to increase the β2-receptor gene and the coupling of the receptor to G proteins, therefore enhancing the effects of LABAs, which are able to increase GR nuclear translocation (Ammit, Burgess et al. 2009; Beck, Vanden Berghe et al. 2009; Chipps 2009; Newton, Leigh et al. 2009; Barnes 2010). Although less effective than in asthma, GCs and combination drugs are also used in the management of COPD and other studies also investigated drugs combining GCs with MAPK or MSK inhibitors (Newton, Leigh et al. 2009).

1.2.5.2. Glucocorticoids' side effects and resistance

Documented since their discovery, GCs' side effects can be quite deleterious. Reviewed many times, the most serious ones comprise osteoporosis, diabetes mellitus and

hypertension but also increased susceptibility to inflammation, suppression of HPA axis, glaucoma and psychiatric disorders. On the other side of the spectrum, less severe but more frequent and distressful for patients can be cited skin thinning and bruising, acne, truncal obesity and fat redistribution or insomnia (Schacke, Docke et al. 2002; Rosen and Miner 2005; McDonough, Curtis et al. 2008). Up-regulation of metabolic genes is thought to be one of the major mechanisms to explain the development of unwanted effects of GCs treatment.

A well known example is the one of the tyrosine aminotransferase (TAT) gene, encoding for a hepatic enzyme involved in glucoconeogenisis (Grange, Roux et al. 1991). Its promoter, as well as the like of glucose-6-phosphatase and the rate-limiting enzyme PEPCK, contain GREs and therefore can be activated by GCs (Hanson and Reshef 1997). Moreover, PEPCK mRNA was also shown to be stabilized by GCs. Up-regulation by GCs of those crucial enzymes for glucose synthesis leads to enhanced glucose metabolism and storage. Coupled to reduced insulin effectiveness, this would explain hyperglycemia and development of diabetes, in patients treated with GCs. Similarly, GCs-induced glaucoma would be the result of transactivation of genes encoding trabecular meshwork inducible glucocorticoid response protein (or myocilin), as well as extra cellular protein type I and IV collagen and fibronectin (Zhou, Zhang et al. 1998). Build up of these proteins would lead to trabecular meshwork changes and subsequent increased intraocular pressure and optic nerve damage (Kersey and Broadway 2005). One reason for hypertension is sodium retention. Because GCs have been shown to transactivate the genes encoding epithelial sodium channel alpha (αENaC) and serum and glucocorticoid-induced protein kinase 1 (SGK1), long-term treatment can cause Na^+ retention and disturb other electrolytes concentrations (SGK1 acting on Na^+ but also K^+ and $2Cl^-$ transport), causing subsequent hypertension (Otulakowski, Rafii et al. 1999; Sayegh, Auerbach et al. 1999; Lang and Cohen 2001; Tessier and Woodgett 2006). Another deleterious adverse effect of GCs treatments is osteoporosis. Linked to decreased bone mineral density and increased spontaneous fractures, it can develop in up to 50% of patients under long-term GCs treatment. GCs can influence the osteoblast/osteoclast metabolic balance. Globally they decrease osteoblasts proliferation and increase their apoptosis, overall decreasing bone formation. On the other hand, GCs can positively regulate osteoclasts survival, resulting in increased bone resorption and unbalanced bone

turnover (Olney 2009). Indeed, GCs can up-regulate osteoprotegerin ligand (OPG-L), an osteoclast differentiation factor, via a transactivation mechanism. GCs are also able to up-regulate the receptor activator of NF-κB ligand (RANKL), factor that stimulates osteoclast activity. To further weight the balance in favor of osteoclast, GCs were shown to down-regulate osteoprotegerin (OPG), an inhibitor of RANKL (Vega, Maalouf et al. 2007). Unbalanced bone metabolism leads to more fragile bones, increasing the risk of fractures. This is also influenced by GCs-induced muscle wasting. (Schacke, Docke et al. 2002; Cooper 2004; Beck, Vanden Berghe et al. 2009; Hardy and Cooper 2010).

Besides, on top of those GCs-induced undesirable effects, a small number of patients experience resistance to GCs therapeutic effects, characterized by poor or no response to even high doses of treatment. It is believed that such resistance can be of several origins, including genetics (familial GC resistance), defective signalling or even environmental factors (such as exposition to allergens or smoking). At the molecular levels, defective signalling can be explained by excessive activation of MAPK pathways and AP-1 or reduced histone deacetylase 2 activity (Barnes 2009), hence less repression of inflammatory genes expression. Resistance can also be attributed to defects at different levels of GR function such as ligand binding, nuclear translocation or GRE binding (Ito, Chung et al. 2006; Barnes 2010).

1.2.6. Dissociated model of regulation of gene expression by glucocorticoids

Representative of a current trend in the literature (De Bosscher, Vanden Berghe et al. 2000; Karin and Chang 2001; Asadullah, Schäcke et al. 2002; Goulding 2004; Buttgereit, Song et al. 2005; Newton and Holden 2007), Van Bogaert recently stated that “it is generally believed that that the process of transrepression has the largest anti-inflammatory impact and that GC-induced side effects rather result from transactivation” (Van Bogaert, De Bosscher et al.). According to this consensus, it should therefore be possible to uncouple the harmful from the beneficial effects of the GC action. Thus, over the past 15 years or so, a lot of effort has been put in the search for a ligand that would be able to promote transrepression and retain anti-inflammatory properties but would not transactivate and therefore avoid side

effects. Such ligands are known as dissociated GR ligands, selective GR agonists (SEGRAs) or modulators (SGRMs).

1.2.6.1. GR^{dim} mouse

It all started with a transgenic strain of mice, knocked-in with a dimerisation-deficient version of GR: the GR^{dim} mice. In these mice, the gene encoding GR was mutated (A458T) in the GR D-loop, preventing GR from dimerising and thus binding to GRE of GCs-target genes (Reichardt, Kaestner et al. 1998). Not only did the homozygous mutants live but after injection of dex, no induction of GRE-dependent genes was observed (such as TAT in the liver), contrarily to what was usually seen in wild type mice. Unable to transactivate, GR^{dim} mice were however able to transrepress the AP-1 genes collagenase 3 and gelatinase B, highlighting molecular separation of transactivation and transrepression (Reichardt, Kaestner et al. 1998). Repression by GCs of other AP-1 (Tuckermann, Reichardt et al. 1999) and NF-kB (Reichardt, Tuckermann et al. 2001) dependent inflammatory genes were later shown to be also independent of GR dimerisation, supporting the idea that anti-inflammatory properties did not require DNA binding. Thus a GR-ligand able to act only via this DNA binding-independent mechanism might be sufficient for the treatment of inflammatory diseases. From then on, the search for a "perfect GC" able to retain all its anti-inflammatory while putting aside the evil side effects was launched and is reviewed below. A decade later however, new evidence started to undermine the paradigm, showing that expression of some prednisolone-induced genes was still present in GR^{dim} mice (Frijters, Fleuren et al. 2010), while Rauch and colleagues reported a GR dimerization-independent mechanism of loss of osteoblast differentiation, accounting for part of GCs-induced osteoporosis (Rauch, Seitz et al. 2010).

1.2.6.2. Selective Glucocorticoid Receptor Modulators

Since the discovery of GCs and report of adverse effects, alternative drugs retaining the anti-inflammatory effects with fewer side effects have been considered as "the Holy Grail of the steroid pharmacology" (Belvisi, Brown et al. 2001). In that quest, SGRMs were promising candidates and should be able to functionally dissociate the therapeutic effects from the side effects of conventional balanced GCs like dex. Such compounds should therefore improve benefit/risk ratio, by selectively inducing transrepression rather than transactivation (Figure 1.3). Several new compounds with a high affinity for GR have been

described in the past years and have been extensively reviewed (De Bosscher ; Schacke, Berger et al. 2007; Löwenberg, Stahn et al. 2008; Newton, Leigh et al. 2009; Berlin 2010). Among the few dozen described, can be cited RU24858 (Vayssiere, Dupont et al. 1997), Compound A (CpdA) (Louw and Swart 1999; De Bosscher, Berghe et al. 2005), AL-438 (Coghlan, Jacobson et al. 2003), ZK 216348 (Schacke, Schottelius et al. 2004), LGD-5552 (Lopez, Ardecky et al. 2008) and ZK 245186 (Schäcke, Zollner et al. 2009).

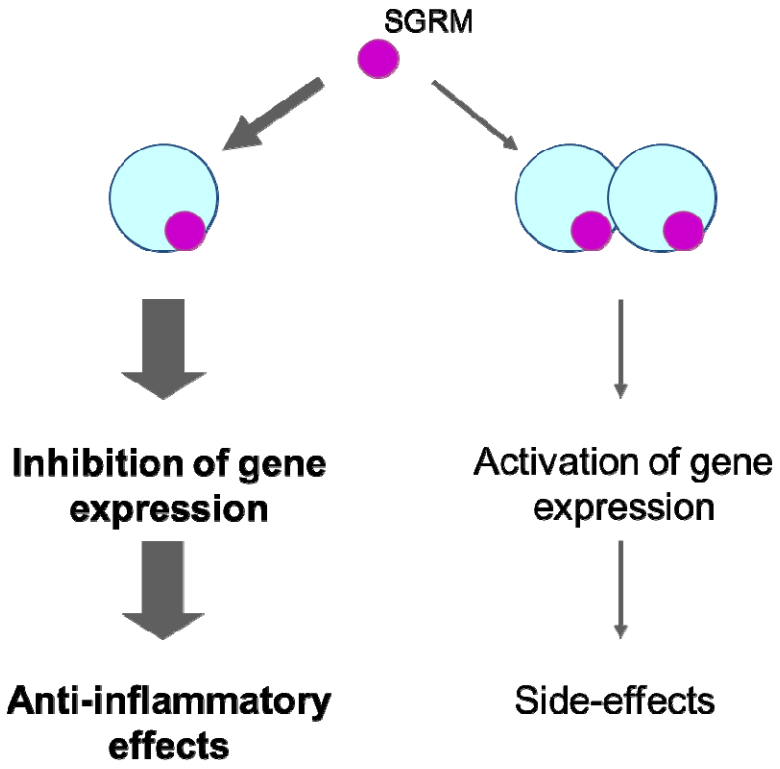


Figure 1.3. Illustration of the "dissociated GR ligand" concept.
SGRMs are developed with the aim of increasing the benefice/risk ratio of the GCs action, by dissociating the anti-inflammatory effects of GR, classically associated with transrepression, from the unwanted side effects, associated with transactivation.Such compounds are expected to retain GCs' anti-inflammatory properties while transactivation mechanisms are less affected.

Typically, SGRMs have been identified *in vitro* from drug libraries or chemical scaffold adjustments, firstly on the basis of affinity for GR and secondly on the basis of reporter constructs. Transactivation is classically tested against well known GC target genes such as TAT, or against constructs that contain well characterised GC responsive promoters or multimerised GR binding sites. Transrepression, on the other hand, is tested using promoters that contain AP-1 and/or NF-κB binding sites and are activated by pro-inflammatory stimuli. Alternatively, reporters containing multimerised AP-1 or NF-κB binding sites may be used. Transactivation and transrepression properties are then compared to those of classic GR-ligands such as dex or prednisone. To finally assess the dissociation, common anti-inflammatory effects and side effects of GCs are usually also investigated, both *in vitro* and *in vivo*.

RU24858 was among the first dissociated GR-ligands unveiled (Vayssiere, Dupont et al. 1997; Chivers, Gong et al. 2006). With its steroidal structure, it showed some good *in vitro* dissociation: similar AP-1 and NF-κB dependent transrepression to those of dex and prednisolone; weaker transactivation, with a maximum of 25% compared to the 100% of dex. Moreover, this compound also showed some good anti-inflammatory activities *in vivo* in rodents but failed to show any improved effects/side effects profile (Belvisi, Wicks et al. 2001). However, later studies (Chivers, Gong et al. 2006; Janka-Junttila, Moilanen et al. 2006) showed that this first generation of dissociated glucocorticoids were also able to induce GILZ, DUSP1, annexin 1 or AMP N gene expression as much as dex, suggesting a potential role for those genes in the anti-inflammatory effects observed.

Similarly, compound Abbott-Ligand 438 (AL-438) (Coghlan, Jacobson et al. 2003) was identified from a bank of potential dissociated candidates, screened for their binding afinity to GR, abilities to induce a MMTV reporter construct (containing multiple GREs) and repress a E-selectin reporter construct (containing both AP-1 and NF-κB binding sites). In those tests, AL-438 (compound 18) showed binding affinity to GR similar to the one of prednisolone and a strong preference for transrepression in the MMTV/E-selectin system. This non-steroidal compound was also able to repress IL1β-induced IL-6 protein expression to the same extent as prednisolone, whereas it was weaker at inducing aromatase expression, a direct GR target believed to be involved in some of GCs metabolic side effects.

Moreover, contrarily to prednisolone, AL-438 did not inhibit osteocalcin expression, a bone formation marker. In the same study (Coghlan, Jacobson et al. 2003), AL-438 had some good *in vivo* anti-inflammatory activities in a rat model of acute inflammation and showed some reduced side effects regarding glucose and bone metabolism. These effects on bone metabolism were also reported in a later study (Humphrey, Williams et al. 2006), where the authors demonstrate significant inhibition of OPG and low RANKL induction after AL-438 treatment. Moreover, AL-438 was also shown to have different cofactors interaction compared to those of prednisolone, suggesting a different way of altering GR function. Later investigations (Owens and Keyse 2007) supported the less likeliness of AL-438 to induce osteoporosis. Indeed, AL-438 did not reduce cell proliferation of murine chondrogenic cells and did not affect metatarsal bone growth, compared to dex or prednisolone.

Another SGRM, CpdA (De Bosscher, Berghe et al. 2005), was not discovered out of chemical libraries. It is a stable analogue of the hydroxyl phenyl aziridine precursor found in a Namibian desert shrub. Challenged for GR binding, it showed an affinity for the receptor slightly higher than the one of dex. In further *in vitro* tests, CpdA showed a perfectly dissociated profile, with no transactivation of multimerised GRE-dependent nor MMTV constructs and effective repression of E-Selectin, IL-6 and IL-8 reporter constructs, to a similar extent than dex (De Bosscher, Berghe et al. 2005). The same group also investigated the *in vivo* effects of CpdA. In a murine zymosan-induced inflamed paw model, pre-treatment with CpdA showed similar anti-inflammatory effects as dex, represented by a significant decrease of paw swelling (De Bosscher, Berghe et al. 2005). Blood glucose levels after CpdA were, however, similar to those observed in vehicle-injected animals, and significantly less than they were after dex injection, supporting the dissociation idea (De Bosscher, Berghe et al. 2005). Since these early observations, CpdA has been proven to have anti-inflammatory effects in collagen-induced arthritis (CIA) (Dewint, Gossye et al. 2008; Gossye, Elewaut et al. 2009) and multiple sclerosis murine models (Wüst, Tischner et al. 2009; van Loo, Sze et al. 2010). Common GCs-induced side effects were also addressed, with CpdA shown to have reduced hyperglycemia/hyperinsulinemia (Dewint, Gossye et al. 2008; van Loo, Sze et al. 2010), reduced HPA axis suppression (van Loo, Sze et al. 2010), no acquired resistance (Gossye, Elewaut et al. 2009) and a potential positive bone marker profile (Rauner, Goettsch et al. 2011). A recent study also demonstrated that CpdA could

inhibit GR dimerisation and that it could be responsible for the dissociation properties of this compound (Robertson, Allie-Reid et al. 2010). It is worth noting, however, that in all studies, similar effects than dex were only obtained with a dose of CpdA 5 to 15 times higher and some authors (Wüst, Tischner et al. 2009) reported toxic to lethal effects of CpdA *in vivo*, due to its conversion into pro-apoptotic agents, reflecting a narrow and potentially dangerous therapeutic window for this compound.

LCG-5552 (Lopez, Ardecky et al. 2008) also is a non-steroidal GR-ligand. Transrepression activity was tested using E-selectin and IL-6 promoters constructs, showing a similar repression efficacy to the one of prednisolone and dex, whereas weaker transactivation was observed in the MMTV-luciferase reporter assay. Moreover, *in vivo* studies using a rheumatoid arthritis and a multiple sclerosis rat models showed strong anti-inflammatory effects (inhibition of COX-2 and MCP-1) and a smaller disease incidence compared to prednisolone (Lopez, Ardecky et al. 2008). In the same publication, it was also reported that LGD-5552 was able to induce the anti-inflammatory cytokine IL-10, which could therefore contribute to the efficacy of this compound. Common side effects (high blood pressure, bone metabolism and body weight) were also investigated, showing less impact of LGD-5552 than when treated with prednisolone.

Two other SGRMs, ZK 216348 (Schacke, Schottelius et al. 2004) and ZK 245186 (Schäcke, Zollner et al. 2009) were described by Bayer Schering Pharma. With its non-steroidal structure, ZK 216348 was found to bind to GR with a similar affinity as dex. *In vitro*, transactivation was assessed by measuring the induction of TAT expression, transrepression with the repression of the pro-inflammatory cytokine IL-8. ZK 216348 was more than 300 times less potent than dex at inducing TAT in liver cells. It also exhibit lower efficacy. ZK 216348 was able to repress LPS-induced IL-8 expression in a monocytic cell line, but with lower efficacy and potency in comparison to dex and prednisolone. In human monocytes, ZK 216348 was less potent than dex and prednisolone but had similar efficacy on repression of LPS-induced TNFα and IL-12p70 (Schacke, Schottelius et al. 2004). *In vivo*, in rodent models of skin inflammation, the same group reported that ZK 216348 had comparable anti-inflammatory effects to prednisolone after topical application in mice but those effects where significantly less in rats (Schacke, Schottelius et al. 2004). TAT expression was

significantly less after ZK 216348 than prednisolone and similar observations were made when considering skin atrophy markers and blood glucose levels. In another study (Humphrey, Williams et al. 2006), ZK 216348 was also reported to repress OPG to a similar extent than dex and prednisolone, while being a poor activator of RANKL expression. ZK 216348 therefore seemed to display an encouraging improved effects/side effects profile.

Few years later, ZK 245186 (also known as BOL-303242-X or mapracorat) was described as a new non-steroidal GR agonist with potential use as topical agent for skin inflammatory diseases (Schäcke, Zollner et al. 2009). *In vitro*, ZK 245186 bound to GR with similar affinity to two well described topical GCs (mometasone furoate, MF and medroxyprogesterone acetate, MPA) and was able to repress AP-1-dependent collagenase promoter activity as well as expression of pro-inflammatory cytokines IL-12p40 and INF-γ (Schäcke, Zollner et al. 2009). Tested in the same study for its transactivation abilities, ZK 245186 was less able than its GCs counterparts to induce MMTV-promoter and TAT activity, suggesting a preference for transrepression. *In vivo*, the compound had anti-inflammatory activity in irritant and allergic contact dermatitis in rodents, with comparable efficacy and potency to MPA. Moreover, lack of TAT induction by ZK 245186 as well as its positive effects on blood sugar levels and skin atrophy suggested that this compound was also dissociated *in vivo*. Later studies by Bausch & Lomb investigated the effects of ZK 245186 in ocular inflammatory conditions, with encouraging anti-inflammatory effects in human corneal epithelial cells (Cavet, Harrington et al. 2010), reduced myocilin expression in monkey trabecular meshwork cells (Pfeffer, DeWitt et al. 2010) and anti-inflammatory effects and reduced effects in intraocular pressure and body weight in a rabbit model of ocular disease (Shafiee, Bucolo et al. 2011). A recent study in rabbits and monkey (Proksch, Lowe et al. 2011) reported that ZK 245186 had a “favourable pharmacokinetic profile (...) supporting further clinical investigations” and that daily doses of the compound may be efficient as anti-inflammatory therapy for ophthalmic diseases. Following up conclusive cells and animal studies, ZK 245186 is currently under clinical investigation for its use as topical agent in ocular inflammation after cataract surgery (phase III), allergic conjunctivitis (phase II), dry eye syndrome (phase II) and atopic dermatitis (phase II).

Altogether, those studies suggest that dissociated GR-ligands can have similar anti-inflammatory properties but fewer side effects than the classic glucocorticoids. However, the degree of dissociation is not yet perfect and it is therefore still possible that some of the anti-inflammatory effects are mediated by the induction of gene expression such as IL-10 by LGD-5552, as well as GILZ, DUSP1, CXCR4 or annexin 1 by RU24858 (Chivers, Gong et al. 2006; Janka-Junttila, Moilanen et al. 2006). Thus, we can wonder if the anti-inflammatory effects of those compounds are still dependent on transrepression alone or if they are as much dependent on transactivation. Also, and it is worth noting, several groups have reported that some of the side effects of the GCs were the result of gene transrepression. For example in GC-induced osteoporosis, the inhibition of bone resorption has been attributed to the inhibition of osteocalcin transcription, a protein secreted by osteoblasts and favourising bone mineralisation (Meyer, Carlstedt-Duke et al. 1997). Another example of side effect mediated by GC-induced transrepression is the suppression of the HPA axis, *via* the negative feedback exerted by GCs on genes such as pro-opiomelanocortin (POMC) (Bilodeau, Vallette-Kasic et al. 2006). Transrepression of “side-effects genes” by GCs therefore highlights again the need for extreme caution when one is trying to establish a GR ligand as dissociated. Indeed, the concept of dissociation of anti-inflammatory effects from unwanted side effects based on the favourisation of transrepression over transactivation might not be as clear and simple as it once seemed to be.

1.3. Dual Specificity Phosphatase 1

1.3.1. Dual Specificity Phosphatases

MAPKs phosphatases (MKPs) belong to the dual-specificity phosphatase (DUSP) family. These enzymes are negative regulators of MAPK signalling, dephosphorylating and thus inactivating MAPKs, simultaneously on the threonine and tyrosine residues contained within the TXY activation motif (Patterson, Brummer et al. 2009). To date, about 10 phosphatases comprising a DUSP domain and a MAPK binding domain have been identified. According to their sequence similarities, structure, substrate specificity and localisation, they can be divided into three groups. The first one comprises DUSP1, DUSP2, DUSP4 and DUSP5; all highly inducible nuclear phosphatases, able to inactivate JNK, ERK and p38 MAPK. The second includes DUSP6, DUSP7 and DUSP9, which are cytoplasmic phosphatases with a preference for ERK. Finally, the third group contains DUSP8, DUSP10 and DUSP16, localised in both the nuclear and cytoplasmic compartments and with a preference for JNK and p38 MAPKs (Dickinson and Keyse 2006; Owens and Keyse 2007). As discussed earlier in this chapter, MAPKs play a crucial role in the regulation of inflammatory gene expression, acting at both transcriptional and post-transcriptional level. For this reason, inactivation of these kinases by DUSPs has been widely studied, as a mean to better understand the signalling mechanisms regulating immune reactions. With proven anti-inflammatory effects and regulation by both glucocorticoids and pro-inflammatory stimuli, my focus is set here on dual-specificity phosphatase 1. Along the next few pages I will review DUSP1's history, regulation and role in an inflammatory context. I will also discuss its potential utilisation as a therapeutic target.

1.3.2. Dual Specificity Phosphatase 1

Dual-specificity phophatase-1 (DUSP1, also called MKP-1) is the archetypal DUSP, first cloned as a product of the immediate early gene 3CH134 in fibroblasts (Sun, Charles et al. 1993). As the other members of the DUSP family, DUSP1 is able to dephosphorylate the threonine and tyrosine residues of the Thr-X-Tyr of MAPKs' activity domains. It has been shown that DUSP1 preferred substrates were JNK and p38 MAPK rather than ERK (Franklin

and Kraft 1997). This has been confirmed when *Dusp1* knockout (*Dusp1*$^{-/-}$) mice showed no altered ERK activity (Dorfman, Carrasco et al. 1996) and was further supported by the work of Wu et al. where p38 MAPK and JNK were hyper activated after different types of stress in mouse embryonic fibroblasts from *Dusp1*$^{-/-}$ mice, compared to their wild type (WT) counterparts (Wu and Bennett 2005). A year later Abraham et al. demonstrated that JNK and p38 MAPK inhibition by GCs was abrogated in bone marrow macrophages derived from *Dusp1*$^{-/-}$ mice (Abraham, Lawrence et al. 2006), corroborating the critical role of DUPS1 in the regulation of JNK and p38 MAPK activities and subsequent inflammation processes.

1.3.2.1. DUSP1 is critical in the regulation of inflammatory processes

1.3.2.1.1. DUSP1 is induced by inflammatory stimuli and glucocorticoids

DUSP1 is induced by a wide variety of stimuli including LPS, TNFα, IL-10, IL-1 and dexamethasone (dex, a synthetic glucocorticoid), in many cell types such as macrophages, mast cells, osteoblasts and T cells (Chen, Li et al. 2002; Lasa, Abraham et al. 2002; Hammer, Mages et al. 2005; Zhao, Shepherd et al. 2005; Abraham and Clark 2006). Such induction has been reported as transient in response to pro-inflammatory stimuli, as illustrated in the case of LPS (Abraham, Lawrence et al. 2006) or TNFα (Quante, Ng et al. 2008; King, Holden et al. 2009) challenges. We (Lasa, Abraham et al. 2002; Abraham, Lawrence et al. 2006; Tchen, Martins et al. 2010), and others (Toh, Yang et al. 2004; Fürst, Zahler et al. 2008; Johansson-Haque, Palanichamy et al. 2008; Shipp, Lee et al.), also extensively described how DUSP1 was upregulated by glucocorticoids, and how this induction was potentiated in the presence of pro-inflammatory stimuli, supporting a role for DUSP1 in negative control of inflammatory responses. Moreover, by its inhibitory effects on p38 and JNK MAPKs, DUSP1 was shown to contribute to the downregulation pro-inflammatory genes expression such as TNFα, IL-6 or IL-8 (Chen, Li et al. 2002; Abraham, Lawrence et al. 2006; King, Holden et al. 2009) and thus has been considered for now quite a while, as a key anti-inflammatory molecule.

1.3.2.1.2. DUSP1 protects against endotoxic shock

Although a little 'disappointing' at the start, because they were viable, fertile and appeared normal (Dorfman, Carrasco et al. 1996), *Dusp1* deficient mice's detailed phenotype, as partly described previously, soon confirmed the endogenous role of DUSP1 as a regulator of inflammatory processes. Indeed, these mice also exhibited a pattern of strongly overexpressed inflammatory cytokines such as IL-6, TNFα, IFNγ and IL-10 at the time of an LPS challenge, when compared to WT animals (**Hammer**, **Mages** et al. 2006; Salojin, Owusu et al. 2006; Zhao, Wang et al. 2006). Considering the predominant role of IL-6 and TNFα in septic shock (Guidet, Staikowsky et al. 1993), it is not surprising that *Dusp1*$^{-/-}$ mice are more prone to lethal endotoxin shock (**Hammer**, **Mages** et al. 2006; Zhao, Wang et al. 2006; Frazier, Wang et al. 2009). On top of sensitivity to LPS's toxicity, *Dusp1*$^{-/-}$ mice were also shown to be over responsive to TNF insults leading to dramatic inflammation, cell death and activated MAPK activity (Libert, oral communication, 3rd Benelux Nuclear Receptor Meeting, 2010). These mice were also described as much more sensitive to Gram-positive bacterial infection, as challenge with *Staphylococcus aureus* induced a substantially higher mortality rate in the null animals, compared to WT littermates (Wang, Meng et al. 2007). Altogether these data support a protective effect of DUSP1 against damaging and lethal consequences of excessive production of pro-inflammatory cytokines.

1.3.2.1.3. DUSP1 and inflammatory diseases

Considering its crucial role in immune responses, it is not surprising to see that DUSP1's involvement in autoimmune and inflammatory disease has been extensively studied. In a chicken collagen induced arthritis model (Salojin, Owusu et al. 2006), *Dusp1*$^{-/-}$ mice, compared to WT littermates, exhibited notably accelerated disease progression and increased severity, in agreement with increased levels of pro-inflammatory cytokines observed in these same animals. In fibroblast-like synoviocytes (FLS) from RA patients, DUSP1 was expressed at time 0 and increased after stimulation with dex and IL-1β. Such induction was correlated with subsequent dephosphorylation of JNK and p38 MAPK, suggesting induction of DUSP1 as a potential therapeutic target for RA (Toh, Yang et al. 2004). Studies in our lab also identified a spontaneous inflammatory phenotype in a colony

of *Dusp1*$^{-/-}$ mice, characterised by swelling of the distal phalanx (S. Abraham, PhD Thesis, 2007) as well as impaired therapeutic responses to dex in collagen-induced arthritic *Dusp1*$^{-/-}$ mice (Y. Vattakuzhi, unpublished results). These results emphasise once again the important role of DUSP1 in maintaining a balanced inflammatory state.

In asthma, *DUSP1* polymorphisms were recently associated with clinical response to inhaled corticosteroid therapy (Jin, Hu et al. 2010). Indeed, in these ethnically diverse cohorts of patients, a *DUSP1* polymorphism which appeared to modify the relationship between steroid use and bronchodilator response was also associated with a symptomatic response to inhaled corticosteroid therapy. These findings might be useful in the future to identify asthmatic patients more likely to respond to inhaled corticosteroid treatments. DUSP1 expression has also been investigated by an Australian group in airway smooth muscle (ASM) cells. In this model, repression of IL-6 by GCs was shown to occur via upregulation of DUSP1 (Quante, Ng et al. 2008). The authors showed that TNF-α acted via a p38 MAPK-dependent pathway to stabilize the IL-6 mRNA transcript and, using siRNA, that lack of DUSP1 reversed the inhibition of TNF-α–induced IL-6 secretion by dex.

According to Wu et al, DUSP1 is involved in metabolic control, as *Dusp1*$^{-/-}$ mice were resistant to diet-induced obesity thanks to enhanced energy expenditure. However, these mice succumbed from glucose intolerance caused by high-fat diet (Wu, Roth et al. 2006). Other studies have shown that DUSP1 was overexpressed in the livers of C57BL/6 mice following high fat diet (Reddy, Nguyen et al. 2004) as well as in those of type1 diabetic rats (Chin, Ramirez et al. 1995). More recently, microarray analysis in peripheral leukocytes from rats with high-fat diet-induced insulin resistance showed an increase in DUSP1 mRNA levels at basal level and 3 h after glucose administration (Fujimoto, Mochizuki et al. 2010), supporting a role for DUSP1 in metabolic homeostasis.

Over the past few years, DUSP1 has also been shown to be involved in the induction and development of certain cancers. Reviewed many times (Keyse 2008; Bermudez, Pagès et al. 2010; Haagenson and Wu 2010), DUSP1 is recognised as over-expressed in many human cancer types, including colon, prostate, bladder, non-small-cell lung carcinoma, ovarian and breast malignancies. In the case of prostate and breast cancer, DUSP1 high expression levels

were correlated with lower JNK activity; JNK having been established to play a role in tumour suppression. In ovarian cancer, DUSP1 has even been associated with shorter progression-free survival. Resistance to chemotherapy agents, have equally been linked with DUSP1 overexpression and reduced JNK activity, as well as decreased treatment-induced apoptosis. DUSP1 would therefore appear to be an attractive target in cancer therapy, to overcome chemoresistance and enhance performance of conventional chemotherapeutic strategies.

1.3.2.2. Involvement of DUSP1 in regulation of anti-inflammatory gene expression by GCs

In HeLa cells COX-2 mRNA stability was regulated positively by p38 MAPK and negatively by dex (Ridley, Dean et al. 1998; Lasa, Brook et al. 2001). In this cell system dex inhibited the function of p38 MAPK and up-regulated the expression of DUSP1 (Lasa, Abraham et al. 2002). It was not yet proved that DUSP1 was responsible for the inhibition of p38 or the destabilisation of COX-2 mRNA, because RNA interference experiments to knock down DUSP1 expression were unsuccessful. In macrophages from a *Dusp1*$^{-/-}$ mouse, dex could no longer inhibit p38 MAPK (Abraham, Lawrence et al. 2006). Interleukin 1β (IL-1β) expression was inhibited by dex in WT macrophages but not affected by dex in *Dusp1*$^{-/-}$ macrophages. The p40 subunit of interleukin 12 (IL-12p40) was equally inhibited by dex in WT and *Dusp1*$^{-/-}$ macrophages. COX-2 was between these two extremes; its inhibition by dex was impaired but not completely lost in KO macrophages (Abraham, Lawrence et al. 2006). Another group (Quante, Ng et al. 2008) showed, in airway smooth muscle cells, that glucocorticoids were inhibiting TNFα-induced IL-6 expression *via* reducing its mRNA stability and that this occurred through DUSP1 induction. Taken together, these results suggest that glucocorticoids have at least two ways to affect the expression of inflammatory genes. One way is to up-regulate DUSP1 and to inhibit p38 MAPK (this could influence gene expression at the transcriptional level, the post-transcriptional level or both). The second, unidentified mechanism is independent of DUSP1. Genes can be inhibited by dex via DUSP1-dependent mechanisms (*e.g.* IL-1β), via DUSP1-independent mechanisms (*e.g.* IL-12p40) or via a combination of DUSP1-dependent and -independent mechanisms (*e.g.* COX-2).

1.4. Aims of the project

This PhD project was designed around two main questions, aiming at understanding the involvement of DUSP1 in (1) the post-transcriptional regulation of pro-inflammatory gene expression by glucocorticoids and (2) the anti-inflammatory effects of novel GR ligands, or SGRMs.

The aim of the project was to investigate the anti-inflammatory effects of the classic and dissociated glucocorticoids, their dependency on DUSP1 and the impact that dependency on gene activation might have on the "dissociation concept" and the way novel GR ligands are discovered.

My project was divided into two main sub-projects:

1.4.1. To study the regulation of inflammatory mRNA stability by glucocorticoids and the involvement of DUSP1

Some anti-inflammatory effects of glucocorticoids can be dependent or partly dependent on DUSP1, a phosphatase able to inactivate MAPKs such as p38. Moreover, some pro-inflammatory mediators are regulated by glucocorticoids at the post-transcriptional level and mRNA destabilisation has been shown to involve p38 MAPK and the ARE binding protein TTP.

Our hypothesis was that glucocorticoids would inhibit inflammatory gene expression at a post-transcriptional level by both increasing the expression of TTP and increasing its mRNA destabilising activity. Glucocorticoids may (1) upregulate TTP expression; (2) upregulate DUSP1 expression; (3) as a consequence inhibit the function of p38 MAPK; (4) as a consequence increase the mRNA destabilising function of TTP and cause more rapid degradation of mRNAs that are targeted by TTP.

So, the aims of this part of the project were:

- To determine if glucocorticoids induced DUSP1 and TTP in our *in vitro* model of inflammation.
- To determine if glucocorticoids increased the degradation rates of inflammatory mRNAs.

- To determine whether the post-transcriptional effects of glucocorticoids were dependent on DUSP1.

1.4.2. To study the anti-inflammatory effects of two experimental SGRMs and the involvement of DUSP1 in those effects

Cpd1 and Cpd2 are two novel, non-steroidal GR-ligands. Those compounds have been shown to have *in vitro* preference for transrepression and, *in vivo*, to have an improved effects/side effects profile. Experiments in the laboratory have shown that Cpd1 and Cpd2 have the ability to activate the DUSP1 promoter (J. Martins, unpublished).

Our hypothesis was that, even if they were not supposed to have a great transactivational activity, the anti-inflammatory effects of those compounds would involve DUSP1 induction.

Therefore, several questions needed further investigation:

- How dissociated Cpd1 and Cpd2 really were?
- Were they able to induce DUSP1 at both the mRNA and protein levels?
- What were their anti-inflammatory effects compared to those of classic glucocorticoids?
- Were their anti-inflammatory effects dependent on DUSP1?
- How would this dependency, if any, impact the way novel GR ligands are discovered and their properties understood?

Chapter 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. General reagents and materials

- Acrylamide 30% (w/v)/bis-acrylamide 0.8% (w/v) was from Severn Biotech Ltd (UK);
- Agar was from Difco (USA);
- Agarose, SeeBlue Pre-Stained Standard, One Shot Top10 competent cells and Gibco heat inactivated Foetal Calf Serum (FCS) were from Invitrogen (USA);
- Bio-plex beads, Western blotting chambers and the GS-710 densitometer were from Bio-Rad Laboratories (UK);
- Cryovials were from Simport (Canada);
- Culture flasks, plates and dishes were from BD Falcon and Sterilin (UK);
- DMSO, Dexamethasone, Actinomycin D and all the reagents used in the making of the different buffers were from Sigma-Aldrich (UK), unless otherwise specified;
- DTT, mycrocystin and lipopolysaccharide (LPS) were from Enzo Life Sciences (UK);
- Dual-luciferase Reporter Assay Kit, Passive Lysis Buffer, Luciferase Assay System, Reporter Lysis Buffer, renilla, pGL3-Basic and pGL3-Promoter reporter vectors were from Promega (UK);
- Enhanced chemiluminescence (ECL) Western Blotting Detection Reagents kit was from GE Healthcare (UK);
- G-418, DMEM, RPMI, Penicillin/Streptomycin 100X and Trypsin/EDTA 1X were obtained from PAA Laboratories (UK);
- IL-1α and β were homemade in the laboratory by L. Rawlinson;
- Murine macrophage colony stimulating factor (M-CSF) and recombinant human TNFα were from Peprotech (UK);
- One-Step RT qPCR Mix was from Eurogentec (Belgium);
- Polyscreen PVDF Transfer membrane and MicroBeta Luminometer were from Perkin Elmer Life Sciences (UK);
- Re-Blot Plus Strong Solution 10X and filter 96 wells plates were from Millipore;
- RNeasy Mini Kit, RNase-free DNase Set, QIAshredder columns, Superfect® Transfection Reagent and Plasmis *Plus* Maxi Kit were from Qiagen (UK);

- RO4553059 (Cpd1) and RO4638375 (Cpd2) were a gift from Roche (UK);
- Rotor-Gene 6000 Series was from Corbett Research (Australia);
- SB 202190 and CpdA were from Calbiochem;
- Super RX medical X-ray films were from Fujifilm;
- TaqMan probes (see table 2.2) were from Applied Biosystems (USA);
- White 96 wells microtiter plates were from Thermo Scientific (ABgene, UK);
- Yeast extract and Tryptone for bacterial culture were from Biogene (UK).

2.2. Buffers

4X SDS sample buffer

0.4 M Tris-HCl pH = 6.8, 0.4% (w/v) SDS, 40% (v/v) glycerol, 1% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue.

Blocking buffer

5% dried skimmed milk and 1:1000 sodium azide in PBS.

Luria-Bertani broth

1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1% (w/v) tryptone, in autoclaved distilled water.

PBS-T

0.05% of Tween 20 in PBS.

Phosphate Buffer Saline (PBS)

10% PBS 10X (Prolabo) in distilled water.

Radio-immunoprecipitation assay (RIPA) lysis buffer

20 mM Tris-HCl pH = 7.5, 150 mM NaCl, 1% (v/v) NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% (v/v) SDS.

Running buffer

10% of Ultra Pure Tris/Glycine/SDS 10X (National Diagnostics) in distilled water.

TBS-T

0.05% of Tween 20 in Tris-Buffered Saline: 75 mM NaCl, 10 mM Tris pH = 7.4, 1 mM EDTA, in distilled water.

Transfer buffer

12 g of Tris, 56.7 g of glycine, 3.2 L of distilled water and 800 mL of 100% methanol. Kept at 4˚C.

Whole cell lysis buffer

Tris-HCL 50 mM (pH = 7.5), NaCl 250 nM, EDTA 3 nM, EGTA 3 nM, 1% Triton, 0.5% NP40 and 10% glycerol. Completed to 50 mL with distilled water. Kept at 4˚C.

2.3. Cell culture

2.3.1. Media

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), containing L-glutamine, sodium pyruvate and 4.5 g/L L-glucose and supplemented with 10% FCS. Primary cells (mouse macrophages) were maintained in Roswell Park Memorial Institute 1640 medium (RPMI) containing L-glutamine and HEPES and supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C.

2.3.2. Bone marrow derived macrophages

All animal procedures were performed under United Kingdom Home Office regulations and with local Ethical Review Committee approval. Wild type (WT) and DUSP1 knock-out (KO) mice were maintained in group cages, food and water *ad libitum*.

Age- and sex-matched animals were sacrificed by exposure to a rising concentration of CO_2, using an appropriate gas chamber. The femur and tibia were dissected, cleaned and placed in ice cold RPMI medium. In a Class II Biological Safety Cabinet, the bones were placed in a Petri dish with a small volume of RPMI. Using a scalpel, the ends of the bones were chopped off and discarded. A 20 mL syringe was then filled with 15 mL of culture medium containing M-CSF and attached to a 25–gauge hypodermic needle. The bone marrow was flushed out in a new 50 mL Falcon tube by inserting the needle into one end of the bone. The flushed bones were discarded. Next, 15 mL of RPMI containing M-CSF and 10% (v/v) FCS were added in the tube to the bone marrow. The cell suspension was mixed by vortexing for about one minute and poured in a 14 cm Petri dish. The cells were incubated for 5 days. The bone marrow stem cells adherents to the plate were differentiated macrophages. These were

trypsinised, resuspended in medium and seeded at a density of $1x10^6$ cells per well of a 12 well plate. Cells were rested overnight prior to experimental treatments.

2.3.3. Cell lines

2.3.3.1. General maintenance

Cell types of both murine and human origin were used for the experiments described in this report. All cells were grown to confluence in 150 cm^2 flasks. To passage cells, medium was aspirated and cells washed once with serum free medium. Apart from the RAW 264.7 cells which were scraped, all cell types were trypsinised for 5 to 10 min, suspended in 10 mL serum free medium and centrifuged at 4000 rpm for 5 min. The supernatant was discarded and the cell pellet resuspended in culture medium supplemented with 10% (v/v) FCS. Unless otherwise specified, cells were seeded at high density in 12 wells plates in 2 mL of medium or in 6 wells plates in 3 mL medium. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C, in 150 cm^2 flasks and split at a ratio of 1:5.

2.3.3.2. Murine cell lines

RAW 264.7 cells are a murine monocyte/macrophage cell line derived from male Balb/c mice infected with the Abelson leukaemia virus. These cells are very sensitive to lypopolysaccharide (LPS) and produce a vast range of cytokines and chemokines, for which reason they are widely used to study inflammatory cell signalling. RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC).

Mouse embryonic fibroblats (MEFs) were obtained from mouse embryos by R. Perelli. Briefly, pregnant $DUSP1^{+/+}$ and $DUSP1^{-/-}$ females were sacrificed at 13 days post coitum and each embryo was separated from the placenta and the surrounding membranes. Brain, liver and spleen were cut away, washed thoroughly and the remaining embryos were minced and trypsinised for 15 min. Cells were recovered by centrifugation and resuspended in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin. When confluent, cells were passaged according to the 3T3 protocol (Todaro and Green 1963).

2.3.3.3. Human cell lines

HeLa cells are from a human cervical epidermal adenocarcinoma origin. They were the first continuous cancer cell line established, in 1951 by George Gey from John Hopkins hospital in Baltimore. They were derived from a biopsy of a patient's cervix called Henrietta Lacks. Over the past 60 years, HeLa cells have been thoroughly used across all fields of biomedical research: cancer research, establishment of the polio vaccine, cloning, HIV, tuberculosis, telomerase discovery... HeLa cells respond particularly well to IL-1 stimulation and are widely used as a model for human response to inflammation *in vitro*.

A549 cells are carcinomic human alveolar basal epithelial cells. The line was established from a lung tumor in the 1970s by D. Giard. They are also sensitive to IL-1 stimulation and used in the study of inflammatory diseases such as asthma or COPD.
Both cell types were obtained from ATCC.

2.4. Transfections

2.4.1. Stably transfected A549 cells

A549 reporter cell lines were a gift from Dr R. Newton (University of Calgary).
They comprised A549 cells, stably transfected with either a GRE- or NFκB-dependent luciferase reporter. GRE-dependent reporter cells contained two copies (underlined) of a GRE consensus site derived from the rat TAT gene (5'-GCT GTA CAG GAT GTT CTA GGC TGT ACA GGA TGT TCT AG-3'), upstream of a β-globin minimal promoter and a luciferase coding sequence (pGL3.neo.TATA.2GRE) (Chivers, Cambridge et al. 2004). The NF-κB-dependent reporter 6κBtkluc (referred to as 6κB-luc), contained three tandem repeats of the sequence 5'-AGC TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GA-3', which contained two copies of the decameric NF-κB site (underlined), derived from the human *HIV* enahncer, upstream of a minimal thymidine kinase promoter driving a luciferase gene (Newton, Hart et al. 1998; Chivers, Cambridge et al. 2004). The NF-κB-dependent reporter 3κBu-luc, or pGL3.neo.TATA.3κBu (referred to as 3κB-luc), consisted of a double-stranded

oligonucleotide 5'-AGG GGA TTC CCT AGG GGA TTC CCT AGG GGA TTC CCT-3' containing three copies of the upstream NF-κB site (underlined) derived from the human *COX-2* promoter (Newton, Kuitert et al. 1997) and a minimal β-globin promoter driving luciferase (Catley, Cambridge et al. 2004; Holden, Gong et al. 2007).

Reporter cells were maintained in DMEM supplemented with 10% (v/v) FCS and 0.2 mg/mL G-418. Prior to experiments, the reporter cells were seeded to confluence ($3x10^5$ cells per well of a 12 well plate, 2 mL per well) and were incubated overnight in G-418-free culture medium. Cells were harvested in Reporter Lysis Buffer 6 h after treatment for luciferase assay. Firefly luciferase activity was measured using the Luciferase Assay System and Microbeta luminometer. As each well was seeded with the same number of cells and all the cells contain the reporter construct, we and others (Newton, Seybold et al. 1998) find the reporter activity to be highly reproducible and the normalisation to another reporter unnecessary.

2.4.2. Bacterial culture and DNA purification

E.coli One Shot TOP10 competent cells were typically transformed with 2 µL of the appropriate DNA construct using manufacturer's instructions. Briefly, the mix was incubated on ice for 30 min, heat-shocked at 42°C for 45 sec in a water bath and transferred back on ice for 2 min. 400 µL of Luria-Bertani (LB) broth were then added, and the cells were left to recover for 1h at 37°C with gentle agitation. 20 to 200 µL of the mix was then spread onto LB-agar plates [1.6% (w/v) agar] containing ampicillin for selection of the transformants. Plates were incubated at 37°C overnight and colonies picked up the following day. Single colonies were cultured overnight in 4 mL LB containing ampicillin, at 37°C with gentle shaking. The following day, 1 mL of the mix was transferred to 100 mL ampicillin containing LB and cultured overnight in the same conditions.

Stocks were prepared using 200 µL 100% glycerol mixed to 800 µL of freshly grown bacterial culture in sterile Cryovials. The vials were left on dry ice for 2 min before being transferred to -80°C.

For DNA extraction, the 100 mL of freshly cultured bacteria were centrifuged at 5000 x g for 10 min and DNA extracted using the Plasmid *Plus* Midi Kit, according to the manufacturer's guidelines. After purification, DNA concentrations were estimated by measuring the absorbance at 260nm (A_{260}), and DNA purity was assessed using the A_{260}/A_{280} ratio.

2.4.3. Transient transfections

A549 and HeLa cells were transiently transfected using SuperFect Transfection Reagent. Briefly, cells were seeded at low density in 6 wells plates and left to recover overnight, then washed in 1X sterile PBS and supplemented with 500 µL of FCS containing DMEM. Each well was transfected with 200 ng of the indicated Firefly luciferase construct, 100 ng of pRenilla-CMV plasmid (Promega, UK) and 700 ng of pBluescript (total of 1 µg/well). The volume was brought up to 105 µL for each sample, by adding DMEM and 5 µL of SuperFect. The mix was vortexed 30 sec and incubated for 15 min at room temperature then 105 µL was added onto the cells and left to incubate at 37°C/5% CO2 for 3 h. At the end of the incubation period, cells were washed, supplemented with 3 mL of FCS containing DMEM and left to recover overnight.

The following day the cells were stimulated with the specified reagents for the appropriate amount of time and harvested in 200 µL of Passive Lysis Buffer. Cells were actively lysed by 2 rounds of freezing/thawing and then centrifuged at 13000 rpm at 4°C for 1 min. 20 µL of supernatant were loaded on a 96 wells white microtiter plate and luciferase activities were measured using the Dual Luciferase Reporter Assay Kit and Microbeta luminometer. For each sample, values were normalised against the Renilla luciferase readings.

2.5. Protein detection

Cells were grown in 12 well plates. Cell culture supernatants and lysates were collected and stored at -20°C until used for measurement of protein expression.

2.5.1. Western blotting

2.5.1.1. Cell harvesting and preparation of samples

All steps were performed on ice. The medium was removed and the cells washed twice in PBS. 100 µl of whole cell lysis buffer (Tris-HCL 50 mM (pH = 7.5), NaCl 250 nM, EDTA 3 nM, EGTA 3 nM, 1% Triton, 0.5% NP40 and 10% glycerol) supplemented with protease and phosphatase inhibitors (1 µl/mL mycrocystin, 2 µl/mL dithiothreitol (DTT), 1 µl/mL proteinase inhibitor E64, 1 µl/mL pepstatin, 5 µl/mL aprotinin, 5 µl/mL phenylmethylsulfonyl fluoride (PMSF) and 10µl/mL sodium fluoride (NAF)) were added to lyse the cells. Cells were scraped, the lysates transferred to 1.5 mL ependorfs and centrifuged at 13000 rpm for 1 min at 4˚C to precipitate the cell debris. Using a spectrophotometer, protein concentrations were determined by Bradford assay. The volume corresponding to 30 to 70µg of protein was calculated and the samples mixed with the appropriate volume of 4X SDS sample buffer (containing 40 µl/mL of DTT) to make the final volume of all the samples equal. Samples were then boiled for 5 min and quickly spun before gel loading.

2.5.1.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by a discontinuous stacking/separating gel electrophoresis system, following the method originally described by Laemmli (Laemmli 1970).

Stacking gel layer

5% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 125 mM Tris-HCl pH = 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.06% (v/v) TEMED.

Separating gel layer

10 or 12% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 380 mM Tris-HCl pH = 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.04% (v/v) TEMED.

Typically, to allow protein sizing, 15 µl of markers ranging 4-250 kDa (SeeBlue Pre-Stained Standard) were loaded alongside the protein samples. Gels were usually electrophoresed for 3h at 120V with running buffer.

2.5.1.3. Transfer onto PVDF membrane

Once electrophoresis was complete, the gel was removed from the apparatus. The stacking part was cut off and the rest of the gel was assembled into a transfer "sandwich" as follows: three sheets of Whatman 3MM paper (wetted with transfer buffer), PVDF membrane previously soaked in 100% methanol for 2 min, rinsed in distilled water and soaked in transfer buffer, gel and three sheets of 3MM Whatman paper (wetted with transfer buffer). The "sandwich" was then placed inside a transfer cassette between two sponges (wetted with transfer buffer) and this was placed into a Western blotting chamber. Proteins were transferred from the gel to the membrane by electrophoresis in transfer buffer at 100V for 1 to 1.4 h, at 4˚C.

2.5.1.4. Immunodetection

According to the antibody to be used, the membrane was blocked for 45 min, 1h or overnight at room temperature in blocking buffer. The primary antibody (table 2.1), at the appropriate dilution in blocking buffer, was then added and incubated overnight at 4˚C. Next, the membrane was washed three times 10 min in PBS-T and incubated for 1 or 1.5 h with the appropriate IgG/horseradish peroxidase-conjugated secondary antibody, diluted in blocking buffer. The membrane was washed four times 10 min in PBS-T and the antigen detected by enhanced chemiluminescence (ECL), according to the manufacturer's instructions.

When necessary, membranes were stripped using 1X Re-Blot Plus Strong Solution for 15 min at room temperature. The membrane was blocked and then blotted with a different antibody.

Protein bands intensity was quantified by densitometric analysis using a calibrated imaging densitomer (GS-710) and the Phoretix ID software.

Antibody	Dilution	Species	Company	Secondary antibody dilution
Tubulin	1:10000	mouse monoclonal	Sigma	1:2000
DUSP1 (MKP-1 V15)	1:500	rabbit polyclonal	Santa Cruz	1:2000
DUSP1 (MKP-1 M18)	1:100	rabbit polyclonal	Santa Cruz	1:1000
DUSP1	1:1000	rabbit polyclonal	Millipore	1:5000
DUSP1	1:250	goat polyclonal	LifeSpan Biosciences	1:2000
COX-2	1:200	rabbit polyclonal	Cayman Chemical Company	1:1000
iNos	1:200	rabbit polyclonal	BD Transduction Laboratories	1:2000
TTP (SAK21)	1:1000	rabbit polyclonal	"homemade"	1:2000
Rabbit IgG HRP conjugated	–	swine polyclonal	Dako	–
Mouse IgG HRP conjugated	–	rabbit polyclonal	Dako	–
Goat IgG HRP conjugated	–	rabbit polyclonal	Dako	–

Table 2.1. Details of primary and secondary antibodies used for Western blotting.
For each antibody is indicated its specie of origin, its dilution and the dilution of the corresponding secondary antibody.

2.5.2. Enzyme linked immunosorbent assay

Murine IL-12p40 protein production was measured from cell culture supernatants by standard sandwich ELISA technique, using an eBioscience kit, according to the manufacturer's instructions. The technique involves the immobilisation of an antibody onto an inert plastic microtiter plate, the capture of the antigen by this antibody and the detection or the antigen by a second antibody, biotin coupled. Briefly, after coating the plate overnight with the capture antibody anti-IL-12p40, samples and standards were incubated for 2 h then detected with a biotin-conjugated antibody. The avidin-HRP enzyme was incubated on the plate for 30 min, the substrate solution added, the reaction stopped after about 15 min and the plate read for absorbance at 450 nm, using the FLUOstar Omega equipment from BMG Labtech. Values were normalised against the standard curve and further analysed using the Prism software.

2.5.3. Multiplex detection of proteins from a single biological sample

IL-6, TNFα and CXCL-1 proteins were detected simultaneously from cell culture supernatant using the xMap Technology (Dunbar 2006) from Luminex and filter 96 well plates. Briefly, color-coded Bio-plex beads, or microspheres, were coupled to antibodies against IL-6, TNFα and CXCL-1 (R&D Systems) using an amine coupling kit from Biacore Life Sciences. Beads were coated with 50µg/ml of the primary antibodies; standards and samples were then added and let overnight at 4°C. After addition of 0.5 µg/ml of the corresponding biotinylated secondary antibodies (Peprotech) and streptavidin-PE at 2 µg/ml (Europa Bioproducts), protein expression was detected by the levels of fluorescence, with laser excitement of each internal dye identifying the different microspheres, using the Luminex 100 Total System. Values were normalised against the standard curve obtained for each protein and further analysed using the Prism software.

2.6. Quantitative real time-polymerase chain reaction (RT-PCR)

RT-PCR is a method to quantify gene expression at the mRNA level. This method measures mRNA levels via the fluorescence emitted from a dye on specifically designed TaqMan probes after excitation by laser scanning technology.

2.6.1. mRNA extraction

Cells were lysed using 350 µl of RLT buffer containing 100 µl/mL of DTT. The lysates were homogenised by centrifugation through a QIAshredder column and total RNA was extracted using RNeasy Mini Kit, according to the manufacturer's instructions and including the additional DNase digestion step.

2.6.2. Detection

RT-PCR reactions were set up in a total volume of 10 µl and each reaction contained 2.45 µl of RNase-Free water, 5 µl of 2X Reaction Buffer, 0.05 µl of EuroScript RT enzyme (all from the Reverse Transcription qPCR mastermix No ROX, Eurogentec), 0.5 µl of TaqMan probe (table 2.2) and 2 µl of template. Each sample was set up at least in duplicate. The RT-PCR procedure was performed using Rotor-Gene 6000 Series. The detector was programmed as follow:

- Reverse transcription step: 48˚C for 30 min.
- Deactivation of the enzyme: 95˚C for 10 min.
- PCR amplification: cycles of 95˚C for 3 seconds (denaturation) and 60˚C for 30 seconds (extension).

TaqMan probe	Murine	Human
COX-2 (PTGS2)	Mm00478374_m1	Hs00153133_m1
DUSP1	Mm00457274_g1	Hs00610256_g1
GAPDH	Mm99999915_g1	Hs99999905_m1
GILZ	–	Hs00932202_g1
IL-10	Mm00439614_m1	–
IL-12p40	Mm01288990_m1	–
IL-1α	Mm00439620_m1	–
IL-6	Mm00446190_m1	Hs00985641_m1
iNos	Mm00440485_m1	–
TNFα	Mm00443258_m1	–
TTP (ZFP36)	Mm00457144_m1	Hs00185658_m1

Table 2.2. Details of the Taqman probes used for mRNA measurement.

2.6.3. Analysis

Relative quantification was performed using the $\Delta\Delta C_t$ method. This method uses arithmetic formulas to calculate the relative abundance of the gene of interest. The number of PCR cycles required for the fluorescence associated with the reaction to be greater than the baseline fluorescence is called the threshold cycle (C_t). Since this method is very sensitive, small changes in concentration of template between samples will result in large differences in the calculated abundance. To control this, the results are normalised against the level of a housekeeping gene, GAPDH. An assumption is made that the levels of GAPDH mRNA do not vary with treatment. ΔC_t is the difference between the averages of threshold cycles for the reference gene (GAPDH) and the target gene. $\Delta\Delta C_t$ determines a relative value (an arbitrary constant) to which other values are normalised. To establish the fold difference between sample mRNA contents, value $2^{-\Delta\Delta Ct}$ was used. This value is the amount of target normalised to an endogenous reference and relative calibrator (control), where $2^{-\Delta\Delta Ct}$ value for control mRNA (usually unstimulated point) equals 1.

2.7. Statistics

Statistical analysis was performed using analysis of variance with the Bonferroni post-test for multiple comparisons or t-tests for columns comparisons. All tests were performed using Prism software version 5 (GraphPad). A *p* value <0.05 was considered significant.

Company name	Website
American Type Culture Collection (ATCC)	www.lgcstandards-atcc.org
Applied Biosystems	www.appliedbiosystems.com
BD Falcon	www.bdbiosciences.com
Biogene	www.biogene.com
Bio-Rad Laboratories	www.bio-rad.com
BMG Labtech	www.bmglabtech.com
Calbiochem (now Merck Millipore)	www.merckmillipore.com
Cayman Chemical Company	www.caymanchem.com
Corbett Research	www.corbettlifescience.com
Dako	www.dako.com
Difco (now BD Diagnostic Systems)	www.bd.com/ds
Elmer Life Sciences	www.perkinelmer.com
Enzo Life Sciences	www.enzolifesciences.com
Eurogentec	www.eurogentec.com
Europa Bioproducts	www.europa-bioproducts.com
Fujifilm	www.business.fujifilm.co.uk/medical
GE Healthcare	www.gehealthcare.com
GraphPad	www.graphpad.com/welcome.htm
Invitrogen	www.invitrogen.com
Life Span Bioscience	www.lsbio.com
Luminex	www.luminexcorp.com
Millipore	www.millipore.com
PAA Laboratories	www.paa.com
Peprotech	www.peprotech.com
Promega	www.promega.com
Qiagen	www.qiagen.com
R&D Systems	www.rndsystems.com
Roche	www.roche.com
Santa Cruz Biotechnology	www.scbt.com
Severn Biotech Ltd	www.severnbiotech.com
Sigma-Aldrich	www.sigmaaldrich.com
Simport	www.simport.com
Sterilin	www.sterilin.co.uk
Thermo Scientific	www.thermoscientific.com

Table 2.3. Contact details for supplying companies.

Chapter 3

POST-TRANSCRIPTIONAL REGULATION OF INFLAMMATORY GENES BY GLUCOCORTICOIDS

3. POST-TRANSCRIPTIONAL REGULATION OF INFLAMMATORY GENES BY GLUCOCORTICOIDS

3.1. Introduction

As mentioned previously in the main introduction to this work, several reports indicate that glucocorticoids are able to regulate inflammatory gene expression. In their study from 2006, Smoak and Cidlowski showed that TNFα expression is controlled by posttranscriptional mechanisms involving TTP. Using A549 cells they demonstrated that TNFα mRNA was decreased by dex through its 3'UTR and that this decrease was abrogated when adding siRNAs targeting TTP (Smoak and Cidlowski 2006). In the same study, TTP protein and mRNA expression was significantly up-regulated by dex both *in vitro* (A549 cells) and *in vivo* (rat tissus). A few years later TTP protein and mRNA expression was also shown to be induced by dex alone or in combination with IL-1β (King, Kaur et al. 2009). However treatment with dex in an IL-1β-induced context led to repressed TTP expression compared to IL-1β treatment on its own. This would be the result of p38 MAPK signalling pathway repression by dex.

Regarding another pro-inflammatory mediator, IL-1α, its regulation by GCs was shown to be dependent on DUSP1, as demonstrated by a severely impaired response to dex in macrophages from *Dusp1*$^{-/-}$ mice, alongside that of COX-2 and IL-1β (Abraham, Lawrence et al. 2006). Yet this DUSP1 dependent inhibition by dex was not proven to be occurring via posttranscriptional mechanisms.

iNOS expression, on the other hand has been long proven to be regulated by GCs at the posttranscriptional level in C6 glioma cells (Jun Shinoda 2003), hepatocytes (Ozaki, Habara et al.) and macrophages (Korhonen, Lahti et al. 2002). However, work from our group suggested that iNOS inhibition by GCs was independent of DUSP1, at least at the protein level in murine bone marrow derived macrophages (Abraham, Lawrence et al. 2006). Very recently that same pro-inflammatory mediator was shown to be regulated by DUSP1 via p38 MAPK in human A549 cells (using siRNA) and murine cells (macrophages from WT and

DUSP1 KO mice) (Turpeinen 2011). Whether dex regulates iNOS expression in a posttranscriptional manner and through DUSP1 still remains to be investigated.

Finally, circumstantial evidence supports the hypothesis that glucocorticoids destabilise COX-2 mRNA via the up-regulation of DUSP1 and inhibition of p38 MAPK. The p38 MAPK pathway stabilises COX-2 mRNA in several cell types (Ridley, Dean et al. 1998; Dean, Brook et al. 1999; Jang, Sanchez et al. 2000; Lasa, Mahtani et al. 2000; Faour, He et al. 2001; Bachelor, Silvers et al. 2002) and is likely to do so by preventing COX-2 mRNA deadenylation, the rate limiting step in mRNA degradation (Dean, Sarsfield et al. 2003). Moreover, it was shown that Dex inhibits COX-2 expression largely at the post-transcriptional level. This effect involves accelerated mRNA deadenylation (Ristimaki, Narko et al. 1996; Newton, Seybold et al. 1998). Dex also inhibits p38 MAPK function by inducing expression of DUSP1 (Abraham, Lawrence et al. 2006) and inhibits COX-2 expression largely via the up-regulation of DUSP1 (Abraham, Lawrence et al. 2006).

These observations are from a variety of different cell types, and do not conclusively prove a role of DUSP1 in the regulation of pro-inflammatory mRNA stability by glucocorticoids. The experiments described in this section were designed to address the hypothesis directly by testing post-transcriptional effects of dex in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells.

3.2. DUSP1 is up-regulated by glucocorticoids

To begin with, commercially available anti-DUSP1 antibodies were tested for their ability to detect DUSP1 protein in extracts from cell types commonly used in the laboratory (murine bone marrow derived macrophages, RAW264.7 cells, MEFs, HeLa and A549 cells). Macrophages obtained from DUSP1 deficient mice were used as a negative control: the antibody tested should not detect DUSP1 in these extracts. All antibodies used are described in

Table *3.* and were tested using the manufacturer's recommendations, protocols commonly used in the laboratory or any combination of the previous. When having problem with an antibody, I tried to vary the length of blocking (30 min up to 20 h), the incubation time of

the primary and/or secondary antibody (1 to 20 h), their concentrations (up to ten times the manufacturer's recommendation), the composition of the washing (PBS-T versus TBS-T) and blocking (3% versus 5% milk, BSA) buffers and even the exposure time of the film (from 30 sec to overnight exposure). V-15 from Santa Cruz worked well for detection of DUSP1 in RAW264.7 cells (Figure 3.2a) but did not give consistent results with other cell types (data not shown). Batch J3007 of the anti-DUSP1 antibody M-18, also from Santa Cruz, consistently detected DUSP1 in all cell types tested, whereas batches I0705 and G2307 of the same antibody did not work. M18-J3007 was used in all of the experiments presented here, with the exception of Figure 3.2. Unfortunately the stock of this particular batch of antiserum ran out in the spring of 2009 and I had to go back to antibody testing. None of the other M-18 batches gave consistent results. I also tried the goat polyclonal antibody from Lifespan and the rabbit polyclonal from Sigma. Despite trying different protocols and varying experimental conditions I managed to detect DUSP1 only inconsistently with the former, not at all with the latter. Finally, the anti-DUSP1 from Millipore turned out to be unspecific. A clear band was detected (Figure 3.1) at around 40kDa, but in extracts from both *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMDMs. The cells were stimulated with 10 nM of dex for 0, 1 or 4 hours. No variation of expression was detected between the different conditions. The positive control included in the experiment was a 3T3 cell extract provided by the manufacturer, which also contained an antigen detected by the antibody. All together, the data support the non-specificity of the Millipore antibody. The protein detected could be DUSP4, as its molecular weight is also around 40kDa, is not regulated by dex and its expression does not differ between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMDMs (S. Abraham, unpublished observations). To this day we are still trying to find appropriate antibodies with which to detect DUSP1 protein.

Antibody	Specie	Lot number	DUSP1 detection?
H-66 (sc-10796)	rabbit polyclonal	D0104	NO
V-15 (sc-1102)	rabbit polyclonal	D241	NO
		G2205	± (RAW)
M-18 (sc-1199)	rabbit polyclonal	I0705	NO
		G2307	±
		J3007	**YES all**
		F1808	±
		E2109	±
Lifespan (LS-B2736)	goat polyclonal	16752	±
Millipore (07-535)	rabbit polyclonal	26730	Non specific
Sigma (M-3787)	rabbit polyclonal	074K4801	NO

Table 3.1. Comparison of the different antibodies used for DUSP1 protein detection by Western blotting.

The antibodies referred to as "sc" were from Santa Cruz. All antibodies have been tested in a variety of conditions for their ability to detect DUSP1 protein in a strong and reproducible way. ± indicates that the antibody was able to detect DUSP1 at least once but in non-reproducible manner.

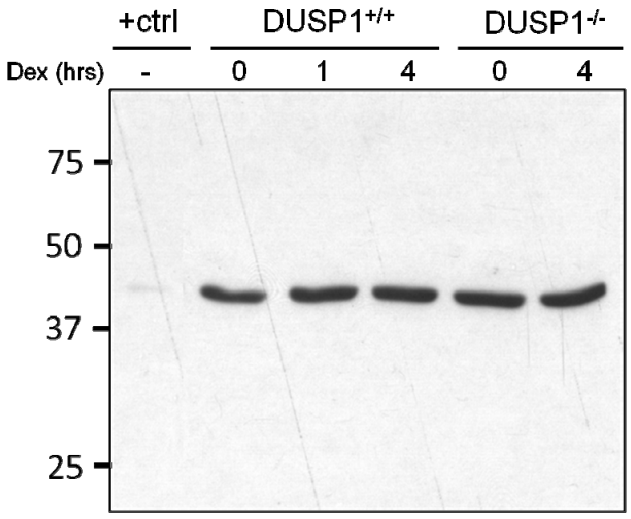


Figure 3.1*. Unspecificity of the DUSP1 Millipore antibody.*

The anti-DUSP1 antibody from Millipore (see Table 3.) was tested in BMDMs from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice. Cells were treated with 10 nM of dex for 0, 1 or 4 h. "+ctrl" indicates the positive control given alongside the antibody (3T3 cells extract). Cell lysis and Western blotting were performed according to the manufacturer's instructions.

RAW264.7 cells and murine bone marrow derived macrophages were treated with LPS alone (10 ng/ml), dex alone (100 nM) or a combination of both for 1, 2, 4 and 8 h. Total mRNA from both cell types and proteins from RAW264.7 cells were then extracted from the cells lysates and RT-PCR and Western blotting for DUSP1 were performed. DUSP1 mRNA results are presented as a percentage of the maximum induction, to make the comparison between the different treatments and cell types easier.

In RAW264.7 cells, DUSP1 protein was induced by LPS alone with a maximum expression after 1 h treatment (Figure 3.2a). The expression of DUSP1 protein then decreased, but was still detectable at 8 h. On the other hand, there was little response to dex alone, other than a possible weak expression of the DUSP1 protein after 4 and 8 h. However, the combination of LPS and dex strongly induced DUSP1 protein expression with a maximum at 1h and a sustained expression at later time points. Dex therefore appeared to both increase and prolong the expression of DUSP1 protein in response to LPS. At the mRNA level (Figure 3.2b), the same type of response pattern could be observed. LPS alone induced DUSP1 mRNA strongly but quite transiently, with a return to near basal levels within 8 h. Dex alone induced DUSP1 mRNA relatively weakly. When combined with LPS, dex both increased and prolonged the expression of DUSP1 mRNA. An analysis of variance on LPS or dex alone compared to the combination of LPS and dex showed highly significant differences, confirming the increased peak of expression and sustained expression of DUSP1 in response to the combined treatment. Therefore, at both mRNA and protein levels dex and LPS cooperated to regulate the expression of DUSP1. At late (4 and 8h) time points, the cooperative effects of dex and LPS could be described as synergistic.

The expression of DUSP1 mRNA in BMDMs followed a similar pattern (Figure 3.2c). LPS and dex separately increased DUSP1 expression at 1 h, returning to lower expression levels at later time points. In particular, in LPS stimulated cells, DUSP1 mRNA was back to its basal levels at 8 h. Used in combination, both reagents were able to strongly induce DUSP1 mRNA expression at 1 h, with a highly significant increase, compared to the effects of LPS or dex alone ($p<0.001$). This induction was sustained over time, with still significant cooperative effects of dex and LPS at 4 and 8 h. Just as in RAW264.7 cells, the effect of dex and LPS could also be considered as synergistic in mouse bone marrow derived macrophages.

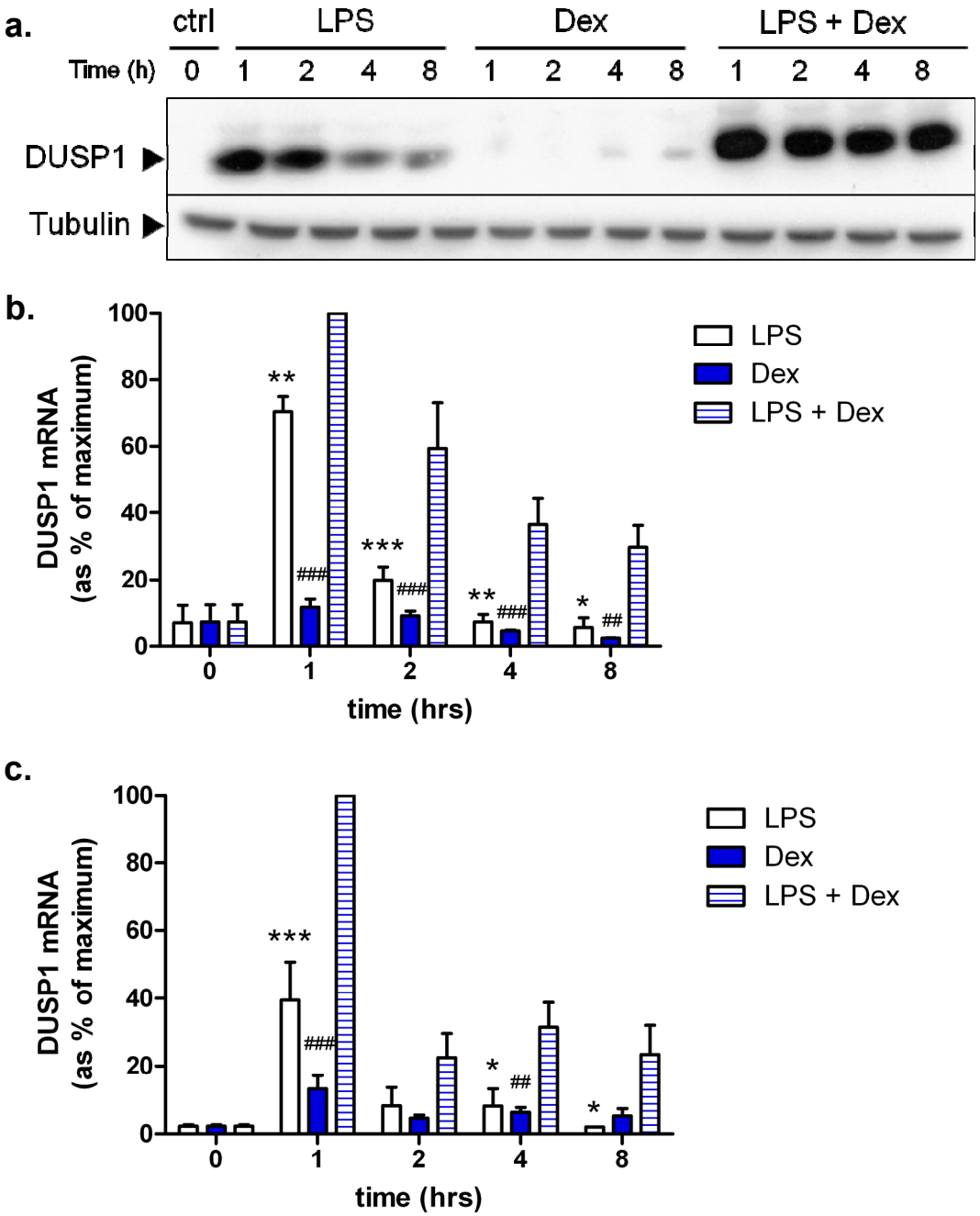


Figure 3.2. ***Time course of DUSP1 expression.***

Cells were treated for the times shown with LPS alone (10 ng/ml), dex (100 nM) or both reagents. ***(a)*** *DUSP1 and tubulin proteins were detected in RAW264.7cells extract by Western blotting; tubulin is used here (and in all the following Western blots) as a loading control. A representative experiment is shown. DUSP1 mRNA was quantified by real-time PCR and expressed as a percentage of its maximum in* ***(b)*** *RAW 264.7 cells and* ***(c)*** *bone marrow derived primary macrophages. Graphs indicate means ± SEM (error bars) from at least three independent experiments. Statistical analysis was performed using analysis of variance for LPS versus LPS + dex (*) and dex versus LPS + dex (#). Significance was taken as follow: */#, p < 0.05; **/##, p < 0.01and ***/###, p < 0.001. Data shown were obtained in collaboration with H. Cunliffe, a PhD student under my direction.*

3.3. Effects of glucocorticoids on the expression of COX-2, iNOS and TTP are partly dependent on DUSP1

3.3.1. Regulation of COX-2 and iNOS expression

Experiments in primary mouse macrophages showed that DUSP1 protein expression also appeared to be dependent on the dose of dex. Macrophages from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice (Figure 3.3) were pre-treated with increasing doses of dex for 2h and then stimulated with 10ng/ml of LPS for 4h. Proteins were extracted using whole cell lysis buffer, quantified and Western blotted for DUSP1, COX-2, iNOS and tubulin. Under those conditions, DUSP1 protein was weakly induced by LPS in WT cells. Note that, as in RAW264.7 cells (Figure 3.2), LPS transiently up-regulates DUSP1 protein in mouse macrophages. DUSP1 peaks at 1 h and declines thereafter (S. Abraham, unpublished observations) (Hu, Chen et al. 2007; Turpeinen 2011). The time point selected for this experiment is therefore well after the peak of expression of DUSP1 protein in response to LPS alone. The LPS-induced expression of DUSP1 was potentiated in a dose dependent manner by the addition of 10nM to 1µM of dex. In effect, this reveals the same prolongation of gene expression that was described in RAW264.7 cells. In the same experiment, dex dose-dependently decreased the expression of COX-2 and iNOS, in parallel with the increased expression of DUSP1. DUSP1 protein was not detected in *Dusp1*$^{-/-}$ macrophages, confirming the specificity of the antibody. In comparison to the *Dusp1*$^{+/+}$, dex inhibited COX-2 expression very weakly in the absence of DUSP1. This suggested that dex inhibits the expression of COX-2 in a manner that is largely dependent on DUSP1, consistent with a previous report from our laboratory (Abraham, Lawrence et al. 2006). iNOS expression appeared to be quite strongly dose-dependently inhibited in both *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells, although the inhibition was a little weaker in the *Dusp1* deficient macrophages, suggesting a role for DUSP1 in the regulation of iNOS protein expression (Turpeinen 2011). However, the impairment of response to dex in *Dusp1*$^{-/-}$ cells was less striking in the case of iNOS than in the case of COX-2. This also agrees with previous findings from our group (Abraham, Lawrence et al. 2006).

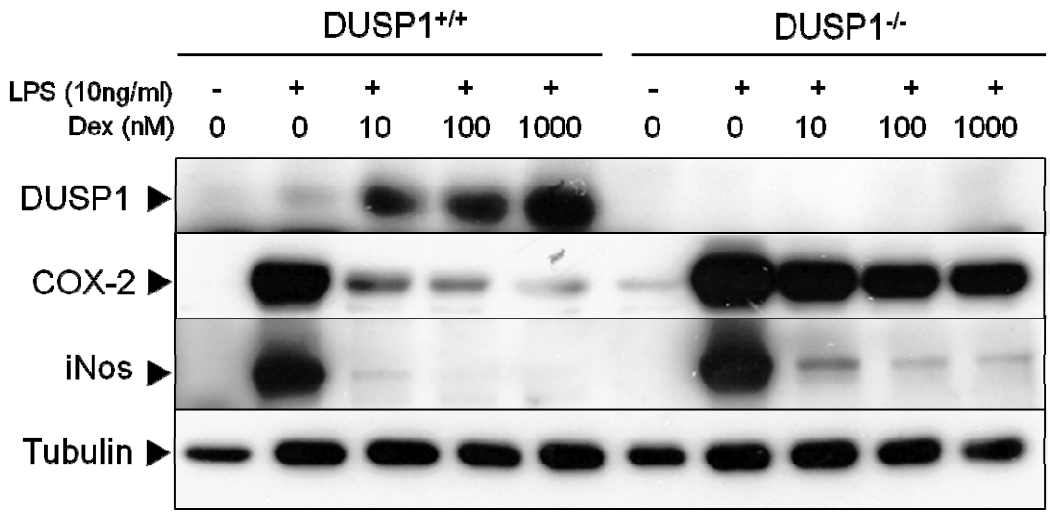


Figure 3.3. ***Induction of DUSP1 and DUSP1-dependent inhibition of COX-2 and iNos proteins expression by dex in a dose dependent manner.***

Primary macrophages from Dusp1$^{+/+}$ or Dusp1$^{-/-}$ mice were pre-treated with increasing concentrations (10nM to 1μM) of dex for 2h then challenged with LPS (10ng/ml) for 4h. Proteins were detected by Western blotting. A representative experiment of three repeats is shown.

3.3.2. Regulation of TTP expression

p38 MAPK pathway has been shown to stabilise both TTP mRNA (Tchen, Brook et al. 2004) and protein (Brook, Tchen et al. 2006; Hitti, Iakovleva et al. 2006; Janka-Junttila, Moilanen et al. 2006) . Previous work in our group showed that macrophages from *Dusp1* deficient mice exhibited enhanced and prolonged activation of p38 MAPK (Abraham, Lawrence et al. 2006). Finally, it has been suggested that glucocorticoids were able to destabilise pro-inflammatory mRNAs by up-regulating tristetraprolin (TTP) (Smoak and Cidlowski 2006). In this section, it was therefore decided to investigate the effects of dex on the expression of TTP protein and mRNA. In this perspective, bone marrow macrophages derived from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice were pre-treated for 2 h with increasing concentrations (10nM to 1µM) of dex then stimulated for 4 h with 10ng/ml of LPS. TTP protein was detected by Western blotting using the "homemade" antiserum SAK21 (Figure 3.4 a) (Mahtani, Brook et al. 2001). mRNA was detected by real-time PCR, using a Taqman probe for the *Zfp36* mRNA (encoding the TTP protein, also known as zinc finger protein 36 homolog) (Figure 3.4 b and c).

In *Dusp1*$^{+/+}$ macrophages, TTP was strongly induced by LPS, at both the protein and mRNA level. TTP protein expression was dose-dependently inhibited by dex. Likewise at the mRNA level, with a decrease of the *Zfp36* mRNA levels in response to dex treatment, which becomes significant at the higher dose of 1000 nM ($p < 0.05$), when compared to LPS treated cells. In *Dusp1*$^{-/-}$ cells, TTP protein and mRNA expression was also induced by LPS. Protein expression appeared to be much stronger in the macrophages from *Dusp1* deficient animals than in those from *Dusp1*$^{+/+}$ mice. However, this increase was not observed at the mRNA level; although it cannot be ruled out that these cells significantly overexpress *Zfp36* mRNA at earlier time points. These observations are consistent with the fact that *Dusp1*$^{-/-}$ cells have enhanced and prolonged p38 activation, which may lead to stabilisation of TTP protein and accumulation (Brook, Tchen et al. 2006; Hitti, Iakovleva et al. 2006). This of course, remains to be proven by further work. When the cells from *Dusp1*$^{-/-}$ mice were treated with increasing concentrations (10 and 1000 nM) of dex, no Zfp36 mRNA decrease could be seen (Figure 3.4 b and c), when a relatively weak dose-dependent inhibition of TTP protein was observed (Figure 3.4 a).

Whether it is at the protein or mRNA level, experiments described in this section reflect differences in the regulation of TTP expression, between macrophages from *Dusp1*$^{+/+}$ and *Dups1*$^{-/-}$ mice. Altogether this suggests that dex may decrease TTP expression at least partly *via* DUSP1.

The inhibition of *Zfp36* mRNA and TTP protein by dex in the *Dusp1*$^{+/+}$ cells argues against the idea of up-regulation of TTP as a mechanism for the anti-inflammatory effects of dex (Smoak and Cidlowski 2006). Moreover, although dex may up-regulate TTP in the *Dusp1*$^{-/-}$ but not in *Dusp1*$^{+/+}$ cells, the anti-inflammatory effects of dex are weaker in the former than in the latter (Figure 3.3), suggesting that under the conditions of this experiment, the inhibition of COX-2 gene expression by dex does not appear to involve an increase in the expression of TTP. Rather, the strength of anti-inflammatory effect of dex seems to be correlated with how well it decreases TTP expression.

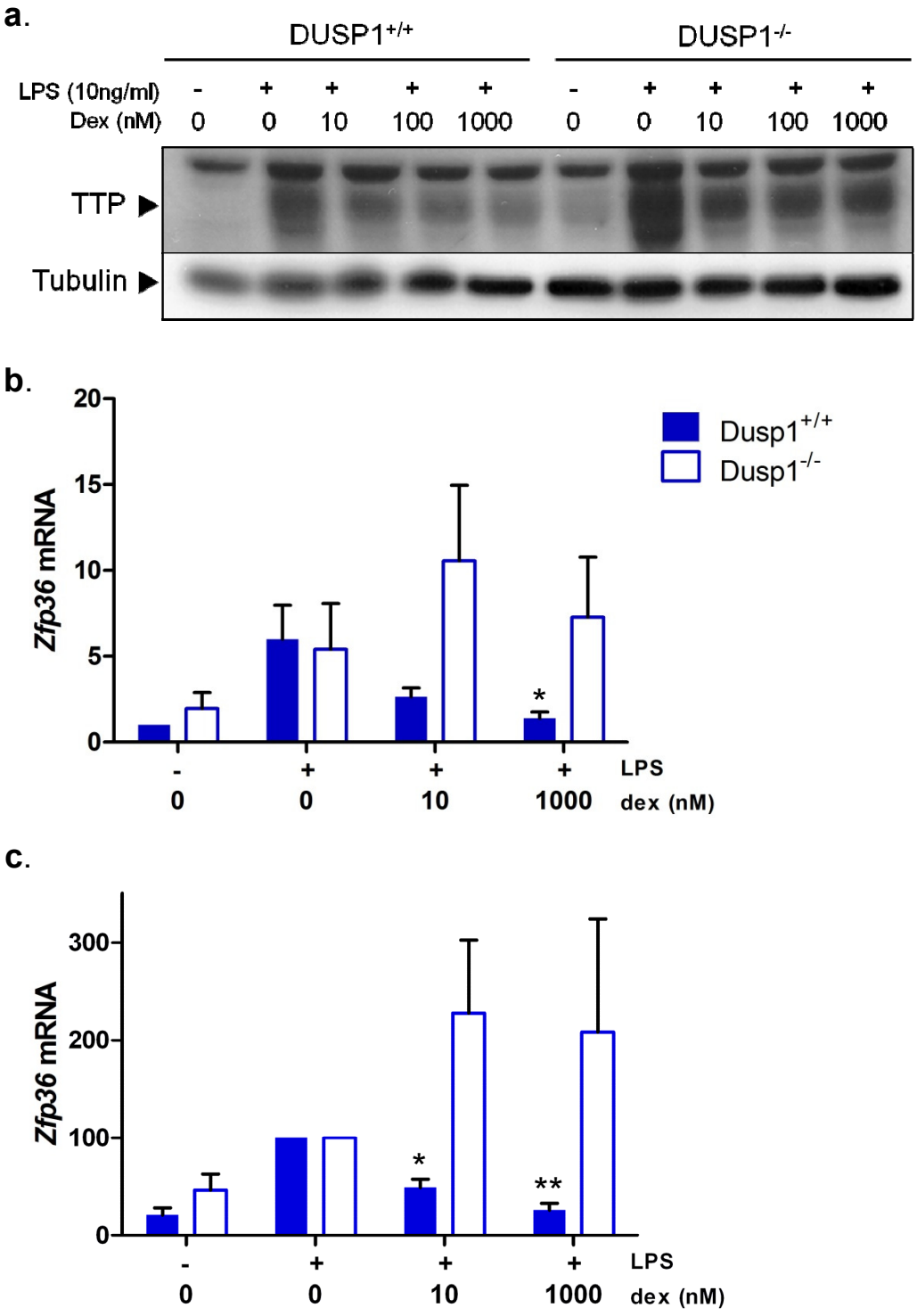


Figure 3.4. ***Dose dependent inhibition of TTP expression by dex may be partly dependent on DUSP1 in primary mouse macrophages.***

Cells from Dusp$^{+/+}$ and Dusp$^{-/-}$ mice were pre-treated with increasing concentrations (10 nM to 1 µM) of dex for 2 h then challenged for 4 h with LPS (10 ng/ml). ***(a)*** *TTP protein was detected by Western blotting. Zfp36 mRNA expression was measured by RT-PCR and normalised against the house keeping gene GAPDH.* ***(b)*** *Results are expressed as fold changes over mRNA levels in vehicle-treated Dusp1$^{+/+}$ cells.* ***(c)*** *Results are expressed as a percentage of the induction by LPS (set as 100%). For each treatment, graphs indicate mean ± SEM (error bars) from n = 3 independent experiments. Significance was taken as follows: *, $p < 0.05$ and **, $p < 0.001$.*

3.4. Anti-inflammatory effects of glucocorticoids in mouse embryonic fibroblast.

Because of their limited availability and the time taken to generate them, an alternative cell type to the bone marrow derived mouse macrophages was considered, in order to extend the investigation of the anti-inflammatory effects of glucocorticoids in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells. Immortalised mouse embryonic fibroblasts (MEFs) had previously been generated in the laboratory by R. Perelli, from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice and were known to show an induction of COX-2 by pro-inflammatory stimuli (Yamamoto, Yamazaki et al. 2004). I therefore chose to investigate their response to both pro-inflammatory stimuli and glucocorticoids before to decide if they were a good candidate to use in complement to the BMDMs to extend the study without having lengthy cell preparation.

MEFs were pre-treated with increasing concentrations of dex for 2h then challenged with pro-inflammatory stimuli for 24h, as a time course of 4, 12 and 24h showed that 24h of stimulation was the best for inducing a response in those cells (not shown). Different types of pro-inflammatory stimuli were tested: 20 ng/ml of IL-1β, 100 ng/ml of PMA or a combination of both. A combination of IL-1β and PMA was able to induce a stronger response in COX-2 expression in the MEFs than PMA or IL-1β alone (not shown). It was therefore decided to stimulate the cells with both compounds.

DUSP1, COX-2 and iNOS proteins were detected by Western blotting (Figure 3.5). As shown on the upper panel, DUSP1 protein expression was induced by dex in a dose dependent manner, only in the *Dusp1*$^{+/+}$ MEFs. In this same cell type COX-2 protein expression was strongly induced by IL-1β and inhibited by dex but not as much as in the BMDMs (Figure 3.3). In the *Dusp1*$^{-/-}$ MEFs, induction of COX-2 by IL-1β was also powerful and dex was capable of some inhibition, but to a lesser extent than in the *Dusp1*$^{+/+}$ cells, suggesting a role for DUSP1 in the regulation of COX-2 expression in MEFs.

I then went to assess the behaviour of another pro-inflammatory gene, namely inducible nitric oxide synthase (iNOS) (Figure 3.5). iNOS protein expression was noticeably induced by the pro-inflammatory stimulus in both *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells. However, inconsistent results were obtained regarding the role of DUSP1 in the regulation of iNOS expression. The

results obtained from all 15 experiments conducted could be grouped under three types of patterns, as representatively shown on Figure 3.5. In one experiment dex inhibited iNOS expression more strongly in *Dusp1*$^{+/+}$ than *Dusp1*$^{-/-}$ cells, in another there was no difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells in the sensitivity of iNOS to inhibition by dex, and in a third iNOS was more strongly inhibited by dex in the *Dusp1*$^{-/-}$ cells. Because of the inconsistent pattern of response of the *iNos* gene to dex in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ fibroblasts, this investigation was discontinued.

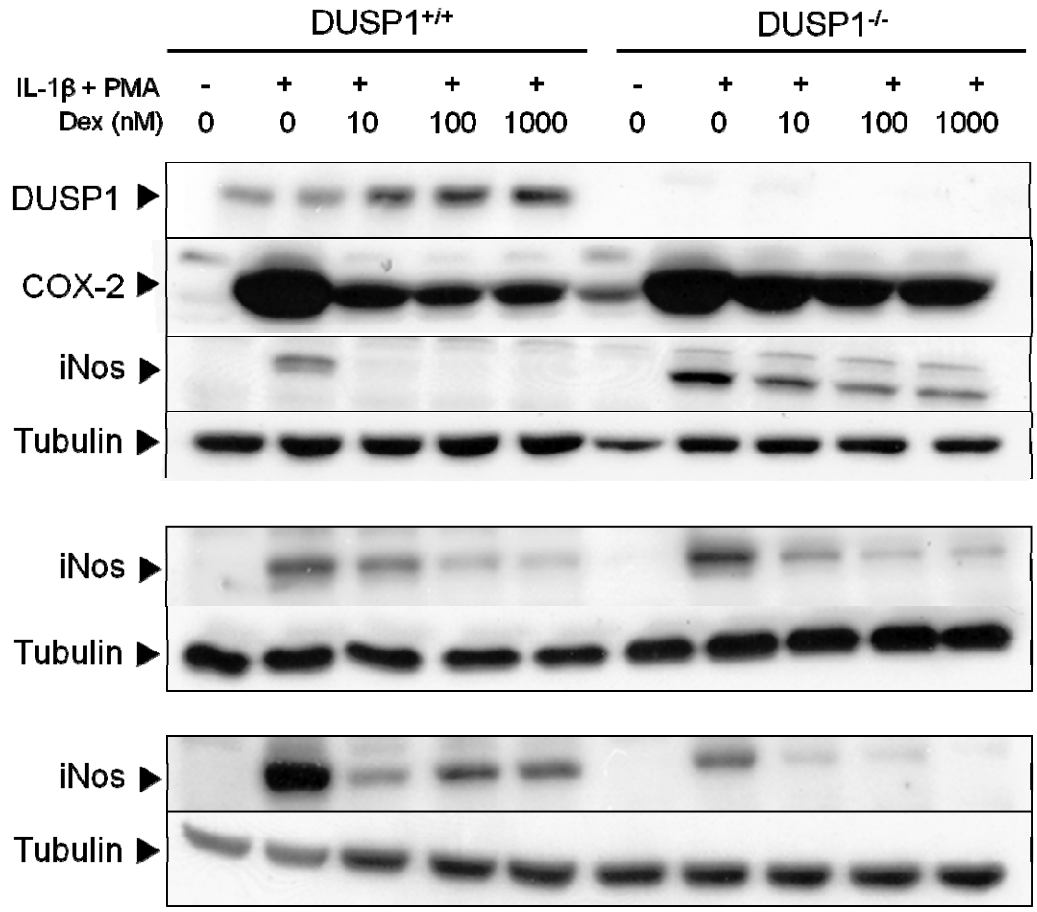


Figure 3.5. ***Effects of dex on DUSP1, COX-2 and iNOS proteins expression in MEFs.***
Dusp1$^{+/+}$ *and Dusp1*$^{-/-}$ *cells were pre-treated with increasing doses of dex (10nM to 1µM) for 2h than challenged with a combination of IL-1ß (20ng/ml) and PMA (100 ng/ml) for 24h. Proteins were detected by Western blotting.*

3.5. Post-transcriptional regulation by glucocorticoids

3.5.1. Effect of p38 inhibition on COX-2 and TNF mRNA stability

Before testing the effects of dex on mRNA stability, preliminary experiments were carried out using the p38 MAPK inhibitor SB 202190 (SB). SB has been widely used for many years to study the p38 MAPK pathways and has been described by Manthey and colleagues, as "a selective inhibitor of p38 in vitro and in vivo", in LPS-induced monocytes. The compound blocked p38 half maximally at 30 nM and concentrations as high as 30 µM did not inhibit ERK and JNK activity (Manthey, Wang et al. 1998). SB's specificity has also been validated in an inflammatory murine BMDM model, where the authors studied the involvement of the p38 MAPK pathway in *IL-10* mRNA stability (Tudor, Marchese et al. 2009).

The intention was to establish the actinomycin D chase assay and confirm that the stability of pro-inflammatory mRNAs is regulated by the p38 MAPK pathway in bone marrow derived murine macrophages as has been described in several other cell types (Clark, Dean et al. 2003; Tchen, Brook et al. 2004; Jang, Lim et al. 2007).

Macrophages from *Dusp1*$^{+/+}$ animals were stimulated with LPS for 4h then treated with actinomycinD (an inhibitor of transcription) and with SB or vehicle (DMSO). Macrophages were harvested every 30 minutes for 2 h (Figure 3.6a). The moment actinomycin D and SB were added is considered as time 0. mRNA was quantified by RT-PCR, using probes for the pro-inflammatory genes *COX-2* and *TNF*, and normalised against the house keeping gene *GAPDH*. mRNA quantity was expressed as a percentage of that at time 0. For each time point, two-way ANOVA and Bonferroni post-test were performed to compare the mRNA levels between vehicle and SB treated cells. Here the results are also shown as one phase exponential decay curves: a non-linear fit of the remaining mRNA over time. For this representation, statistics were done using unpaired T-test to compare the slope values between vehicle and SB treated cells.

There were significantly lower levels of *COX-2* mRNA in SB-treated than control cells at 60, 90 and 120 minute time points (Figure 3.6b). High levels of significance were reached when comparing *COX-2* mRNA quantities at each time point but also when comparing the slopes of the fitted curves. Therefore p38 MAPK stabilises *COX-2* mRNA in mouse macrophages as in many other cell types (Dean, Brook et al. 1999; Jang, Sanchez et al. 2000; Lasa, Mahtani et al. 2000; Faour, He et al. 2001). This result also confirm the observations made by C. Tudor, who carried out the same type of experiment in BMDMs (Tudor, Marchese et al. 2009).

Inhibition of p38 MAPK also appeared to destabilise *TNF* mRNA (Figure 3.6c), although noticeable, the difference between vehicle- and SB-treated did not reach statistical significance, in spite of a large number of repeats of the experiment (n = 7). This was surprising, as in a similar study in the same cell type, *TNF* mRNA was very strongly destabilised in the presence of SB (Tudor, Marchese et al. 2009).

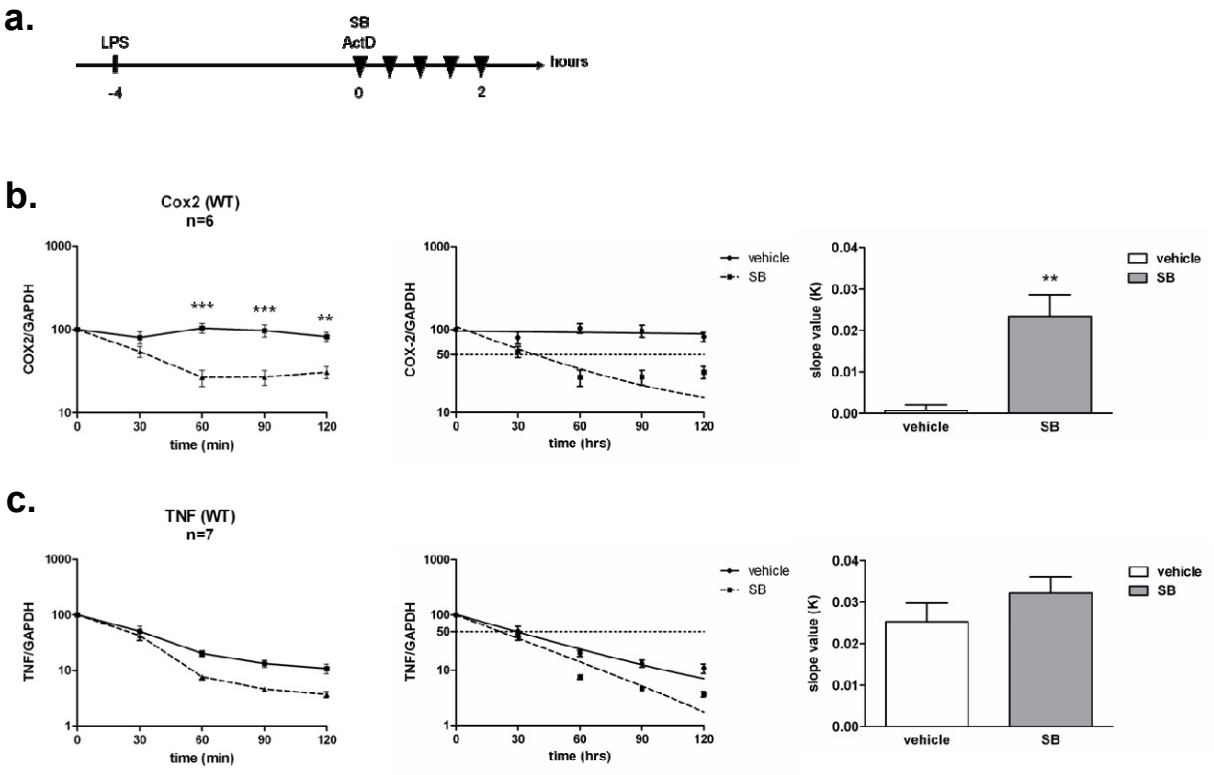


Figure 3.6. ***Effects of SB 202190 on COX-2 and TNF mRNA stability.***

(a) *Primary macrophages form Dusp1$^{+/+}$ (WT) mice were treated with LPS (10 ng) for 4 h then actinomycin D (ActD, 10 µg/ml) and SB 202190 (1 µM) or vehicle (DMSO) were added. mRNA was then harvested every 30 min and* ***(b)*** *COX-2 and* ***(c)*** *TNF mRNA were quantified by real-time PCR.*

In the left graphs mRNA quantity is normalised against the house keeping gene GAPDH and expressed as a remaining percentage of time 0. Statistical analysis was performed using analysis of variance.

Middle graphs represent the same results, fitted as one phase exponential decay curves. Time 0 of addition of ActD is considered as 100% and decay is represented as a percentage of remaining mRNA over time. 50% is indicated by the horizontal dotted line.

The graphs on the right hand side show the averages ± SEM of the slopes values (K) of the curves for each treatment. Statistical analysis was performed using unpaired T-test.

*For each time point and treatment, graphs indicate mean ± SEM (error bars) from n independent experiments. Significance was taken as follows: **, p<0.01 and ***, p<0.001.*

3.5.2. Effect of dex on COX-2, IL-1α and TNFα mRNA stability

Once I had established the actinomycin D chase procedure using RT-PCR to quantify mRNA, and reproduce previous observations on the regulation of COX-2 mRNA stability by the p38 MAPK pathway, the next step was to investigate the post-transcriptional effects of dex and the involvement of DUSP1 in those effects.

For this purpose, macrophages derived from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice were stimulated with LPS for 3h then treated with 100nM of dex or vehicle an hour prior to the addition of actinomycin D. As for the SB experiment, actinomycin D was used to stop transcription and to investigate the effects of dex on mRNA stability. Cells were harvested every 30 minutes for 2h (Figure 3.7a) and COX-2 (Figure 3.7), IL-1α (Figure 3.8) and TNF (Figure 3.9) mRNA were quantified and analysed as described previously. Furthermore, the relative levels of mRNA at time 0 were determined for each gene in both *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells that had been treated for the preceding hour with either vehicle or dex (graphs f and g). In this experimental design the delay between addition of dex and addition of actinomycin D was necessary because it was hypothesised that dex decreases mRNA stability via an increase in DUSP1 gene expression. The results are also shown as one phase exponential decay curves: a non-linear fit of the remaining mRNA over time (graphs d and e). For this representation, statistics were done using unpaired T-test to compare the slope values between vehicle and dex treated cells.

COX-2 mRNA appeared to be destablised by dex, although the difference in abundance was significant at only the 60 minute time point (Figure 3.7b). In *Dusp1*$^{-/-}$ cells there was no apparent effect of dex on COX-2 mRNA stability (Figure 3.7c). Indeed, although there were less repeats of the experiment in the BMDMs from *Dusp1*$^{-/-}$ mice, each of the n=3 experiment was carried out independently, there was little experimental variation and no statistical difference between the dex- and vehicle-treated groups. As shown in graphs f. and g., addition of dex three hours after the LPS stimulus caused a strongly significant ($p < 0.001$) decrease in COX-2 mRNA levels over the following hour in *Dusp1*$^{+/+}$ but not in *Dusp1*$^{-/-}$ cells. However, the analysis of decay slope (Figure 3.7d and e) did not show any statistical difference between dex- and vehicle-treated cells (slope comparison in WT: $p = 0.55$).

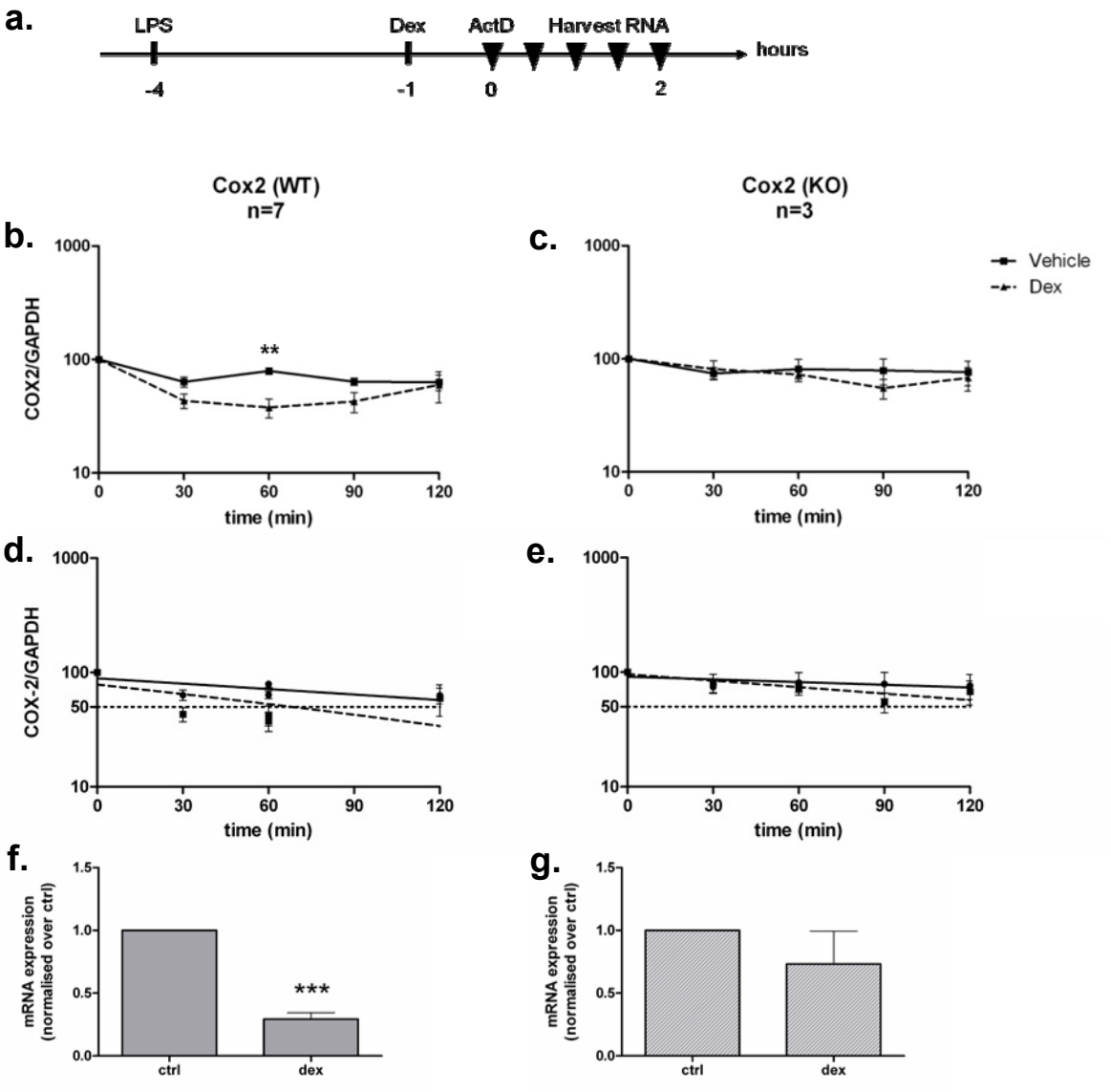


Figure 3.7. ***Effects of dex on COX-2 mRNA stability in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mouse macrophages.***
(a) *Primary macrophages form Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice were treated with LPS (10 ng/ml) for 3 h then dex (100 nM) or vehicle (dry ethanol) and 1 h later actinomycin D (ActD, 10µg/ml) was added. mRNA was then harvested every 30 min and COX-2 mRNA from* ***(b)*** *Dusp1$^{+/+}$ (WT, left hand side) or* ***(c)*** *Dusp1$^{-/-}$ (KO, right hand side) cells was quantified by real-time PCR. mRNA quantity was normalised against the house keeping gene GAPDH and expressed as a percentage of the quantity at time 0. Statistical analysis was performed using analysis of variance (upper graphs).*
Graphs ***(d)*** *and* ***(e)*** *represent the same results but fitted as one phase exponential decay curves. Time 0 of addition of ActD is considered as 100% and decay is represented as a percentage of remaining mRNA over time. 50% is indicated by the horizontal dotted line.*
Graphs ***(f)*** *and* ***(g)*** *represent the mRNA quantity present at time 0 under control (ctrl) and +Dex conditions. Data are presented as a ratio of control values.*
*For each time point, graphs indicate mean ± SEM (error bars) from 0 n=7 (WT) and n=3 (KO) independent experiments. Significance was taken as follows: **, p<0.01 and ***, p<0.001.*

Similarly, IL-1α mRNA appeared to be destabilised by dex, but with a statistically significant effect at only the 60 minute time point (Figure 3.8b). Again, dex had no effect in *Dusp1*$^{-/-}$ cells (Figure 3.8c). Treatment with dex for an hour prior to the start of the actinomycin D chase significantly decreased IL-1α mRNA abundance in *Dusp1*$^{+/+}$ ($p < 0.001$) but not *Dusp1*$^{-/-}$ cells (graphs f and g). As for COX-2, the statistical significance seen at 1 h between vehicle and dex treated cells from *Dusp1*$^{+/+}$ was lost when the results were fitted as a decay curve ($p = 0.19$, Figure 3.8d).

In contrast TNF mRNA was unstable and unaffected by dex in both *Dusp1*$^{+/+}$ (Figure 3.9b and d) and *Dusp1*$^{-/-}$ (Figure 3.9c and e) cells, and treatment with dex for the last hour of the LPS stimulation significantly decreased mRNA levels in both *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells (see panels f and g).

So, in cells from wild type mice, dex seems to have a destabilising effect on COX-2 and IL-1α mRNAs, with the difference to vehicle-treated cells reaching significance at 1 h after the addition of actinomycin D. This effect was not seen any more in the absence of *Dusp1*, hinting at a role of the phosphatase in the regulation of COX-2 and IL-1α mRNA stability by dex. When the results were fitted as mRNA decay curves, a stronger mRNA decline could still be observed after treatment with dex, but no more significance could be found. However, an equivalent decline did not occur in the *Dusp1*$^{-/-}$ cells. Moreover, it is worth noting that in the wild type, but not the *Dusp1* deficient cells, the COX-2 and IL-1α mRNAs levels were significantly reduced after 1 h of treatment with dex (panels f and g, figures 3.7 and 3.8). These observations were consistent with a dex-induced, DUSP1-mediated destabilisation of COX-2 and IL-1α mRNAs but were insufficient to convincingly demonstrate it and were also underlining the critical importance of timing in such experiments. I therefore decided to go back to the experimental design and modify various parameters to determine whether putative post-transcriptional effects of dex could be “captured”.

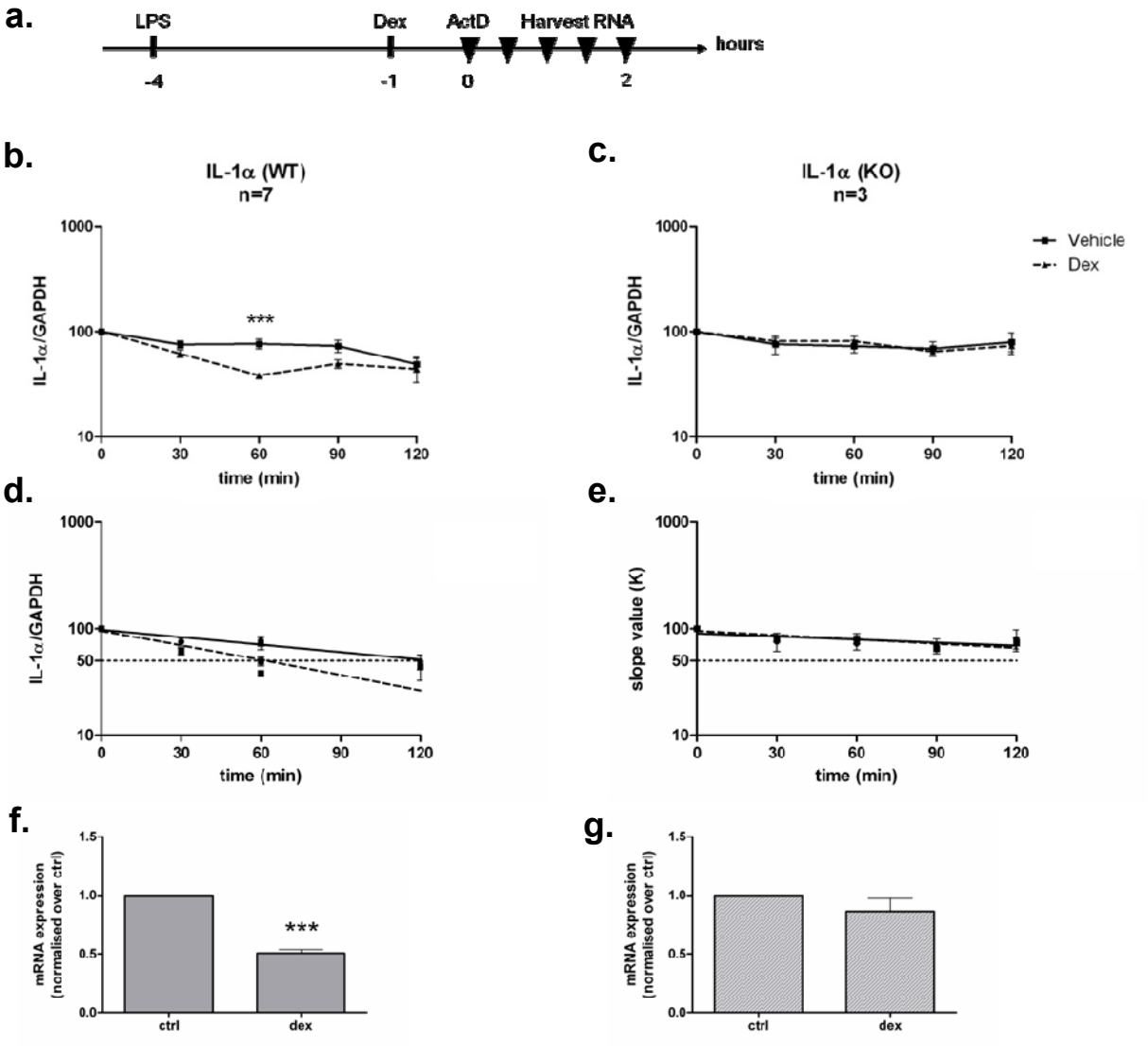


Figure 3.8. ***Effects of dex on IL-1α mRNA stability in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mouse macrophages.***

(a) *Primary macrophages form Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice were treated with LPS (10 ng/ml) for 3 h then dex (100 nM) or vehicle (dry ethanol) and 1 h later actinomycin D (ActD, 10µg/ml) was added. mRNA was then harvested every 30 min and IL-1α mRNA from* ***(b)*** *Dusp1$^{+/+}$ (WT, left hand side) or* ***(c)*** *Dusp1$^{-/-}$ (KO, right hand side) cells was quantified by real-time PCR. mRNA quantity was normalised against the house keeping gene GAPDH and expressed as a percentage of the quantity at time 0. Statistical analysis was performed using analysis of variance (upper graphs).*

Graphs ***(d)*** *and* ***(e)*** *represent the same results but fitted as one phase exponential decay curves. Time 0 of addition of ActD is considered as 100% and decay is represented as a percentage of remaining mRNA over time. 50% is indicated by the horizontal dotted line.*

Graphs ***(f)*** *and* ***(g)*** *represent the mRNA quantity present at time 0 under control (ctrl) and +Dex conditions. Data are presented as a ratio of control values.*

*For each time point, graphs indicate mean ± SEM (error bars) from n=7 (WT) and n=3 (KO) independent experiments. Significance was taken as ***, p<0.001.*

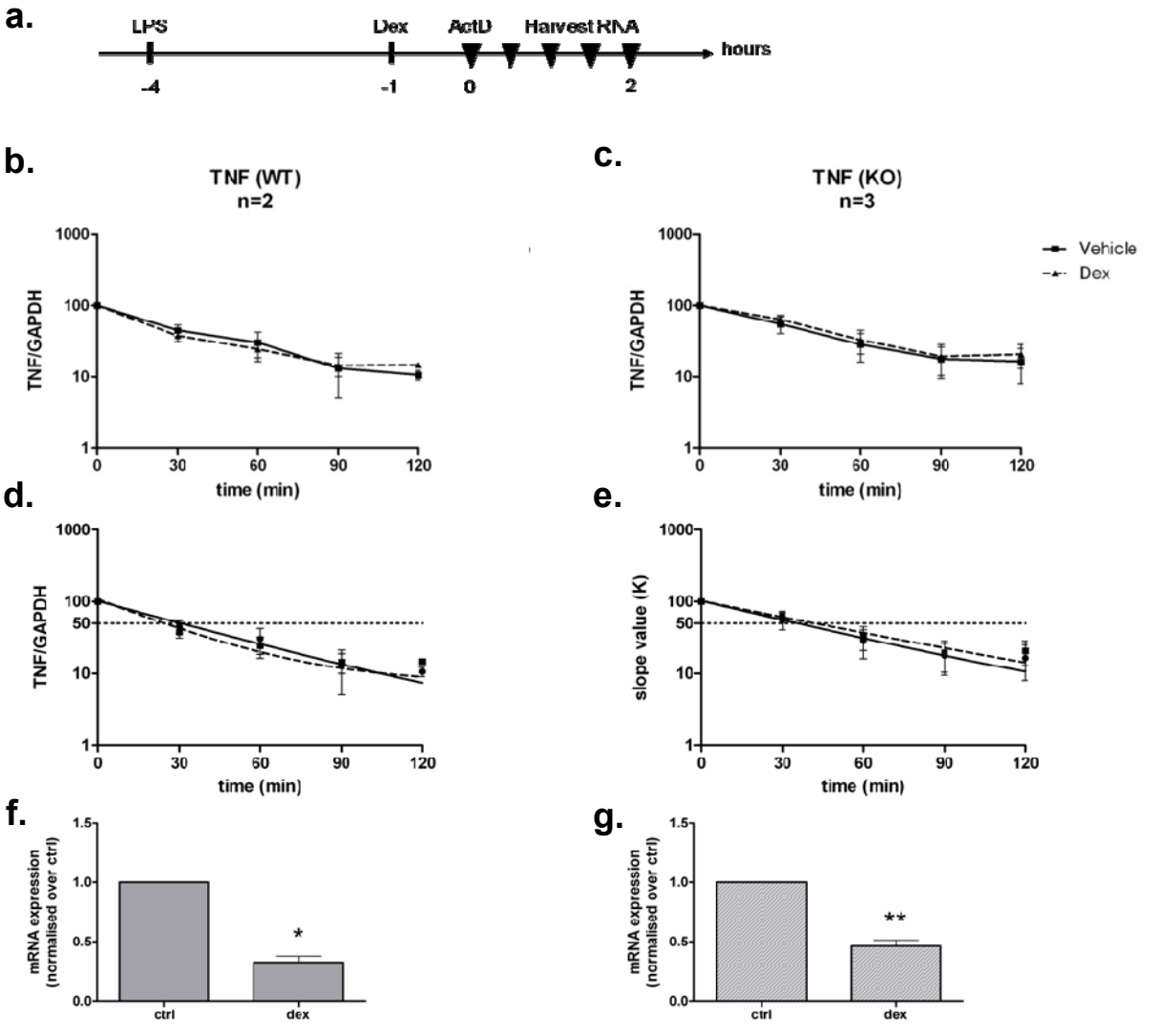


Figure 3.9. ***Effects of dex on TNF mRNA stability in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mouse macrophages.***

(a) *Primary macrophages form Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice were treated with LPS (10 ng/ml) for 3 h then dex (100 nM) or vehicle (dry ethanol) and 1 h later actinomycin D (ActD, 10µg/ml) was added. mRNA was then harvested every 30 min and TNF mRNA from* ***(b)*** *Dusp1$^{+/+}$ (WT, left hand side) or* ***(c)*** *Dusp1$^{-/-}$ (KO, right hand side) cells was quantified by real-time PCR. mRNA quantity was normalised against the house keeping gene GAPDH and expressed as a percentage of the quantity at time 0. Statistical analysis was performed using analysis of variance (upper graphs).*

Graphs ***(d)*** *and* ***(e)*** *represent the same results but fitted as one phase exponential decay curves. Time 0 of addition of ActD is considered as 100% and decay is represented as a percentage of remaining mRNA over time. 50% is indicated by the horizontal dotted line.*

Graphs ***(f)*** *and* ***(g)*** *represent the mRNA quantity present at time 0 under control (ctrl) and +Dex conditions. Data are presented as a ratio of control values.*

*For each time point, graphs indicate mean ± SEM (error bars) from n=2 (WT) and n=3 (KO) independent experiments. Significance was taken as follows: *, p<0.05 and **, p<0.01.*

3.5.3. Optimisation of the actinomycin D chase experiments

In line with the idea of optimising the actinomycin D chase experiments, I used two approaches. The first one was to keep the interval between dex and actinomycin D addition constant and to vary the time of stimulation with LPS; from 2 h to 4 or 8 h. The second approach was to vary the time the cells were left in contact with dex, prior to the addition of actinomycin D. As previously mentioned, because it was hypothesised that the post-transcriptional effects of dex on COX-2 and IL-1α required the up-regulation of the *Dusp1* gene, it was important to leave some time between the addition of dex and of actinomycin D. The first round of experiments (figures 3.7 to 3.9) was performed treating the cells with dex for 1 h. Here I tried leaving the dex on for 30 min. To make the investigation of these parameters possible, a single time point was collected 1 h after the addition of actinomycin D, corresponding to the time point at which dex appeared to have its greatest effect.

In the first attempt, to assess the effect of the sole variation of the time of LPS stimulation, dex did not consistently affect COX-2 or IL-1α stability nor was any DUSP1 dependency demonstrated (not shown).

Next, I shortened the interval between dex and actinomycin D addition, from 1 h to 30 min, varying again the time of LPS treatment (Figure 3.10a). COX-2 and IL-1α mRNAs were quantified as previously described. The results (Figure 3.10b) are presented as the percentage of mRNA degradation against the time course of LPS stimulation. Results around or below 0 suggest that no destabilisation occurred.

In the *Dusp1*$^{+/+}$ cells, dex appeared to promote the degradation of COX-2 mRNA over 1 h following the addition of actinomycin D, with the biggest increase in mRNA degradation being observed at 4 h of LPS. These effects were not apparent in the *Dusp1*$^{-/-}$ cells, suggesting a role for DUSP1 in a dex-induced destabilising effect on COX-2 mRNA. In the same experiment, IL-1α mRNA appeared to be more stable than COX-2 mRNA, with no clear differences between the levels of mRNA degradation in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$. However, experimental variation was such that no firm conclusion could be drawn from this experiment.

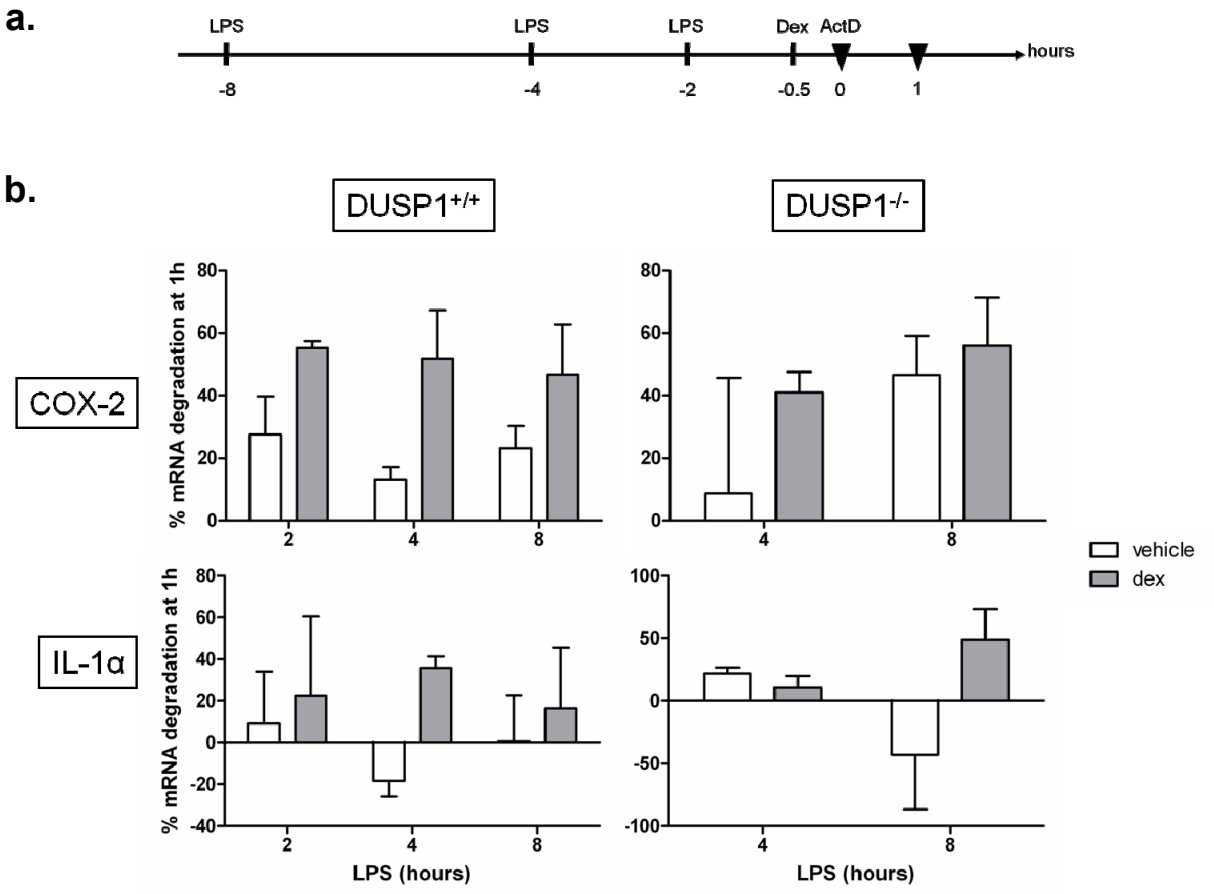


Figure 3.10. ***Effects of an LPS time course and 30 min of dex on COX-2 and IL-1α mRNA stability.*** ***(a)*** *Experimental design. Dusp1$^{+/+}$ and Dusp1$^{-/-}$ BMDMs were stimulated for 2, 4 or 8 h with 10 ng/ml of LPS and 100 nM of dex (or vehicle, dry ethanol) for 30 min prior to the addition of 10µg/ml of ActD. mRNA was harvested at time 0 and after 1 h of the ActD addition.* ***(b)*** *COX-2 and IL-1α mRNAs were quantified using real-time PCR and expressed as a percentage of mRNA degradation (time 0 being considered as 100% of mRNA quantity). Negative values indicate a possible stabilisation of mRNA expression. Note that no values are shown for 2 h of LPS treatment in the Dusp1$^{-/-}$ macrophages, as a technical problem led to their loss. For each time point, graphs indicate mean ± SEM (error bars) from two independent experiments. Statistical analysis was performed using analysis of variance, with all p values being >0.05 (non-significant).*

At a later date, as part of a collaboration with Dr Alaina Ammit, a third variation on the protocol was attempted. *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages were pre-treated with vehicle or 100 nM of dex for 2 h, then with 10 ng/ml of LPS for a further 4 h prior to the addition of actinomycin D. mRNA was collected over a 2 h period (Figure 3.11a). Although S. Abraham stated in her thesis that dex was similarly regulating DUSP1 and COX-2 expression whether it was added before or after LPS, it was another parameter that we could try to vary in the design of the actinomycin D chase experiments.

COX-2 (Figure 3.11b) and TNF (Figure 3.11c) mRNAs were quantified as previously described. For each time point, an analysis of variance was performed to compare the vehicle versus the dex treated cells. With all the p values being over 0.05, no statistical significance was observed. COX-2 mRNA did not appear to be destabilised by dex in the wild type cells, nor in the *Dusp1* deficient BMDMs whereas TNF mRNA appeared to be destabilised in both cell types, regardless of the treatment. However, both mRNAs appeared to be more stable in the *Dusp1*$^{-/-}$ than in the *Dusp1*$^{+/+}$ macrophages.

So, varying the time of LPS challenge and the interval of treatment with dex did not bring any further indications regarding the effects of dex on mRNA stability and the involvement of DUSP1 in these effects, compared to the original experiments. Therefore, no definitive conclusion could be drawn, other than the hint at a dex-induced, DUSP1-mediated destabilisation of COX-2 and IL-1α mRNAs previously described.

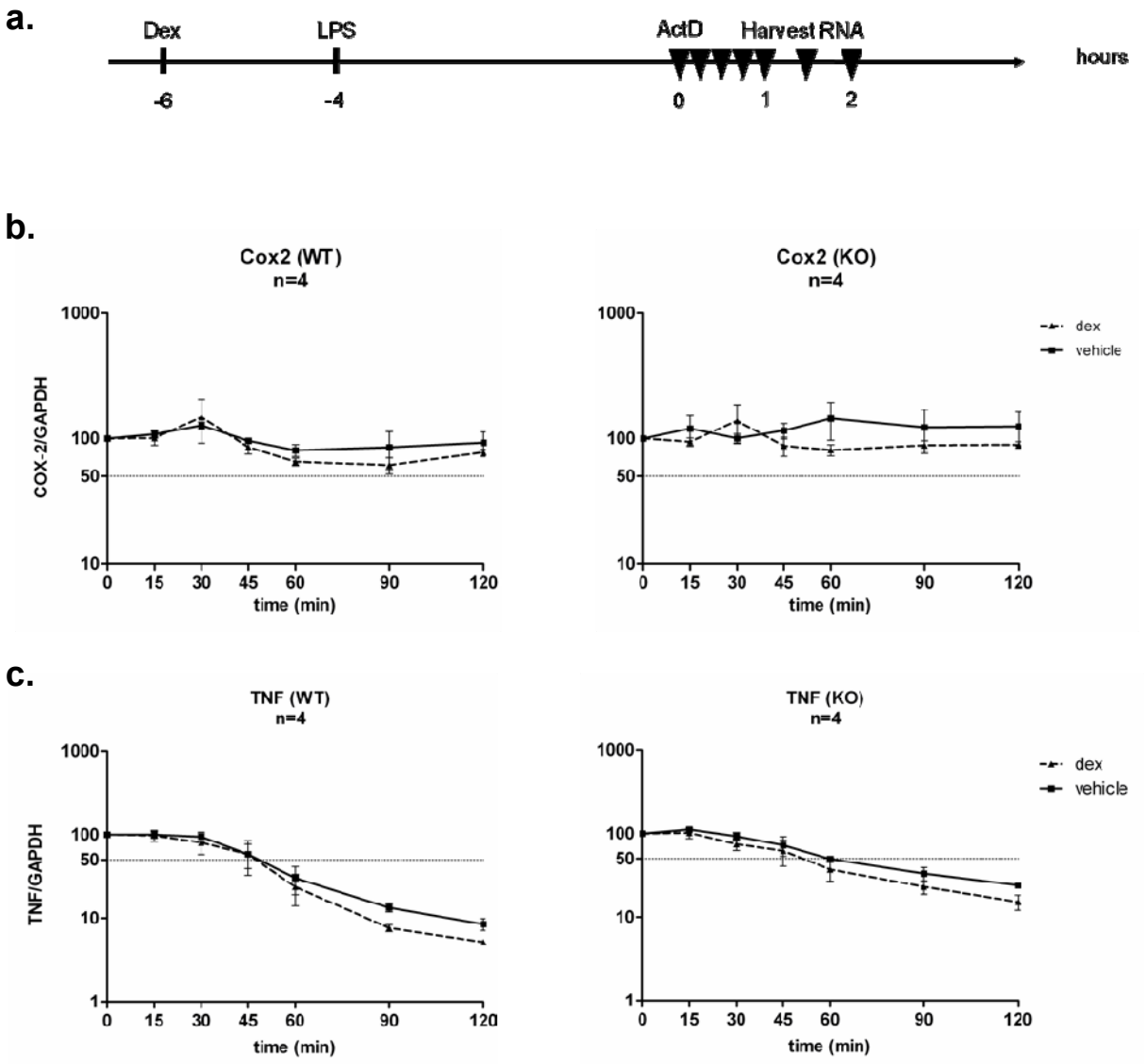


Figure 3.11. ***Effects of the addition of dex prior to LPS on COX-2 and TNF mRNA stability.***
(a) *Experimental design. Dusp1$^{+/+}$ (WT) and Dusp1$^{-/-}$ (KO) BMDMs were stimulated for 2 h with 100 nM of dex (or vehicle, DMSO) then 10 ng/ml of LPS were added, at 4 h prior to the addition of ActD (10 µg/ml) (time 0). mRNA was harvested every 15 min during the first hour of ActD and every 30 min for the second hour.* ***(b)*** *COX-2 and* ***(c)*** *TNF mRNAs were quantified using real-time PCR and expressed as a percentage of remaining mRNA compared to time 0 (being considered as 100%). For each time point, graphs indicate mean ± SEM (error bars) from 4 independent experiments. Statistical analysis was performed using analysis of variance, with all p values being >0.05 (non-significant). Experiments were performed by Dr A. Ammit.*

3.6. Discussion

The aim of this chapter, and more generally, of this project, was to confirm and expand previous results of DUSP1 expression regulation in response to GCs, as well as to develop our knowledge of the role of the phosphatase in the regulation of inflammatory genes. Here, I was particularly interested in determining the role of DUSP1 in the post-transcriptional regulation of pro-inflammatory gene expression.

The first challenge I came across was to find a commercially available antibody which would specifically and consistently detect DUSP1 protein, in most of the cell types used for this project. Despite extensive testing in various conditions, only one particular batch of a Santa Cruz anti-DUSP1 antibody was able to give reproducible results. This antibody was used in most of the experiments presented in this report. However, stock shortage forced us to resume antibody testing. Apart from identifying a non specific antibody, the search was not fruitful and, to date is still ongoing.

To 'warm up' to the project and validate my experimental models, I went on to try to reproduce some of the results previously obtained in the lab. I successfully performed experiments which were in agreement with what had already been observed by R. Perelli (unpublished observations) and Dr. S. Abraham (Abraham, Lawrence et al. 2006). In RAW cells and primary macrophages, a time course showed that not only was DUSP1 expression transiently induced in response to LPS or LPS + dex, but also the combination of pro-inflammatory stimuli and GCs resulted in an increased and, most importantly, prolonged DUSP1 expression, compared to either treatment on their own. Here, more than just a stronger expression of DUSP1, cooperation of LPS and dex leaded to prolongation of gene expression. In presence of both treatments, the shape of the entire time course was modified, compared to what was seen in response to LPS alone. Sustained and prolonged DUSP1 expression, especially at later time points is in agreement with the concept of DUSP1 being part of the anti-inflammatory action of GCs.

Next, what I was interested in was to prove that in my BMDM model, dex was able to induce DUSP1 protein expression and to confirm that COX-2 expression was dependent on DUSP1.

That was successfully demonstrated using *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMDMs, in which DUSP1 was dose dependently induced by increasing concentrations of dex in wild type cells, whilst COX-2 expression was dose dependently inhibited. COX-2 expression regulation in *Dusp1*$^{-/-}$ macrophages clearly confirmed that control of this pro-inflammatory mediator expression by dex was dependent on DUSP1, in agreement with what we reported previously (Abraham, Lawrence et al. 2006). I also wanted to look at iNOS protein expression. There again, it was in agreement with previous work from our group, suggesting some involvement of DUSP1 in the regulation of iNOS expression: strong dose dependent inhibition in *Dusp1*$^{+/+}$, a little less in *Dusp1*$^{-/-}$. Note that these results were also in agreement with findings from another group, who reported, using *Dusp1* siRNA and *Dusp1*$^{-/-}$ mice, that DUSP1 suppressed iNOS expression by limiting p38 MAPK activity (Turpeinen 2011). The same group also showed that COX-2 expression was also regulated by DUSP1, alongside the expression of IL-6 and IL-8 (Turpeinen, Nieminen et al. 2010). At this stage, it would also by interesting to know if regulation of other pro-inflammatory mediators by GCs was dependent on DUSP1. Results detailed in chapter 5 gave some insights on several of them. Indeed, in BMDMs from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice, IL-6 and TNF protein expression were shown to be strongly dependent on DUSP1, whilst Cox-2 mRNA data confirmed the involvement of the phosphatase in the regulation of the cyclooxygenase expression. In the same experiemental set up it was also determined that DUSP1 was involved, to some extend, in the regulation of KC, IL-10 or IL-1α by dex. It would now be particularly relevant to determine the behaviour of these inflammatory mediators *in vivo* in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ animals and in disease models, in response to glucocorticoids such as dex.

Glucocorticoids have long been known to destabilise several pro-inflammatory mRNAs (Stellato 2004; Newton and Holden 2007; Clark, Martins et al. 2008), but the basis of this effect is not clear. Several reports suggested that glucocorticoids could up-regulate TTP, and that this contributed to destabilisation of pro-inflammatory mRNAs (Smoak and Cidlowski 2006; Ishmael, Fang et al. 2008). Previous studies in our lab showed that dex could increase TTP mRNA by approximately three-fold in A549 and HeLa cells, but the protein could not be detected (Dr. R. Crawford, unpublished observations). In mouse macrophages stimulated with LPS, I found that dex actually decreased rather than increased the expression of TTP protein and mRNA. The same effect was reported in BMDMs by Dr Ammit, where dex was

inhibiting LPS-induced TTP protein and mRNA expression in BMDMs from wild type animals (unpublished data). These results are consistent with a previous report, describing inhibition of LPS-induced TTP expression by dex in a macrophage-like cell line (Jalonen, Lahti et al. 2005). It therefore seems unlikely that up-regulation of TTP is an anti-inflammatory mechanism of glucocorticoids in myeloid cells.

To provide a conclusive answer whether TTP contributes to the destabilisation of pro-inflammatory mRNAs by glucocorticoids, an obvious experiment is to test effects of dex on mRNA stability in $TTP^{+/+}$ and $TTP^{-/-}$ macrophages. However, the outcome of such an experiment might be difficult to interpret. The p38 MAPK pathway stabilises various pro-inflammatory mRNAs by promoting the phosphorylation and inactivation of TTP (Stoecklin, Stubbs et al. 2004; Stoecklin, Tenenbaum et al. 2008). In $TTP^{-/-}$ mouse macrophages, such mRNAs are unusually stable and can no longer be destabilised by inhibiting p38 MAPK (Tudor, Marchese et al. 2009). I hypothesise that dex destabilises mRNA by inducing the expression of DUSP1, inhibiting p38 MAPK and reactivating TTP. In this scenario TTP is an essential downstream target of the glucocorticoid-DUSP1-p38 pathway. Therefore $TTP^{-/-}$ macrophages might be insensitive to glucocorticoids regardless of whether glucocorticoids cause any increase in TTP expression.

In fact dex appeared to decrease TTP expression in a manner that was at least partly dependent on DUSP1. As well as causing inactivation of TTP, the p38 MAPK pathway stabilises both TTP mRNA (Tchen, Brook et al. 2004) and protein (Brook, Tchen et al. 2006; Hitti, Iakovleva et al. 2006; Janka-Junttila, Moilanen et al. 2006). Because of this complex interaction, maximum activation of the p38 MAPK pathway is thought to coincide with maximum levels of TTP protein but weakest mRNA destabilising function. Dex might cause destabilisation of pro-inflammatory mRNAs by up-regulating DUSP1, inhibiting p38 MAPK and increasing the activity of TTP (whilst at the same time decreasing the levels of TTP protein). This is consistent with the pattern of TTP expression shown in Figure 3.4. However, it is very difficult to investigate this hypothesis until it is possible to precisely determine the phosphorylation status of endogenous TTP protein.

In this section, what I really focused on was the role of DUSP1 in post-transcriptional regulation of inflammatory mediators by glucocorticoids. First, I confirmed that the glucocorticoid-mediated suppression of COX-2 in mouse macrophages was strongly dependent on DUSP1 (Abraham, Lawrence et al. 2006). Because the generation of mouse macrophages was time-consuming and numbers of available mice were limited, I tried to use an alternative cell type, namely immortalised MEFs from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice. Unfortunately there was no convincing evidence for a role of DUSP1 in the anti-inflammatory action of dex in these cells. This may reflect the fact that dex has relatively weak effects on the expression of DUSP1 in these cells. Dex up-regulated DUSP1 mRNA by only about six-fold in MEFs, compared to 25-fold or more in human and mouse macrophages, human skin fibroblasts or A549 cells (R. Perelli, unpublished observations). The cooperative regulation of DUSP1 protein expression by dex and IL-1β (Figure 3.5) was also weak in MEFs compared to the cooperative regulation by dex and LPS in mouse macrophages (Figure 3.3). Previous experiments found little effect of dex on p38 MAPK signalling in MEFs (Dr. M. Lasa, unpublished observations). Possibly these cells have some defect in dex-induced gene expression, and are not suitable tools for investigating the role of DUSP1.

Effects of dex on mRNA stability were then investigated in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages. Dex seemed to destabilise COX-2 and IL-1α mRNA in a DUSP1-dependent manner (Figure 3.7 and Figure 3.8), but disappointingly the effect was statistically significant at only one time point. At the time I thought that more convincing post-transcriptional effects of dex might be shown by collecting more time points between 0 and 1 h, for example at 15 minute intervals. This was attempted by Dr. A. Ammit and is shown in Figure 3.11 with no more success. It is worth noting however that in this experimental design, dex was added prior to LPS. Obviously in experiments of this kind it is necessary to leave an interval between the addition of dex and the addition of actinomycin D otherwise dex-induced changes in DUSP1 expression cannot occur. Here the expression of both COX-2 and IL-1α was decreased in a DUSP1-dependent manner within the first hour after the addition of dex. This experimental design may underestimate effects of dex on mRNA stability if significant decay has already occurred before the start of the actinomycin D chase. I attempted varying the interval between the addition of dex and the addition of actinomycin

D, but have not found an optimal experimental design in which consistent effects of dex on mRNA stability can be demonstrated (Figure 3.10). At the time these lines were written, the effects of altering the design of this experiment were still being investigated in our group. It would also be interesting to understand what happens to the expression of DUSP1 under the conditions tested in this part of the report. The rapid DUSP1-dependent decrease in COX-2 and IL-1α mRNA levels in response to dex could perhaps be investigated in an indirect manner. For example if actinomcyin D does not decrease COX-2 mRNA over the same interval, then COX-2 transcription has presumably ceased by this time and the dex-induced decrease in COX-2 mRNA levels must be caused by destabilisation. Finally, it may be worthwhile investigating post-transcriptional regulation of other transcripts that are targets of the p38 MAPK pathway, for example IL-10 and KC.

Chapter 4

DISSOCIATION PROPERTIES AND EFFECTS OF SGRMS ON DUSP1 EXPRESSION

4. DISSOCIATION PROPERTIES AND EFFECTS OF SGRMS ON DUSP1 EXPRESSION

4.1. Introduction

As previously stated, it has been suggested in many reports that side effects of glucocorticoids were largely mediated by transactivation of gene expression, whilst anti-inflammatory effects were largely mediated by transrepression. On this basis, novel glucocorticoid receptor ligands that selectively induce transrepression rather than transactivation should have better safety profiles than classical glucocorticoids. A number of selective glucocorticoid receptor modulators (SGRMs) have been described in chapter 1. However, the degree of dissociation between transrepression and transactivation achieved by these SGRMs was usually imperfect and although they were identified on the basis of impaired transcriptional activation, SGRMs may be quite capable of inducing expression of DUSP1 and other genes with anti-inflammatory roles (Chivers, Gong et al. 2006; Janka-Junttila, Moilanen et al. 2006; Lopez, Ardecky et al. 2008; Newton, King et al. 2010). Therefore, it remains possible that some of the anti-inflammatory effects of these synthetic compounds are mediated by activation of gene expression, in particular DUSP1.

To address this hypothesis, three synthetic compounds (Cpd) were used in this study – CpdA, Cpd1 and Cpd2 – and compared to the classic GR ligand dex.

Commercially available, CpdA, or 2-(4-acetoxyphenyl)-2-chloro-N-methylethylammonium chloride is a stable analogue of the hydroxyl phenyl aziridine precursor of plant origin (Louw and Swart 1999). CpdA is a non-steroidal GR ligand with a binding affinity primarily described as similar to that of dex. In the murine fibrosarcoma L929sA cells, CpdA was indeed shown to have a slightly greater binding affinity, with an IC_{50} about 4-fold lower than that of dex (6.4 nM versus 25.9 nM) (De Bosscher, Berghe et al. 2005). More recently however, Robertson and colleagues showed, in murine hepatoma cells, that CpdA was binding GR with a significantly lower affinity (Kd = 81.8 nm) than Dex (Kd = 1.29 nm) (Robertson, Allie-Reid et al. 2010). It is claimed that CpdA is totally dissociated *in vitro*, with

no transactivation on GRE-driven gene expression but with transrepression of an IL-6-driven luciferase reporter (p(IL6B)350hu.IL6P-luc, L929sA stably transfected cells), as well as inhibition of TNF-induced IL-6 protein expression (De Bosscher, Berghe et al. 2005). *In vivo*, CpdA was shown to have anti-inflammatory effects on a zymosan-induced inflamed paw mouse model, as well as an improved effect/side effects profile (no hyperglycemia) (De Bosscher, Berghe et al. 2005). Other studies demonstrated the beneficial use of CpdA in murine collagen-induced arthritis (Dewint, Gossye et al. 2008), in disease progression in an animal model of multiple sclerosis (van Loo, Sze et al. 2010) as well as on inflammation in osteoblasts, without unfavourably altering the RANKL/OPG ratio (Rauch, Gossye et al. 2011). Wüst and colleagues also demonstrated that low to intermediate amounts of CpdA dissolved in water significantly ameliorated the status of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) . However the same group also showed that administration of high-dose CpdA to mice was lethal and that in buffered solutions CpdA was decomposing into toxic, pro-apoptotic, aziridine intermediates, questioning its stability and the possible use of a compound with such a narrow therapeutic window *in vivo* (Wüst, Tischner et al. 2009).

Cpd1 and Cpd2 were selected from patents registered by two companies that have led research into SGRMs and were obtained via a collaboration with Roche. Cpd1 (Jaroch 2002), originally referred to as RO4553059, is closely related to ZK 216348 (Schacke, Schottelius et al. 2004) and ZK 245186 (Schäcke, Zollner et al. 2009) (Figure 4.1). In THP-1 cells stimulated with lipopolysaccharide (LPS), Cpd1 inhibited the expression of IL-8 with 77% efficacy and IC_{50} of $4.3x10^{-9}$ M. For comparison, prednisolone (pred) inhibited expression of IL-8 with 95% efficacy and IC_{50} of $2.4x10^{-10}$ M (Roche, unpublished observations). In the croton oil ear oedema model, pred and Cpd1 were similarly effective at a dose of 30 mg/kg, inhibiting inflammation by 81 and 84% (Roche, unpublished observations). In the same model, pred induced liver tyrosine aminotransferase (a surrogate marker of metabolic side effects) by 8-fold, Cpd1 by 3.7-fold (Jaroch 2002). Cpd2 (Kym, Kort et al. 2003; Coghlan, Jacobson et al. 2003), originally referred to as RO4638375, is very closely related to LGD-5552 (Lopez, Ardecky et al. 2008) and AL-438 (Coghlan, Jacobson et al. 2003) (Figure 4.1). It is highly selective for GR over progesterone receptor (PR), the respective Ki values being 1.5 nM and 1434 nM (Roche, unpublished observations).

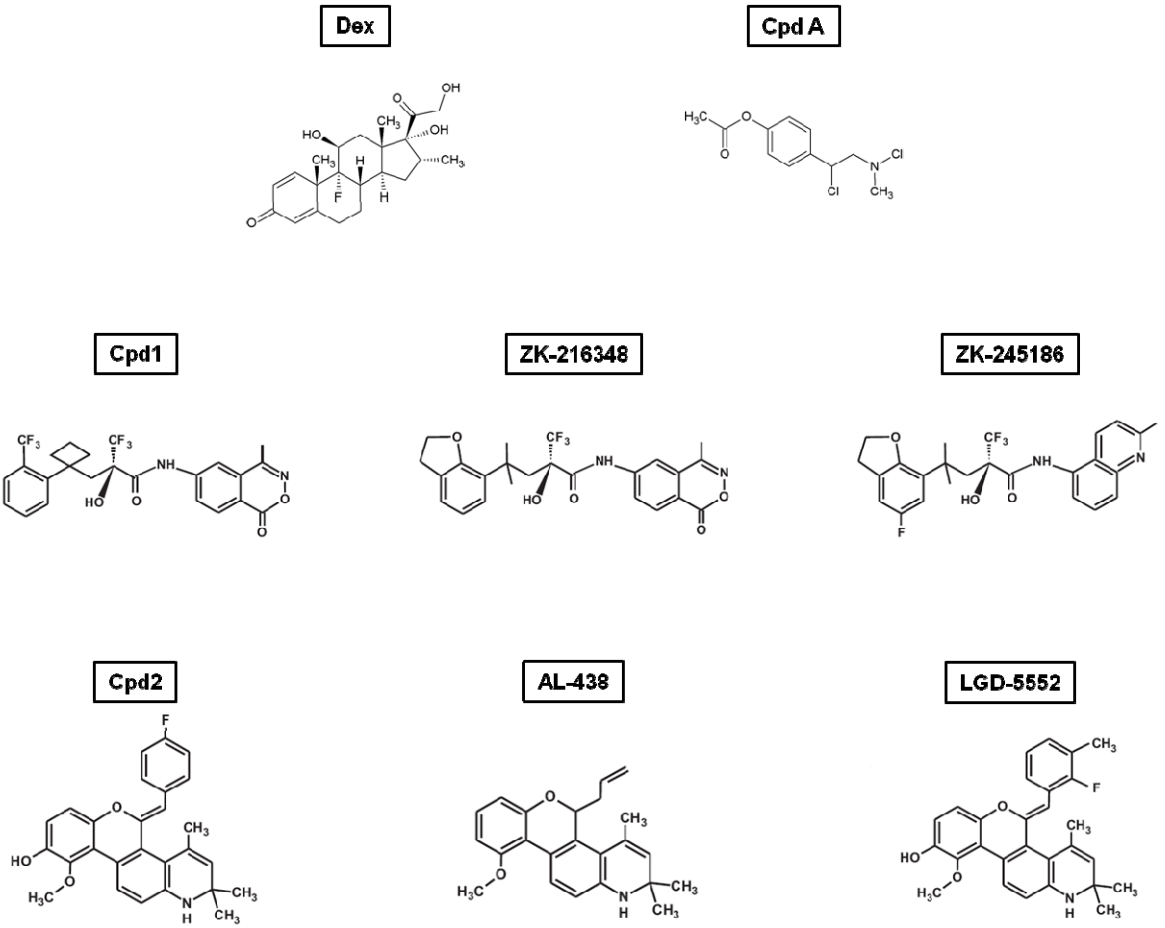


Figure 4.1. Molecular structures of dex, CpdA, Cpd1, Cpd2 and their near relatives.

The aim of this chapter was to determine the dissociation profiles of these three compounds, as well as their ability to up-regulate DUSP1 expression, compared to those of dex. In the next chapter, I investigated whether the anti-inflammatory effects of such compounds – if any – were dependent on the up-regulation of DUSP1.

4.2. Dissociation properties

The ability of dex, Cpd1 and Cpd2 to transactivate or transrepress gene expression was tested using the human-derived A549 pulmonary epithelial cell line, widely used in the study of inflammatory diseases of the lung such as asthma. To assess the transrepression activity of the different compounds, the cells were transfected with constructs consisting of three or six NF-κB binding sites upstream of a minimal promoter and a luciferase coding sequence. Constructs containing two GREs were used to test the ability of dex, Cpd1, Cpd2 and CpdA to transactivate gene expression. The data obtained in these assays were used to calculate a dissociation index for those compounds.

4.2.1. Transrepression

4.2.1.1. Stably transfected cells

To test the transrepression activity of dex, Cpd1 and Cpd2, I first used A549 cells stably transfected with a construct containing three (3κBtkluc) or six (6κBtkluc) κB sites, from of a COX-2 promoter, for the former, or HIV, for the latter. NF-κB motifs are linked to a thymidine kinase minimal promoter and a luciferase coding sequence (Israel, Gougerot-Pocidalo et al. 1992; Newton, Hart et al. 1998). These NF-κB-dependent reporter cells were pre-treated with increasing concentration (1 nM to 1 μM) of the different GR ligands for 2 h, then challenged with 1 ng/ml of IL-1β for 6 h. Cells were then harvested, luciferase and mRNA levels measured and the results expressed as a percentage of those in cells treated with IL-1β alone (set as 100%) (Figure 4.2).

As a control for the ability of the compounds to inhibit the inflammatory response, COX-2 mRNA was quantified in parallel (Figure 4.2b). COX-2 mRNA was strongly induced by IL-1β alone then inhibited by the different compounds in a dose responsive manner (see also figure 5.2 and 5.13), suggesting that the cells were responding well to the treatments. However, no consistent repression of the 3κB-luc reporter was observed, even with dex (Figure 4.2 a). At the highest dose of dex (1 μM), only about 20% inhibition was observed, whilst no repression at all was seen in response to Cpd1 and Cpd2.

A549 cells stably transfected with the construct containing six κB sites showed more inhibition of the luciferase activity in response to dex, to a maximum of about 40% repression (Figure 4.2 c), which was in agreement with a similar study in the same cells (Chivers, Gong et al. 2006), where the authors also observed a 40% repression of luciferase activity after treating the cells with increasing doses of dex and 1 ng/ml of IL-1β. However, this type of response could not be reproduced when treating the cells with dex alongside Cpd1 and Cpd2. Responses to all three compounds appeared to be highly variable and inconsistent.

It was therefore decided to move away from stably transfected cells and consider testing the transrepression abilities of the different GR ligands in A549 cells transiently transfected cells with a reporter construct containing NF-κB sites.

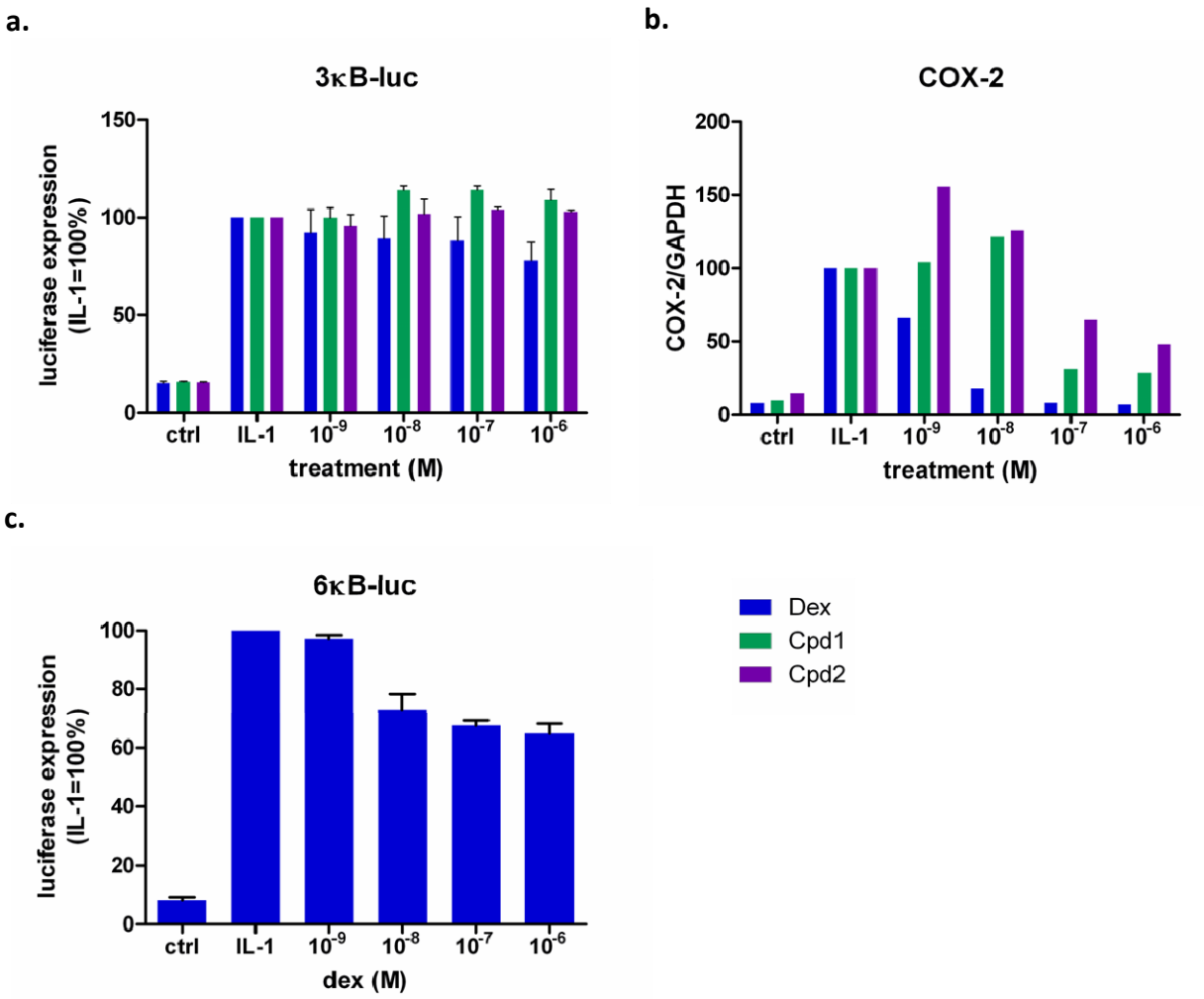


Figure 4.2. Transrepression properties of dex, Cpd1 and Cpd2 in stably transfected A549 reporter cell line.

(a) *A549 cells stably transfected with a reporter containing 3 κB sites were pre-treated with vehicle (0.1% DMSO) or increasing concentrations (1 nM to 1 μM) of dex, Cpd1 or Cpd2 for 2 h then stimulated with IL-1β (1 ng/ml) for 6 h. Cell lysates were harvested and luciferase measured. Graphs represent mean luciferase activity ± SEM, normalised against the one in cells treated with IL-1β alone (set as 100%), n=3.* ***(b)*** *mRNA was collected in one of these transrepression experiments. COX-2 mRNA was measured by quantitative PCR, normalised against GAPDH and plotted as in (a).* ***(c)*** *The transrepression assay was repeated as in (a), but using an A549 cell line stably transfected with a reporter containing 6 κB sites, and omitting Cpd1 and Cpd2.*

4.2.1.2. Transient transfections

In this section dex, Cpd1, Cpd2 but also CpdA were tested for their abilities to transrepress gene expression. For this purpose, A549 cells were transiently transfected, according to the protocol described in chapter 2, with a reporter construct containing 3 κB sites upstream of a minimal IL-6 promoter and a luciferase coding sequence ($(IL\text{-}6\text{-}\kappa B)_3$-50hu.IL6P-luc+, originally described in (De Bosscher, Schmitz et al. 1997)). Once recovered, cells were pre-treated with increasing concentrations of dex, Cpd1, Cpd2 (1 nM to 1 µM) or CpdA (10 nM to 10 µM) for 2 h then stimulated with 1 ng/ml of IL-1β or 25 ng/ml of TNFα for a further 6 h (Figure 4.2). Following stimulation, all cells were harvested and luciferase activity measured. Results are presented as a percentage of luciferase activity, normalised to the one in cells treated with pro-inflammatory stimulus alone (set as 100%).

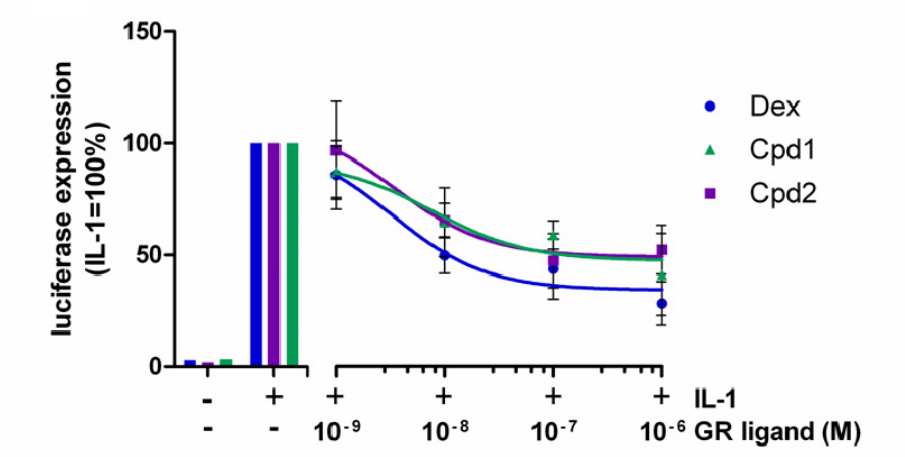
This construct was strongly activated by IL-1β and dose dependently inhibited by dex with an EC_{50} of 3.4 x 10-^{9} M (Figure 4.2 a). However, the extent of inhibition did not exceed 67%. Cpd1 and Cpd2 impaired the activation of the NF-κB dependent reporter with similar efficacy (53% and 54%) and potency (EC_{50} values of 7.8 x 10^{-9} M and 3.5 x 10^{-9} M). Cells treated with increasing doses of CpdA (Figure 4.2 c) showed a decreased luciferase activity only at the highest dose (10 µM), reaching 39% inhibition of the IL-1β induced response. Note that here 10 µM of CpdA were used because it was described to be the efficient dose for this GR ligand (De Bosscher, Berghe et al. 2005), while at similar doses of dex the maximal response was already obtained and such high concentration even lead to cell toxicity.

A similar activation of the construct was seen in cells stimulated with TNFα (Figure 4.2 b) but the pattern of responses to dex, Cpd1 and Cpd2 was very different from the one previously described when the cells were stimulated with IL-1β. Indeed, dex-induced inhibition reached a maximum of 49.5%; Cpd1 was able to repress luciferase activity by only 35% whereas Cpd2 did not exert any repression at all.

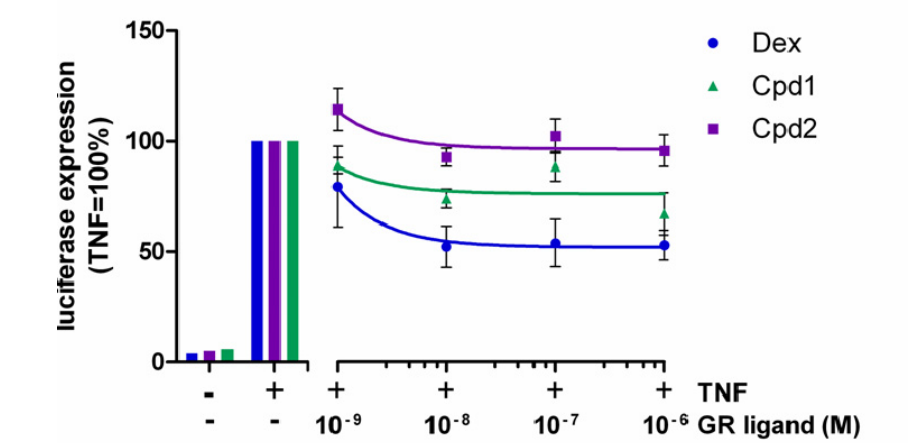
The different patterns of responses in IL-1β and TNFα stimulated cells suggest that the transrepression properties of dex, Cpd1 and Cpd2 appear to be rather dependent on the

stimulus used, with all GR ligands able to repress IL-1β induced luciferase expression, whereas in TNFα stimulated cells, Cpd1 was much weaker than dex and Cpd2 had no effect. The extent of inhibition of the NF-κB dependent reporters by both dex and the SGRMs was less than anticipated; for example it was invariably less than the extent of downregulation of COX-2 mRNA in the same cells (see Figure 5.2). Under none of the conditions described previously was reporter gene expression inhibited by more than 70% (Figure 4.2 and 4.3).

a.



b.



c.

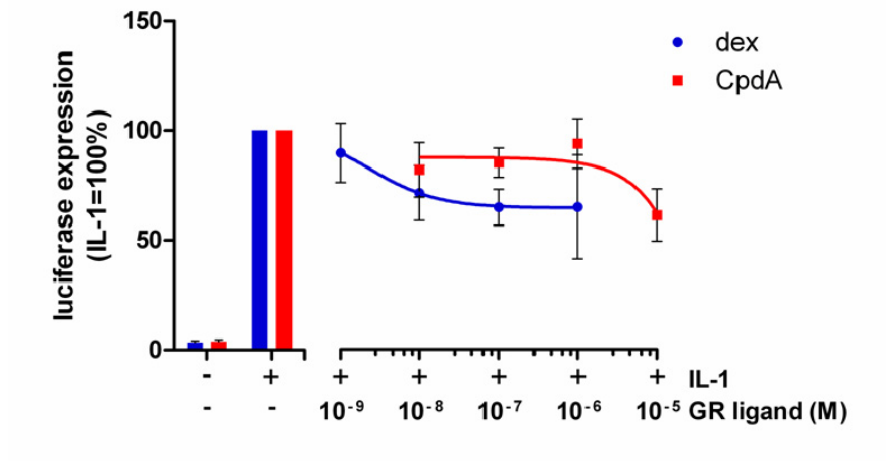


Figure 4.3. Transrepression properties of Cpd1, Cpd2 and CpdA compared to those of dex in A549 transiently transfected cells.

A549 cells were transiently transfected with a NF-κB-dependent reporter containing 3 κB sites upstream of a minimal IL-6 promoter and a luciferase coding sequence. Cells were pre-treated with vehicle (0.1% DMSO) or increasing concentrations (1nM to 10 μM) of dex, Cpd1, Cpd2 or CpdA for 2 h then stimulated with ***(a, c)*** *IL-1β (1 ng/ml) or* ***(b)*** *TNFα (25 ng/ml) for 6 h. Cell lysates were harvested and luciferase measured. Graphs represent mean luciferase activity ± SEM, normalised against that in cells treated with IL-1β alone or TNFα alone (set as 100%).n≥3.*

Next, I wanted to determine if transrepression would be stronger with a weaker pro-inflammatory stimulus. Indeed it was hypothesised that excessive amount of pro-inflammatory stimulus could provide such a strong signal that the GCs could not inhibit it (Irusen, Matthews et al. 2002). To assess whether different doses of the pro-inflammatory stimuli would make a difference to the efficacy of the inhibitory response to dex, Cpd1 and Cpd2, A549 cells were, again, transfected with the NF-κB dependent construct described earlier in this section on transient transfections (4.2.1.2). After recovery, cells were pre-treated with 100 nM of dex, Cpd1 or Cpd2 for 2 h then stimulated with increasing doses of IL-1β (0.04 to 25 ng/ml) or TNFα (0.2 to 25 ng/ml) (Figure 4.4). Following stimulation, all cells were harvested and luciferase activity measured. Results are presented as a percentage of luciferase activity, normalised to that in cells treated with pro-inflammatory stimulus alone (set as 100%).

All IL-1β doses strongly induced reporter gene expression (Figure 4.4a, inset graph), with at least a 60 fold increase. In this dose response experiment, even the lowest dose of IL-1β appeared to induce maximal response of the luciferase construct. When the different GR ligands were added (Figure 4.4a), they were able to repress the IL-1β induced reporter gene expression and no significant difference was found between the responses to the dose used in previous experiments (1 ng/ml) and the four other doses tested.

When transfected cells were stimulated with TNFα (Figure 4.4b), reporter gene expression was also strongly increased, in a dose responsive manner (see inset graph). The strongest response was to 25 ng/ml of TNFα, with over 2000 fold increase. When dex, Cpd1 and Cpd2 were added, some inhibition was seen with dex and Cpd1 but not with Cpd2, confirming what was observed in Figure 4.3. However, no significant difference was observed in the responses to dex and Cpd1, between the dose of stimulus previously used (25 ng/ml) and the three other ones (Figure 4.4b).

The IL-1 experiments shown here are inconclusive, because the signalling pathway for NF-κB activation seemed to be saturated at all of the tested concentrations of this cytokine. However, the results with TNF suggest that decreasing the strength of inflammatory stimulus does not influence the strength of transrepression that can be observed.

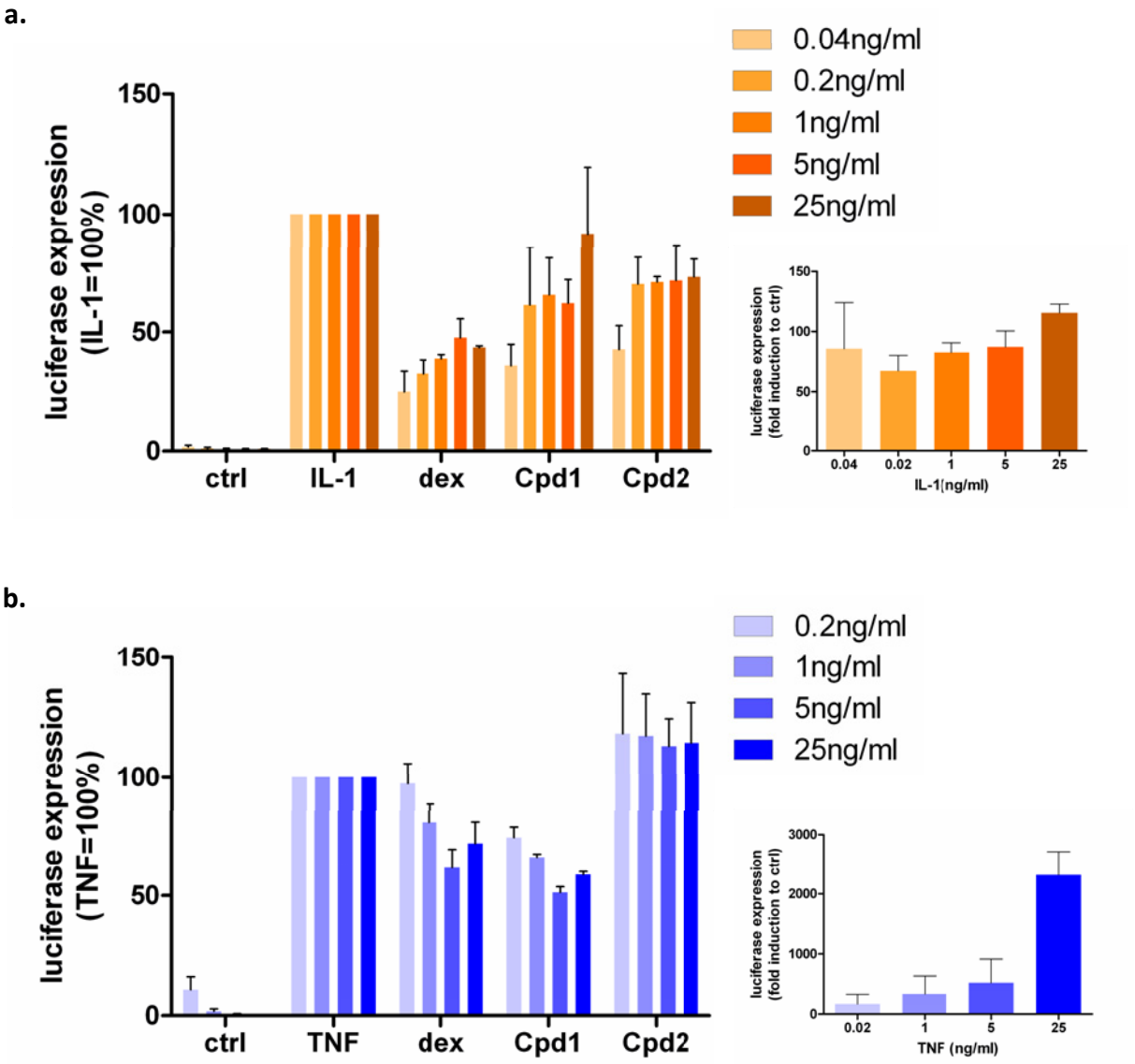


Figure 4.4. Effect of dose variation on the transrepression properties of dex, Cpd1 and Cpd2.
A549 cells were transiently transfected with the NF-κB-dependent reporter, pre-treated with vehicle (0.1% DMSO), dex, Cpd1 or Cpd2 (100 nM) for 2 h then stimulated with increasing doses of ***(a)*** *IL-1β (0.04 to 25 ng/ml) or* ***(b)*** *TNFα (0.02 to 25 ng/ml). Cell lysates were harvested and luciferase measured. Graphs represent mean luciferase activity ± SEM, normalised against that in cells treated with IL-1β alone or TNFα alone (set as 100%).n=3. Inset graphs show dose responses to IL-1β and TNFα, as fold inductions relative to control.*

4.2.2. Transactivation

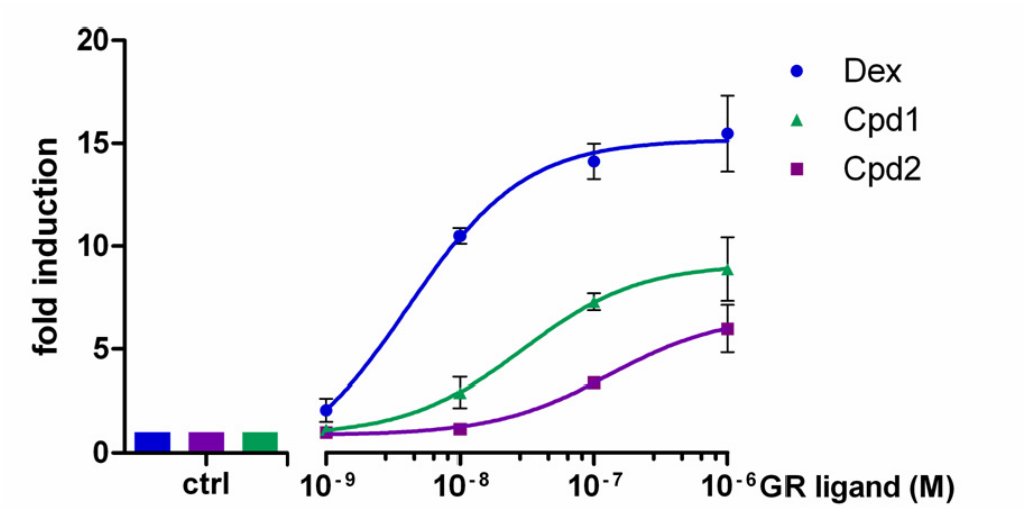
The transactivation potential of the different GR ligands was assessed using the A549 pulmonary epithelial cell line stably transfected with a luciferase reporter containing two tandem GREs derived from the rat TAT gene upstream of a minimal TATA box from the rabbit β-globin gene. Confluent cells were treated with vehicle (0.1% DMSO) or increasing doses of dex, Cpd1, Cpd2 (1 nM to 1 μM) or CpdA (10 nM to 10 μM) for 6 h. Cells were then harvested and luciferase activity measured. Fold induction was calculated by normalising the values from the luciferase reading against the value from the control sample (vehicle-stimulated condition) of each treatment. Results were plotted as means of the fold induction, non-linear regression analysis was then applied and fitting dose response curves were obtained (Figure 4.5).

Luciferase expression was increased in a dose-dependent manner in response to dex, Cpd1 and Cpd2 (Figure 4.5 a). However some differences could be noticed when comparing the three responses, as Cpd1 and Cpd2 appeared weaker, in comparison to the response to dex. For further confirmation, EC_{50} and A_{max} values were calculated (Table 4.). EC_{50} is the concentration required for half-maximal response. This value gives an indication of the potency of a compound: the lower the value is, the more potent the compound is. Here, regarding the EC_{50} values, dex<Cpd1<Cpd2, suggesting than dex is the most powerful at transactivating. The other parameter which can be calculated from a dose-response curve is A_{max}, defined as the maximal activity, or fold induction, obtained for the saturating concentration of ligand. It indicates the efficacy of a compound. The bigger the number is, the more effective the compound is. Regarding this value, dex>Cpd1>Cpd2, confirming the greater ability of dex to transactivate, whereas Cpd1 and Cpd2 had relatively low efficacy and potency in this assay. Although weaker than dex, both Cpd1 and Cpd2 were able to transactivate to some extent, suggesting that these compounds may also be able to induce expression of genes such as DUSP1.

When CpdA was added in the same assay, (Figure 4.5 b) no induction of the luciferase expression could be seen, whereas a strong dose response to dex was again observed. This

result was in agreement with the work of De Bosscher et al., suggesting full dissociation of this compound (De Bosscher, Berghe et al. 2005).

a.



b.

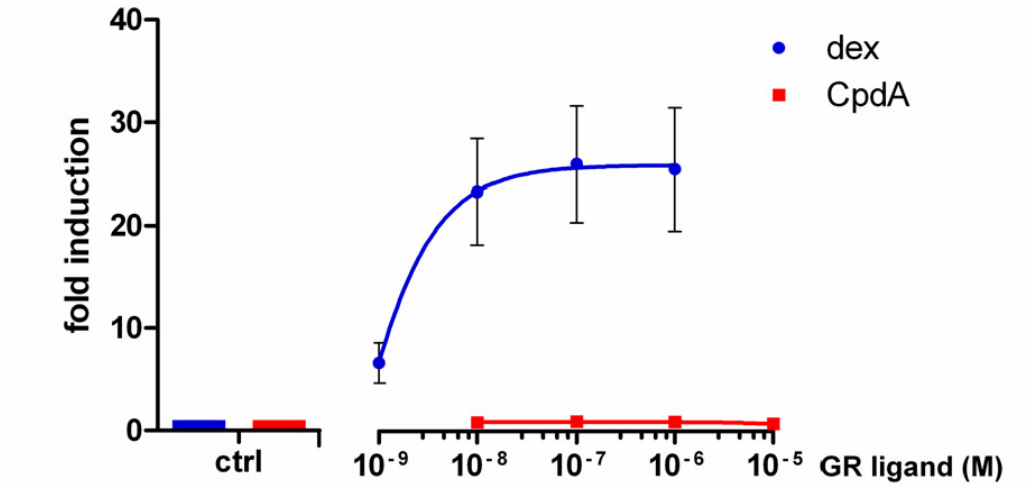


Figure 4.5. **Transactivation properties of dex, Cpd1, Cpd2 and CpdA.**
Stably transfected A549 GRE-dependent reporter cells were incubated for 6h with vehicle (0.1% DMSO) or increasing concentrations (1 nM to 10 µM) of dex, Cpd1, Cpd2 ***(a)*** *or CpdA* ***(b)****. Cell lysates were harvested and luciferase activities measured. Graph indicates mean fold inductions relative to control ± SEM, from at least three independent experiments.*

	EC_{50} (nM)	Amax
dex	4.1	16.4
Cpd1	29	8.4
Cpd2	130	5.9

Table 4.1. Potency and efficacy of dex, Cpd1 and Cpd2 in transactivation of reporter gene expression.
Values were obtained by non-linear regression analysis with the help of the Prism 5.0 software. A_{max} was obtained from a Mickaelis-Menten analysis on each data set. EC_{50} (nM) was obtained after transformation of the x values from each data set in logarithmic values and the subsequent non-linear regression dose response analysis.

Two questions arose from these transactivation assays: to know if the effect seen was dependent on GR and if the compounds themselves could impair the transcriptional activation abilities of a full agonist such as dex.

To address the first question, stably transfected A549 GRE-dependent reporter cells were incubated for 6 h with vehicle (0.1% DMSO) or 10^{-6}M of dex, Cpd1 or Cpd2 and/or the same concentration of the GR antagonist RU486 (RU, mifepristone). At the end of the incubation period, cells were harvested, lysed, and luciferase expression read. Normalised values were then expressed relative to those of dex alone (set as 100%) (Figure 4.6 a). Activation of the GRE reporter by dex, Cpd1 and Cpd2 was clearly blocked by the equivalent concentration of RU, confirming that the SGRMs regulate transcription at least in part via GR.

Since Cpd1 and Cpd2 appeared to function as partial agonists of GR-mediated transcription, it was considered whether they might impair transcriptional activation by a full agonist such as dex. To test this, stably transfected A549 GRE-dependent reporter cells were incubated with vehicle (0.1% DMSO), 10^{-7}M of dex, alone or in the presence of increasing concentrations of the novel GR ligands Cpd1 and Cpd2, as indicated in (Figure 4.6 b). At the end of the 6 h incubation period, cells were harvested, lysed, luciferase expression read and normalised. Values are presented as a percentage of the response to dex alone. The idea behind this experiment was that if Cpd1 or Cpd2 were able to compete with dex, then dex-induced responses would be impaired in the presence of the SGRMs, going down to the levels of transcription observed in response to the compounds alone. To investigate this hypothesis, molar excess (10^{-6} M), equivalence (10^{-7} M) or defiance (10^{-8} M) of Cpd1 and Cpd2 over dex were tested in their ability to induce transactivation.

In Figure 4.6 b, dex, Cpd1 and Cpd2 were all able to transactivate a GRE-dependent reporter. Responses to Cpd1 and Cpd2, however, were significantly less than the response to dex, which was in agreement with what was observed previously, in Figure 4.6 b. When increasing concentrations (10^{-8} to 10^{-6} M) of Cpd1 were added to dex to stimulate A549 GRE-dependent cells, no statistical difference could be observed, with luciferase response being close to 100% for all three doses of Cpd1, suggesting that the effect described here was the sole result of dex. When cells were treated with a mix containing dex and increasing doses (10^{-8} to 10^{-6} M) of Cpd2, statistically significant impairment of dex-induced transcription was only observed in the presence of a ten-fold molar excess of Cpd2 over dex. So, although Cpd1 and Cpd2 act as partial GR agonist, they are not able to antagonise effect of a classical GR agonist such as dex.

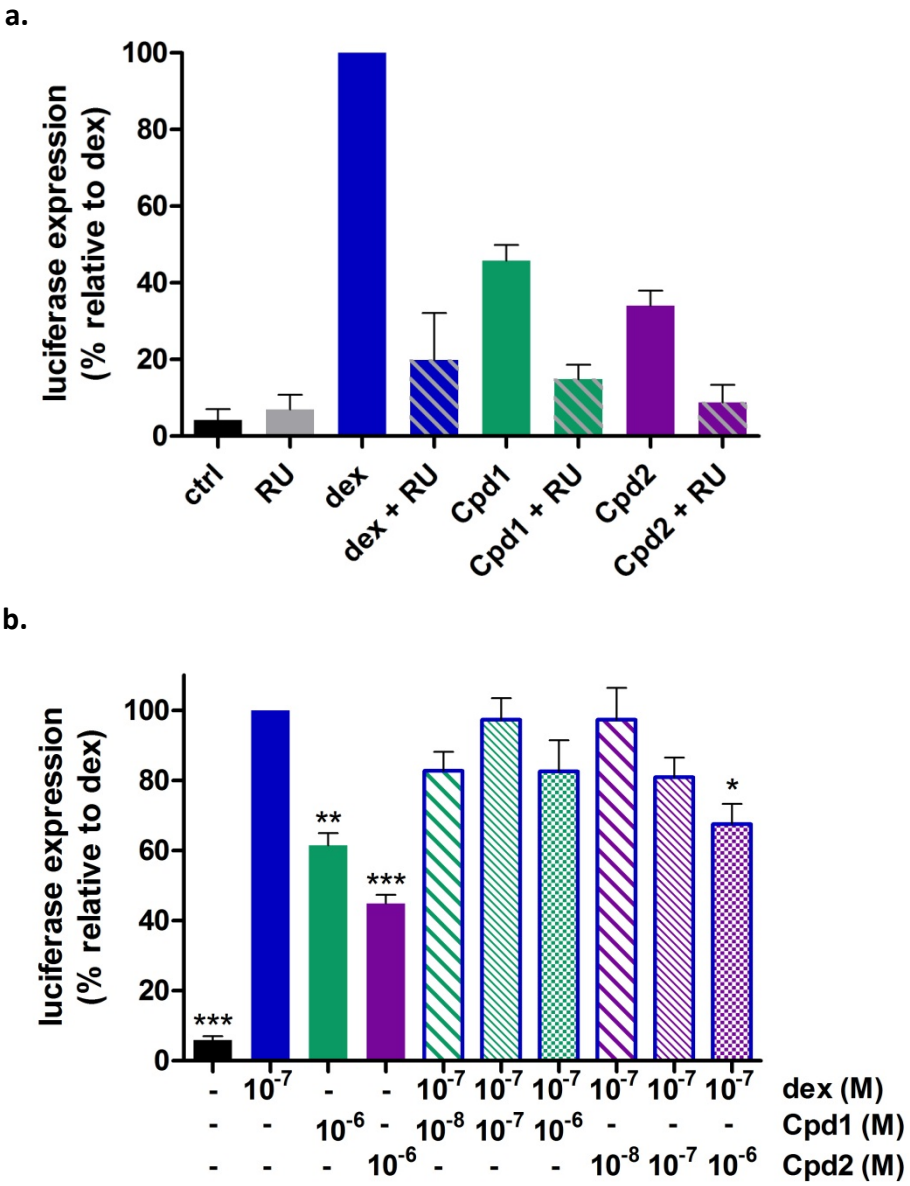


Figure 4.6. GR agonist properties of SGRMs and potential competition with dex.

(a) *Stably transfected A549 GRE-dependent reporter cells were incubated for 6h with vehicle (0.1% DMSO) or 10^{-6} M of dex, Cpd1 or Cpd2 in the absence or presence of 10^{-6} M of the GR antagonist RU486 (RU). Luciferase activities are expressed relative to that of cells treated with dex alone. The graph shows averages from two independent experiments ± SD.* ***(b)*** *Stably transfected A549 GRE-dependent reporter cells were incubated for 6h with vehicle (0.1% DMSO), 10^{-7}M of dex and/or SGRMs at the concentrations indicated. Luciferase activities are expressed relative to that of cells treated with 10^{-7} M dex alone. The graph indicates averages from three independent experiments ± SEM. ***, $p < 0.005$; **, $p < 0.01$; *, $p < 0.05$ relative to cells treated with 10^{-7} M dex alone. Experiment displayed in panel (b) was completed with the technical support of D. Qize.*

4.2.3. Dissociation index

Dissociation indices (DI) were calculated for Cpd1 and Cpd2, based on their EC_{50} values for activation of the GRE dependent reporter and inhibition of the NF-κB dependent reporter, relative to the corresponding values for dex.

$$DI = \frac{EC_{50}\ \text{activation GRE Cpd}}{EC_{50}\ \text{inhibition NF-κB Cpd}} \times \frac{EC_{50}\ \text{inhibition NF-κB dex}}{EC_{50}\ \text{activation GRE dex}}$$

By definition, DI (dex) = 1. Any value greater than unity indicates selective capacity for transrepression over transactivation. Using the above formula, Cpd1 and Cpd2 had dissociation indices of 3.04 and 30.8, respectively. Both compounds therefore appeared to conform to the description of selective GR modulators (SGRMs), with Cpd2 being much more dissociated than Cpd1 and the latter being closer to dex.

On top of the dissociation index calculated based on the different compounds' potencies, DI could also be obtained on the basis of the efficacies of Cpd1 and Cpd2 for activation of the GRE-dependent reporter and repression of the NF-κB-dependent reporter, relative to the corresponding values for dex.

$$DI = \frac{\text{Fold activation GRE Cpd}}{\text{Fold inhibition NF-κB Cpd}} \times \frac{\text{Fold inhibition NF-κB dex}}{\text{Fold activation GRE dex}}$$

Again, a dissociation index greater than unity indicates selective capacity for transrepression over transactivation. Based on their efficacy, DI was 2.53 for Cpd1 and 4.32 for Cpd2, indicating that with this calculation too Cpd1 and Cpd2 are in agreement with the definition of SGRMs.

4.3. Regulation of DUSP1 expression

4.3.1. GC-responsive regions

Our group, and others, previously identified two GC responsive regions (GRRs) within the 5' region of the human *DUSP1*, at -1.3 and -4.6 kb with respect to the transcription start site (Johansson-Haque, Palanichamy et al. 2008; Shipp, Lee et al. 2010; Tchen, Martins et al. 2010). Activation of GRR-4.6 by dex was impaired by mutation of the dimerisation domain of GR, whereas activation of GRR-1.3 by dex was insensitive to this mutation (Tchen, Martins et al. 2010), suggesting that GR may interact differently with the two sites. In the same study, a GC responsive region was also identified in the murine *Dusp1* gene, at -29kb upstream of the transcription start site. Having established that SGRMs were capable of transcriptional activation of a stereotypical GRE reporter (Figure 4.5), we asked whether they could also regulate transcription via GRR-1.3 and -4.6 (Figure 4.7) or GRR-29. For this purpose, four different reporter constructs, generated from genomic DNA by Dr J. Martins (Tchen, Martins et al. 2010), were used and are detailed below

- pGL3b-*Hs*-4.8kb, a reporter construct containing the full length human *DUSP1* promoter, was generated by sub-cloning the -4834 to +211 fragment (with respect to the transcription start site) of *DUSP1* human genomic DNA to the pGL3b vector.
- pGL3p-GRR-1.3-*Hs* and pGL3p-GRR-4.6-*Hs* are reporter constructs which contain the GC responsive region GRR-1.3 (-1366 to -1237) or 4.6 (500 bp fragment, centred on the three putative GC binding sites located 4.6 kb upstream from *DUSP1* transcription start site), known GR binding sites and stretches of flanking DNA that are well conserved between species and appear to play important roles in mediating transcriptional activation by dex (Tchen, Martins et al. 2010).
- pGL3p-GRR-29-*Mm*, the reporter construct containing the murine GC responsive region, located 26854 bp upstream of *Dusp1* transcription start site.

HeLa cells were transiently transfected with the appropriate firefly luciferase construct as well as a *Renilla* luciferase expression vector. After recovery, they were treated with vehicle (0.1% DMSO), 100 nM of dex, Cpd1 or Cpd2 for 20 h, then harvested and luciferase activities were quantified. Firefly activities were normalised against those of *Renilla* and responses to

the different GR ligands calculated. Results are presented as fold inductions relative to control in Figure 4.7.

In the cells transfected with the full length *DUSP1* promoter (Figure 4.7 a), all three GR ligands were able to significantly induce transcription ($p<0.001$). However, as previously observed in the transactivation assay (Figure 4.5), Cpd1 and Cpd2 were less powerful than dex at activating transcription.

The next step was to investigate the ability of dex and the two SGRMs to transactivate via individual GC responsive regions, of human and murine origin (Figure 4.7 b). Dex, Cpd1 and Cpd2 significantly activated transcription via both GRR-1.3 and GRR-4.6. The proximal element (GRR-1.3) was slightly less responsive to the two SGRMs than to dex. The distal element (GRR-4.6) was approximately five-fold more responsive to dex than to either of the SGRMs. It was also established by chromatin IP, that both dex and Cpd1 could promote recruitment of GR to both GRR-1.3 and GRR-4.6 in HeLa cells, whereas Cpd2 did not seem to have much effect (Figure 4.8). In a repeat of this experiment, however, recruitment of GR to GRR-1.3 could be observed in presence of Cpd2 (not shown). Further repeats would therefore need to be performed to establish the abilities of Cpd1 and Cpd2 to recruit GR to GCs-responsive regions in human cells. In another ChIP assay, Dex and Cpd1 were also able to induce gene expression via the murine GRR element (not shown). However, as in human cells, Cpd2 did not seem to have much effect. It is worth noting that murine GRR-29 was less responsive to the different GR ligands than human GRR-1.3 and GRR-4.6. This, and the observation of dex being able to recruit GR at both GRR-1.3 and GRR-4.6 (Figure 4.8), is in agreement with previous observations by our group when studying the response of these constructs to dex (Tchen, Martins et al. 2010).

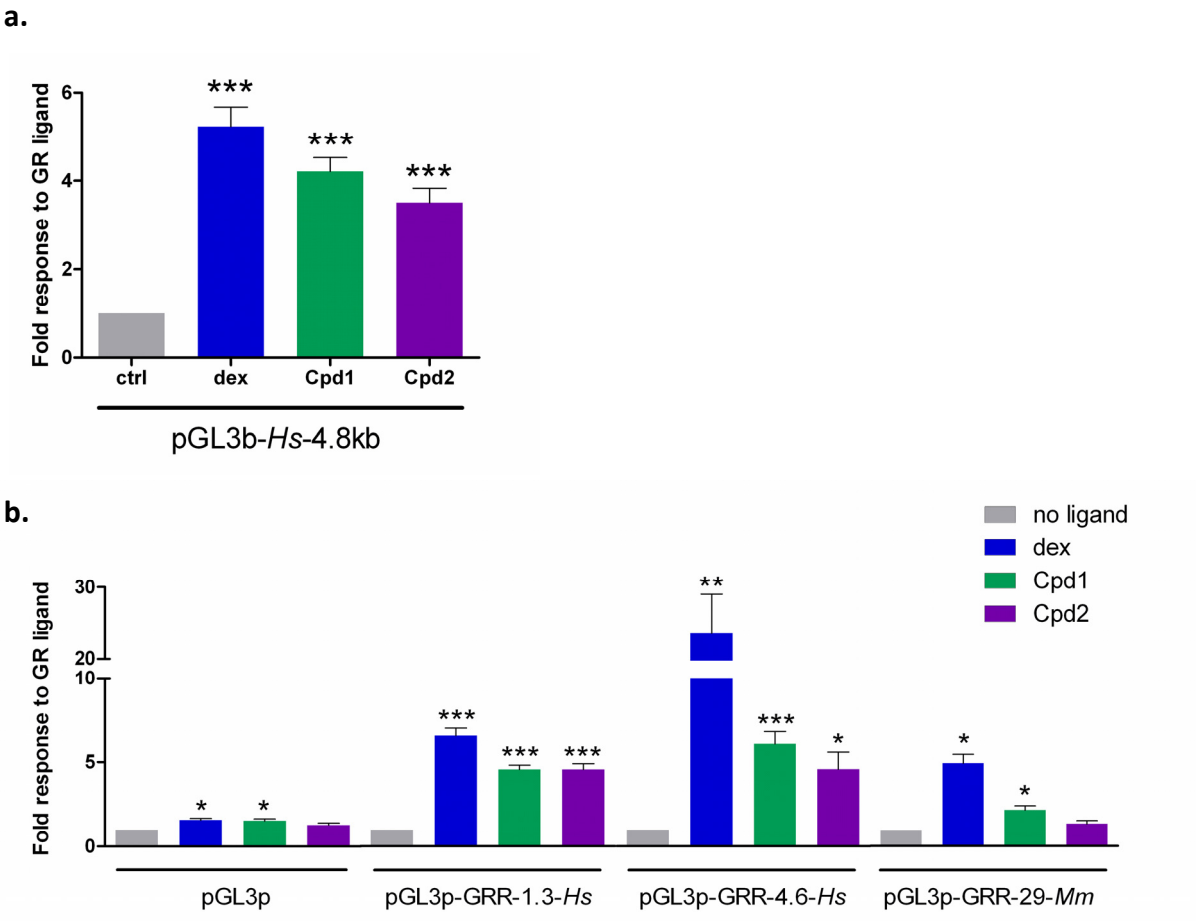


Figure 4.7. Dex, Cpd1 and Cpd2 activate transcription via GC-responsive regions of the DUSP1 gene.

(a) *HeLa cells were transiently transfected with a derivative of the pGL3b construct, containing the full length promoter of the human DUSP1 gene.* ***(b)*** *HeLa cells were transiently transfected with pGL3p or derivatives which contain GRR-1.3 or GRR-4.6 from the human DUSP1 gene; as well as GRR-29 from the murine DUSP1 gene. All cells were treated with vehicle (0.1% DMSO) or 100 nM GR ligands as indicated for 20 h. Cell lysates were prepared, firefly and Renilla luciferase activities measured, and fold responses to ligand calculated. Graphs represent means ± SEM from 3 (pGL3p and pGL3p-GRR-29), 6 (pGL3p-GRR-1.3 and pGL3b-Hs-4.8kb) or 7 (pGL3p-GRR-4.6) independent experiments. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; relative to vehicle-treated cells.*

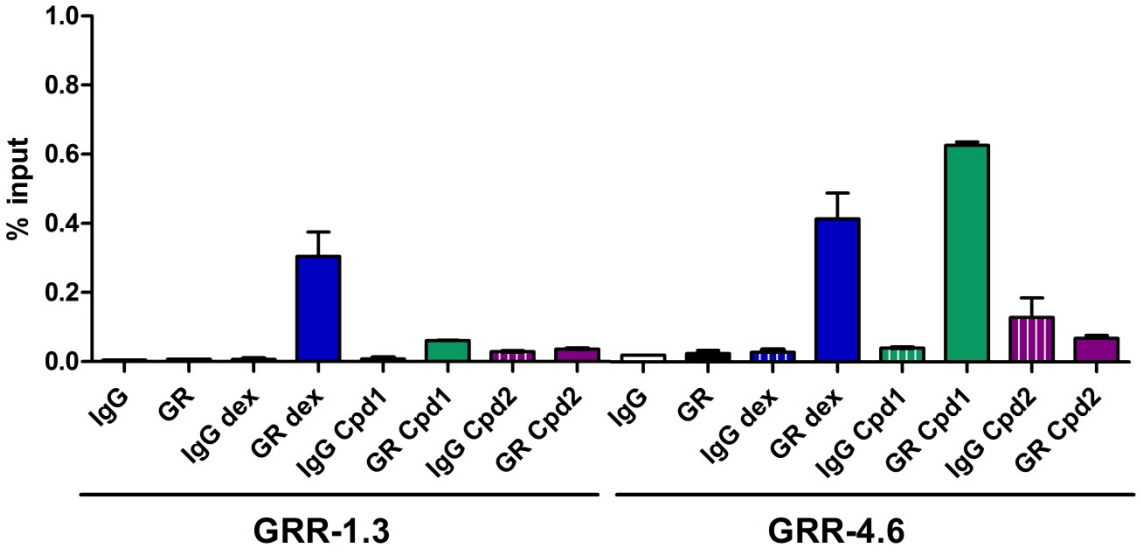


Figure 4.8. Recruitment of GR to functional human GC-responsive regions.

GR recruitment was assessed by ChIP. HeLa cells were treated with vehicle (0.1% DMSO) or GR ligands (100 nM) for 30 min. Samples were then chromatin immunoprecipitated with either IgG or anti-GR antibody. Immunoprecipitated chromatin fragments were detected by quantitative PCR and measurements done in duplicates. Enrichment of specific genomic fragments was calculated as a proportion of the starting material (input). The figure illustrates the means and SD of one experiment. This work was performed with Dr Joana Martins.

4.3.2. DUSP1 expression is induced by dex, Cpd1 and Cpd2 in a dose dependent manner.

The ability of GR ligands to induce expression of DUSP1 mRNA and protein was next tested in a variety of cell types, namely A549, HeLa, RAW264.7 cells and BMDMs (Figures 4.9 to 4.12). As it was previously established that DUSP1 expression peaked after one hour of treatment with dex (Lasa, Abraham et al. 2002); cells were treated for that same interval with increasing concentrations (1 nM to 1 µM) of dex, Cpd1 or Cpd2. mRNA and proteins were then extracted. DUSP1 protein was detected by western blot whereas its mRNA was quantified by RT-PCR.

As detailed in section 3.2 of the preceding chapter, DUSP1 was detected using batch J3007 of the M-18 anti-DUSP1 antibody from Santa Cruz. In all western blots shown in Figures 4.9, 4.10 and 4.11, a 'non-specific' band of higher molecular weight is visible just above DUSP1. Previous observations made by members of our group and unpublished work by Dr S. Abraham identified this band as being DUSP4, a dual specificity phosphatase with a slightly higher molecular weight (42kDa) than DUSP1 (39kDa). This reflects the difficulties met when trying to detect DUSP1 protein and is consistent with the non-specificity of some marketed 'anti-DUSP1' antibodies (chapter 3, section 3.2).

In the experiments detailed below, mRNA results are shown as fitted dose response curves of fold inductions relative to control. In Table 4. are presented the efficacy (A_{max}) and potency (EC_{50}) values of DUSP1 induction in the different cell types investigated here. These values were calculated with the help of the Prism 5.0 software.

In A549 cells, dex (Figure 4.9 a), Cpd1 (Figure 4.9 b) and Cpd2 (Figure 4.9 c) were all able to induce DUSP1 expression at both the mRNA and protein levels. *DUSP1* mRNA expression was induced in a dose dependent manner in response to all treatments. DUSP1 protein was dose-dependently induced by dex and Cpd2 but was not clearly detected in cells treated with Cpd1. The comparison of mRNA levels in the three conditions (Figure 4.9 d) showed that in this cell type, dex was the most potent ligand (EC_{50} dex < Cpd1 < Cpd2) whereas Cpd2 was the most efficient (A_{max} Cpd2 ≈ dex > Cpd1) (Table 4.), even if its curve was shifted to

the right. In this cell type, dex could thus be considered as the most powerful compound in inducing *DUSP1* expression.

In HeLa cells (Figure 4.10), dex, Cpd1 and Cpd2 were all able to induce DUSP1 expression in a dose dependent manner, at both the mRNA and protein levels. Comparing *DUSP1* mRNA expressions in response to the different treatments (Figure 4.10 d), it appeared that in this cell type Cpd2 had the lowest EC_{50} (Cpd2 ≈ dex < Cpd1) whereas Cpd1 had the highest A_{max} (Cpd1 > Cpd2 ≈ dex) (Table 4.).

In RAW264.7 cells (Figure 4.11), dex, Cpd1 and Cpd2 were also all able to induce DUSP1 expression in a dose dependent manner, at both the mRNA and protein levels. However, comparing Dusp1 mRNA induction between the different treatments (Figure 4.11 d), the response to Cpd1 appeared at a lower dose whereas the one to dex appeared stronger. This was confirmed when comparing the EC_{50} and A_{max} values (Table 4.). Regarding EC_{50}, Cpd1 < dex < Cpd2 which indicated that Cpd1 was the most potent compound in inducing Dusp1. On the other hand, when looking at A_{max}: dex > Cpd2 > Cpd1. In those conditions dex was, thus, the treatment with the greatest efficacy in inducing Dusp1 mRNA expression.

In primary mouse macrophages (Figure 4.12), dex, Cpd1 and Cpd2 were able to induce *Dusp1* mRNA expression in a dose dependent manner. However, the increasing response to the increasing drug concentrations was stopped at the 100 nM dose of either dex, Cpd1 or Cpd2. For the highest dose (1 µM), mRNA expression was strongly reduced. No particular explanation for this phenomenon has been found so far but it could be that the viability of the cells was affected at high doses of GR ligands. If focusing on the dose dependent responses (1 to 100 nM), the comparison between the different treatments (Figure 4.12 d) showed that the strongest induction of *Dusp1* mRNA expression was in response to treatment with Cpd1 (A_{max} Cpd1 > dex > Cpd2), whereas dex was the treatment with highest potency for induction of *DUSP1* (EC_{50} dex < Cpd2 ≈ Cpd1).

To summarise, the pattern of response of the DUSP1 gene was highly variable between cell types and was not predictable from the response of the GRE reporter, even within a single cell type. For example, in A549 cells Cpd2 was a poor activator of the GRE reporter, but

activated the endogenous *DUSP1* gene with similar efficacy to dex (although with a rather higher EC_{50}). Rank orders of efficacy and potency of the three compounds for activation of DUSP1 gene expression were not the same for any two cell lines, illustrating the extreme variability of the response.

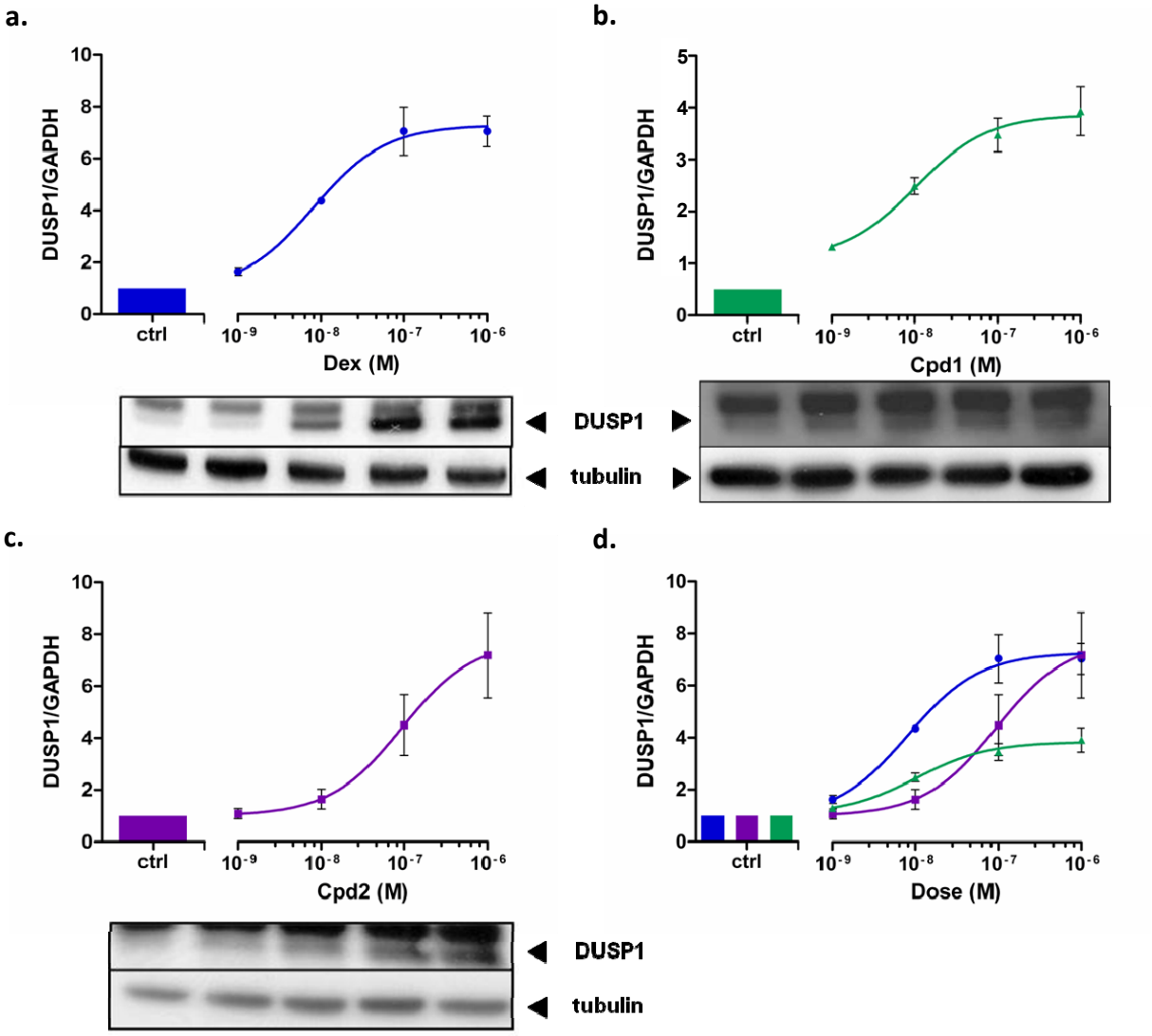


Figure 4.9. DUSP1 is induced in a dose dependent manner by dex, Cpd1 and Cpd2 in A549 cells.
Cells were treated for 1 h with increasing concentrations (1 nM to 1 µM) of ***(a)*** *dex,* ***(b)*** *Cpd1 and* ***(c)****Cpd2. mRNA was harvested and DUSP1 mRNA quantified by real-time PCR (top panels). DUSP1 protein was detected by western blotting (lower panels).* ***(d)*** *Comparison of DUSP1 mRNA dose dependent induction between the different treatments. Results are presented as fold induction relative to cells treated with vehicle alone (0.1% DMSO). Graphs indicate means ± SEM from at least three independent experiments.*

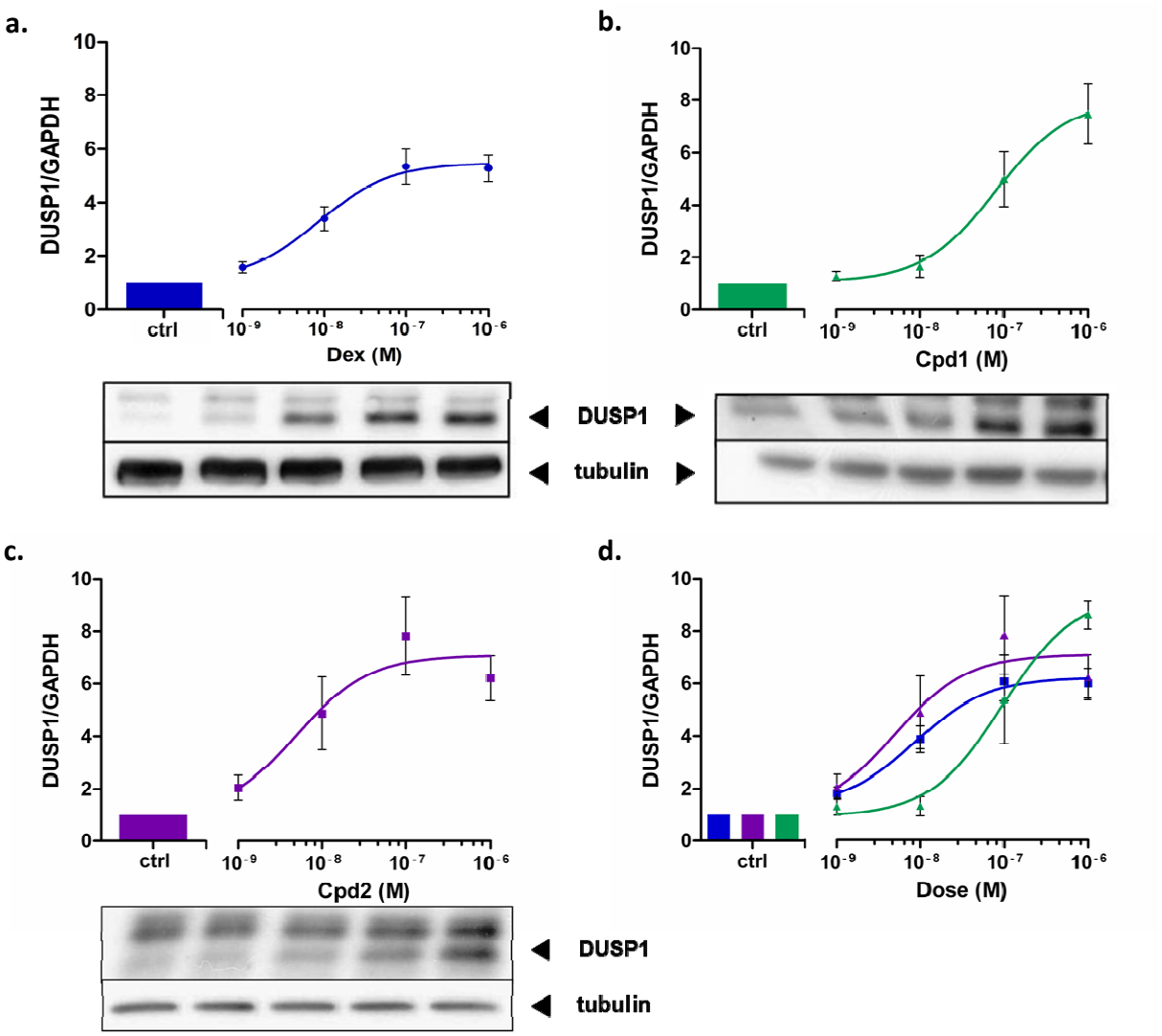


Figure 4.10. DUSP1 is induced in a dose dependent manner by dex, Cpd1 and Cpd2 in HeLa cells. *Cells were treated for 1 h with increasing concentrations (1 nM to 1 µM) of* ***(a)*** *dex,* ***(b)*** *Cpd1 and* ***(c)****Cpd2. mRNA was harvested and DUSP1 mRNA quantified by real-time PCR (top panels). DUSP1 protein was detected by western blotting (lower panels).* ***(d)*** *Comparison of DUSP1 mRNA dose dependent induction between the different treatments. Results are presented as fold induction relative to cells treated with vehicle alone (0.1% DMSO). Graphs indicate means ± SEM from at least three independent experiments.*

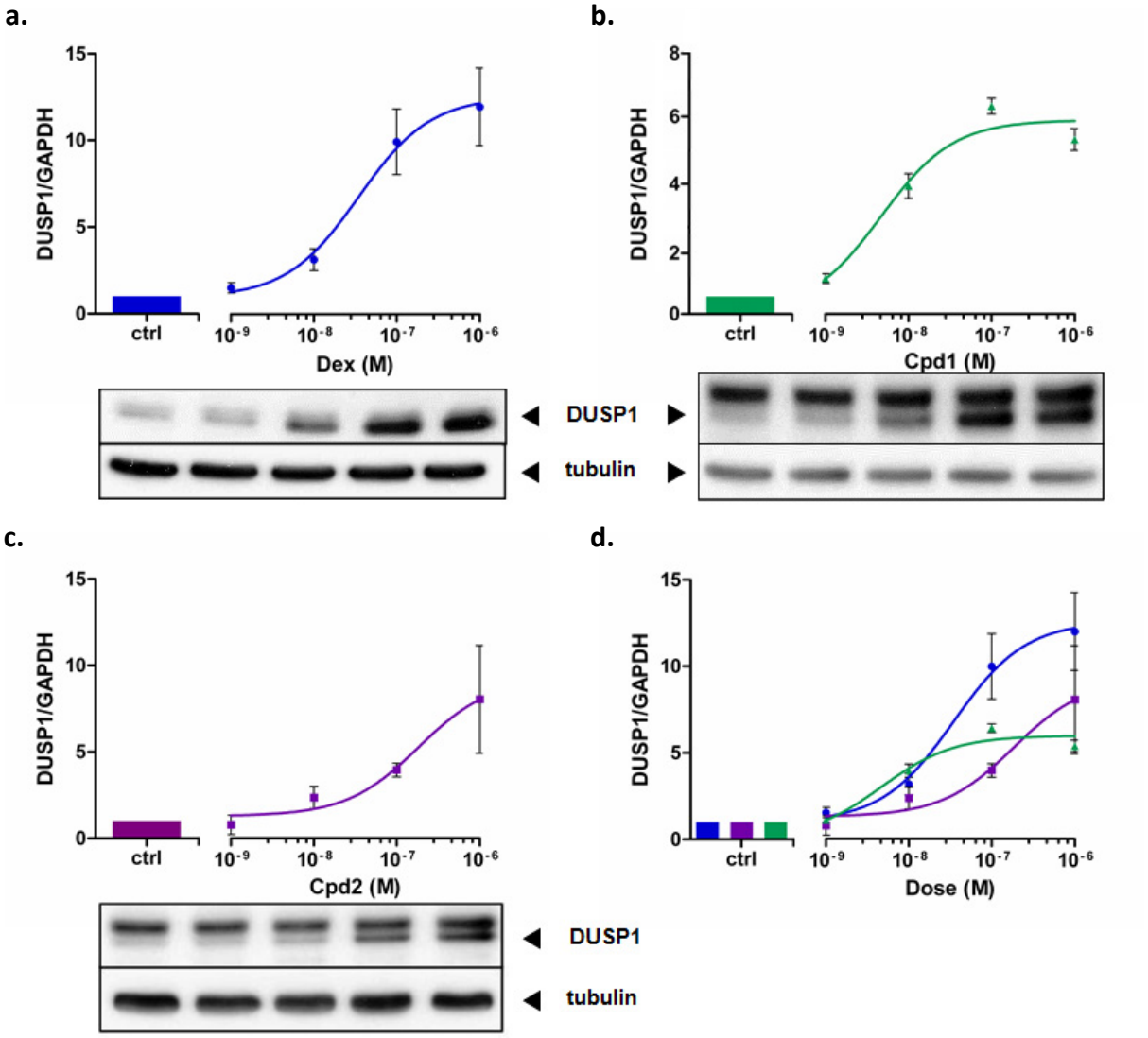


Figure 4.11. DUSP1 is induced in a dose dependent manner by dex, Cpd1 and Cpd2 in RAW cells. *Cells were treated for 1 h with increasing concentrations (1 nM to 1 μM) of* ***(a)*** *dex,* ***(b)*** *Cpd1 and* ***(c)****Cpd2. mRNA was harvested and DUSP1 mRNA quantified by real-time PCR (top panels). DUSP1 protein was detected by western blotting (lower panels).* ***(d)*** *Comparison of DUSP1 mRNA dose dependent induction between the different treatments. Results are presented as fold induction relative to cells treated with vehicle alone (0.1% DMSO). Graphs indicate means ± SEM from at least two independent experiments.*

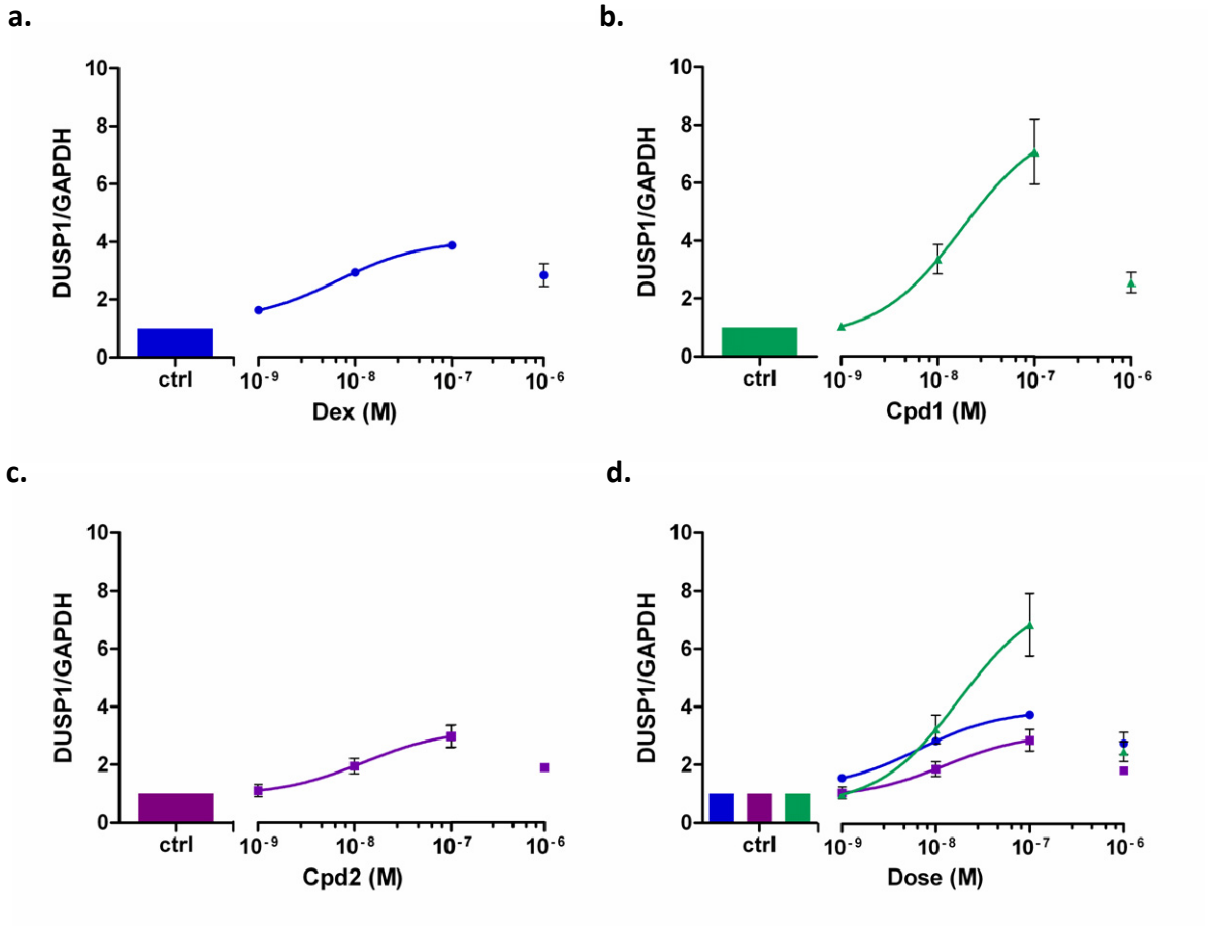


Figure 4.12. DUSP1 is induced in a dose dependent manner by dex, Cpd1 and Cpd2 in BMDMs. *Cells were treated for 1 h with increasing concentrations (1 nM to 1 µM) of* ***(a)*** *dex,* ***(b)*** *Cpd1 and* ***(c)*** *Cpd2. mRNA was harvested and DUSP1 mRNA quantified by real-time PCR.* ***(d)*** *Comparison of DUSP1 mRNA dose dependent induction between the different treatments. Results are presented as fold induction relative to cells treated with vehicle alone (0.1% DMSO). Graphs indicate means ± SEM from at least two independent experiments.*

	A549	HeLa	RAW	BMDMs
	EC_{50}			
Dex	(1) 8.14×10^{-9}	(2) 8.57×10^{-9}	(2) 3.45×10^{-8}	(1) 6.66×10^{-9}
Cpd1	(2) 1.04×10^{-8}	(3) 8.09×10^{-8}	(1) 4.68×10^{-9}	(3) 1.78×10^{-8}
Cpd2	(3) 9.37×10^{-8}	(1) 4.90×10^{-9}	(3) 1.83×10^{-7}	(2) 1.25×10^{-8}
	A_{max}			
Dex	(2) 7.16	(3) 6.00	(1) 12.35	(2) 3.71
Cpd1	(3) 3.65	(1) 7.65	(3) 5.96	(1) 7.92
Cpd2	(1) 7.37	(2) 6.97	(2) 8.51	(3) 2.80

Table 4.2. Efficacy and potency values of DUSP1 induction.
EC_{50} and A_{max} were calculated for the dex, Cpd1 and Cpd2 dose responses in A549, HeLa, RAW cells and BMDMs. Values were obtained by non-linear regression analysis with the help of the Prism 5.0 software. A_{max} was obtained from a Mickaelis-Menten analysis on each data set. EC_{50} (M) was obtained after transformation of the x values from each data set in logarythmic values and the subsequent non-linear regression dose response analysis. Numbers in brackets indicate the rank order of the efficacy and potency of the different compounds.

4.3.3 DUSP1 mRNA is transiently induced by CpdA

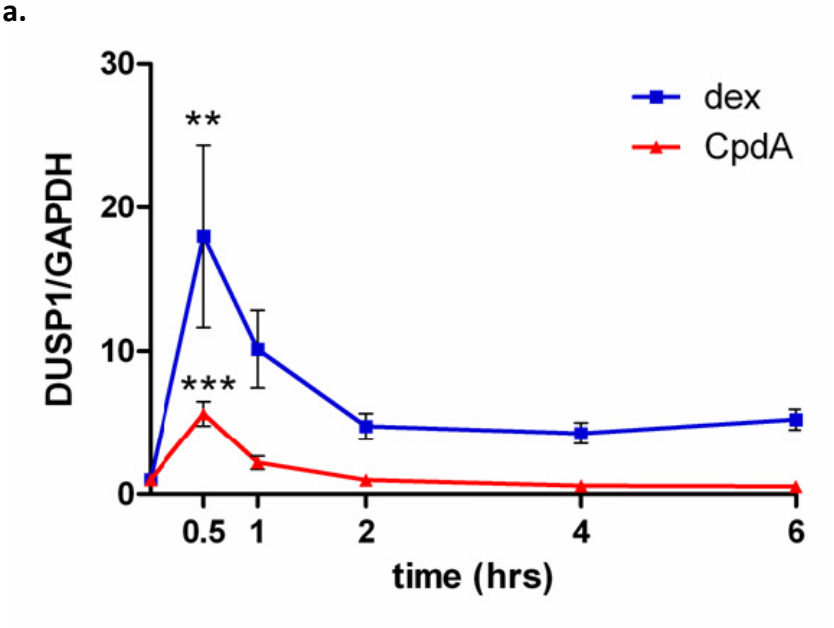
Next, the ability of CpdA to induce DUSP1 mRNA expression was investigated in RAW 264.7 and A549 cells (Figure 4.13). For this purpose, cells were treated with vehicle (0.1% DMSO), dex (100 nM) or CpdA (10 µM) for the indicated time. Cells were then lysed, mRNA harvested and quantified by RT-PCR. Results are presented as a time course of fold induction relative to time 0. Significance was determined, for each treatment, by analysis of variance between each time point and time 0.

In RAW cells, both dex and CpdA significantly induced Dusp1 mRNA expression at 30 min. However, this induction was transient, with much lower levels of DUSP1 seen at 1h and a rapid return to basal levels after 2 h of CpdA treatment. Although non-significant, levels of DUSP1 mRNA expression after 2h of dex treatment showed sustained induction over time, plateauing at around 5 fold increase (4.8 fold at 2 h, 4.3 fold at 4 h and 5.3 fold at 6 h).

In A549 cells, dex and CpdA were also able to induced DUSP1 mRNA expression in a significant manner at 30 min. However, after 1h of CpdA treatment, DUSP1 mRNA levels were strongly reduced, returning to basal levels from 2h onwards. In contrary, DUSP1 mRNA expression appeared sustained over time in response to dex. mRNA levels reached a peak of expression at 1 hour and although lower at 2h, levels of DUSP1 mRNA were still significantly increased by dex at 4 and 6h, confirming previous findings.

In conclusion of this experiment, dex was again able to induce DUSP1 mRNA expression in a significant manner over time whereas CpdA was only able to transiently increase DUSP1 mRNA expression at 30min, in both murine and human derived cells.

a.



b.

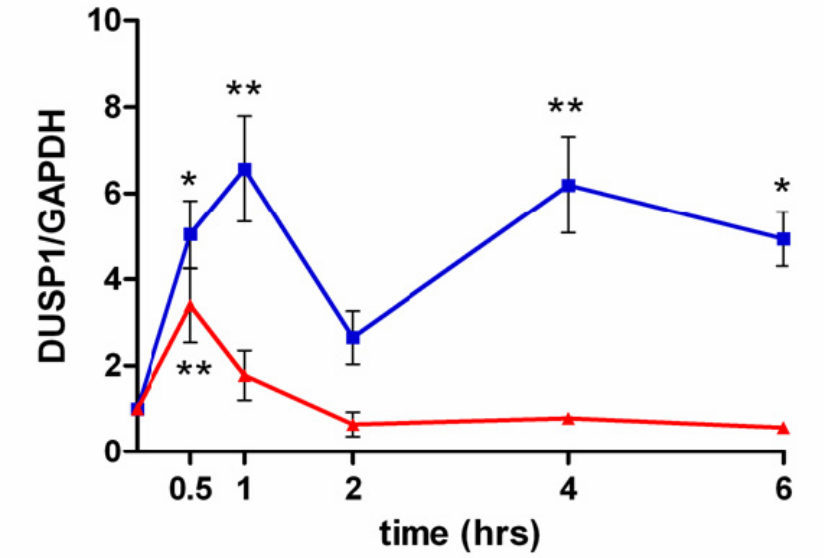


Figure 4.13. Time course of DUSP1 expression in response to dex and CpdA.

(a) *Raw and* ***(b)*** *A549 cells were incubated with dex (100 nM) or CpdA (10 µM) for the indicated times and mRNA was harvested for DUSP1 quantification by real-time PCR. Graphs indicate means ± SEM from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance with the Dunnet correction for comparison of each time point to control (time 0). Significance was taken as follow: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.*

4.4 Discussion

SGRMs ZK 216348, ZK 245186, AL-438 and LGD-5552 were identified by various screen techniques, including receptor binding assays (Schacke, Schottelius et al. 2004; Schäcke, Zollner et al. 2009) and high-throughput screening (Lopez, Ardecky et al. 2008). The common point of these identification methods was their final aim: to identify glucocorticoid receptor ligands with a 'dissociated profile', *i.e.* greater transrepression than transactivation activity; and the expectation that such compounds would retain GR-mediated anti-inflammatory function whilst reducing propensity to undesirable side effects. SGRMs similar to the above compounds, Cpd1 and Cpd2 were identified among other compounds by their originator companies with the same goal in mind and tested for their transrepression and transactivation properties.

It was thus surprising that no convincing evidence for transrepression by Cpd1 or Cpd2 could be found using NF-κB-dependent reporter cells, even when testing the classical GR ligand dex, previously shown to be able to repress both 3κB-luc (King, Holden et al. 2009) and 6κB-luc (Chivers, Gong et al. 2006) constructs expression by about 40%. An explanation for the lack of response of the 3κB-luc reporter could be that either the inhibition of *COX-2* gene expression by dex or other GR ligands did not depend on transrepression of NF-κB in this context, or the reporter did not accurately mirror the transrepression of the endogenous *COX-2* gene. The later seems unlikely, since the reporter construct used three copies of the upstream NF-κB site of the human *COX-2* promoter, which was shown to be necessary for transcriptional induction in response to IL-1β (Newton, Hart et al. 1998).

Similarly, no repression of the 6κB-luc reporter was seen with SGRMs. However, when tested independently, dex was able to inhibit IL-1β-induced reporter expression by almost 40%, consistent with previous results mentioned above. To further investigate transrepression properties of SGRMs, it was decided to move on to transient transfections of A549 cells using that same 6κB-luc reporter. In the initial, IL-1β-stimulated experimental set-up dex, Cpd1 and Cpd2 were able to repress NF-κB-dependent reporter expression but these results were challenged when using a different pro-inflammatory stimulus, namely TNFα. Whilst dex was still able to repress 6κB-luc expression by about 50%, Cpd1 and Cpd2

transrepression ability, or lack of, differed from the results obtained in IL-1β-stimulated cells, suggesting a role for the cellular environment in GR-dependent gene regulation.

As transrepression ability of the different compounds was finally assessed with the help of 6κB-luc transient transfection, one question remained when looking back at this set of experiments: would it be possible that the inhibition of *COX-2* gene expression by dex and other GR ligand does not depend on transrepression of NF-κB in this experimental context, explaining the lack of responsiveness of the 3κB-luc reporter? A study by Reily and colleagues identified, a few years ago, IRF3 as a target of the transrepression activity of GR. NF-κB sequences that recruited IRF3 to activate gene expression were glucocorticoid sensitive, whilst those that did not require IRF3 were relatively insensitive to transrepression by GR (Reily, Pantoja et al. 2006). Also, the expression of COX-2 in LPS-stimulated mouse macrophages was shown to be independent of IRF3 (Ogawa, Lozach et al. 2005). Moreover, in A549 cells stimulated with IL-1β, transcription of *COX-2* was accounted for being mainly due to posttranscriptional mechanisms (Newton, Seybold et al. 1998). These findings suggest that dex does not inhibit COX-2 gene expression by means of transrepression of NF-κB. Furthermore the suppression of COX-2 may not even occur at the transcriptional level. To test this, further work could include the use of a nascent nuclear RNA-based RT-PCR assay to assess the effects of dex on *COX-2* transcription, as well as transient transfections using a *COX-2* promoter construct to determine whether dex may inhibit transcription via sites other than the NF-κB binding sites.

While transrepression assays were challenging, transactivation assessment confirmed that dex was strongly capable of inducing GRE-dependent reporter expression, in agreement with others' findings (Chivers, Cambridge et al. 2004; Kaur, Chivers et al. 2008; Rider, King et al. 2011); and demonstrated that Cpd1 and Cpd2 had relatively low efficacy and potency in this assay of transcriptional activation. Furthermore, RU 486-induced blockade of GRE reporter activation by dex, Cpd1 and Cpd2 confirmed that both SGRMs could regulate gene expression *via* GR and were therefore considered as partial agonists of GR-mediated transcription.

Another parameter that was investigated in the present study was whether Cpd1 or Cpd2 were able to impair transactivation of full GR agonists such as dex. Only a 10-fold molar excess concentration of Cpd2 significantly impaired dex-induced GRE reporter expression whereas equimolar or lower concentrations of Cpd2 and all concentrations tested of Cpd1 had no effect. One explanation, considering that Cpd1 and Cpd2 were shown to exert their effect on transcription activation *via* GR, is that these SGRMs bind to another active site on GR, compared to classical GR ligands. In their recent review, Veleiro and colleagues recount how GR is a flexible protein, capable of adapting to different ligands (Veleiro, Alvarez et al. 2010). The ligand binding domain (LBD) of GR has a distinct ligand binding pocket (LBP) with features that explains ligand selectivity. In the case of dexamethasone, the steroid is enclosed in the LBP, in the bottom half of the GR LBD, occupying 65% of the volume of the GR pocket. Nature of hydrophobic interactions could, according to the authors, be responsible for at least part of the differences in binding affinity. Another interesting feature shown by the crystal structure is the conformational adaptability of GR, to accommodate different ligands, without affecting the structure of the co-activator binding site. On the other hand, different conformation of helix 12 and position of the H1-H3 loop could induce AF-2 conformational changes, possibly altering co-factors binding abilities and resulting in different transcriptional activities (Schoch, D'Arcy et al. 2010; Veleiro, Alvarez et al. 2010). Apart from dex (Bledsoe, Montana et al. 2002), several novel GR ligands have recently been investigated for their properties in fine tuning the AF-2 domain conformation, including some developed by Pfizer (Hu, Du et al. 2011) or other groups (Pecci, Alvarez et al. 2009).

The performance of the experimental SGRMs in transrepression and transactivation assays permitted the calculation of dissociation indices (DI). Whether derived from EC_{50} or fold changes values, DI confirmed the compounds preference for transrepression over transactivation. This was in agreement with the very definition of an SGRM, a compound selectively capable of inducing transrepression rather than transactivation. Hence, and according to popular literature belief, they should retain anti-inflammatory properties of classical GCs whilst having less unwanted secondary effects. This is not as straight forward as it could appear and is to be extensively discussed hereafter.

Before actively challenging the ability of SGRMs to exert anti-inflammatory effects, I first wanted to know if they were able to induce DUSP1 gene expression. A primary aspect of this was to test the compounds in term of their capacity to induce transcription *via* GC-responsive regions of the DUSP1 gene, previously characterised by our group (Tchen, Martins et al. 2010). Although Cpd1 and Cpd2 were able to induce transcription in cells transfected with the full length *DUSP1* promoter as well as those containing individual GRRs, Cpd1 and Cpd2 were less powerful than dex, with Cpd2 being the least powerful. In a chromatin IP assay the same type of pattern was observed, with dex and Cpd1 able to recruit GR to individual GRRs, but Cpd1 being weaker than its classical GC counterpart. Cpd2, on the other hand, did not seem to be able to recruit GR to these regions, which was surprising given the fact that this same compound's transactivation properties were shown to be, at least in part, dependent on GR. Although this experiment would benefit from further repeats, one explanation for this result could be that Cpd2 would recruit GR to other, unknown, region(s) of the *DUSP1* promoter. Another possibility would be that the timing of the experiment did not capture the interaction, that, somehow, the complex GR-Cpd2 detached from its corresponding responsive region. Interaction of transcription factors with binding sites in chromatin are now recognised as being highly dynamic, with occupancy times measured in seconds, rending obsolete a static binding concept. Dynamics of GR, thus, appear crucial in the transcriptional regulation of target genes (George, Schiltz et al. 2009). Differential regulation of cofactors and conformation of GR by novel GR ligand could therefore result in differential occupancy times at binding sites and differential regulation of target genes expression.

Another remark which can be made on this GR recruitment experiment is that recruitment by CPd1 at GRR-4.6 appeared much stronger than at GRR-1.3, suggesting a selection of one site over another and an impact of GR binding sites on the activity of the GR ligand. This concept was discussed recently by Meijsing and colleagues, where they show that GR binding sequences differentially affect GR conformation and co-factors recruitment, allowing tailored regulation of specific target genes (Meijsing, Pufall et al. 2009). Altogether, this and the ability of different GR ligand to differentially affect the receptor's conformation feed into a 'discussion' model where ligand influences GR conformation, impacting on site recognition. In return, selection of certain DNA binding sites would also feed back onto

regulation of receptor conformation and cofactor recruitment, adding an extra layer of gene expression regulation.

To better understand all these different levels of complex regulation, it would be interesting to perform ChIP sequencing, using different GR ligands. In each case, this would allow the identification of the cistrome of DNA-associated proteins and comparison between several compounds would help determine the basis of the differences between classical and novel GR ligands.

Gene induction responses to dex, Cpd1 and Cpd2 are described in terms of A_{max} (maximum response to saturating concentration of ligand) and EC_{50} (concentration required for half maximal response). Studies on different cell types from both mice and human origin showed that the power of the different ligands in inducing DUSP1 expression at either the mRNA or protein level was strongly cell dependent, as different patterns and rank orders of A_{max} and EC_{50} have been observed. As previously described (Simons 2008), these parameters can vary independently of one another in a cell-, gene- and context specific manner. In ways that are not fully understood, transcriptional responses to GR ligands may be influenced by levels of transcription factors and cofactors with which GR interacts, and activation of signalling pathways that modulate GR function. Similar variations in response are shown here. For example, the three compounds had opposite rank orders of A_{max} values in A549 and murine macrophages (Cpd2 > dex > Cpd1 in A549; Cpd1 > dex > Cpd2 in macrophages). There were also opposite rank orders of EC_{50} values in HeLa and RAW264.7 cells (Cpd2 < dex < Cpd1 in HeLa; Cpd1 < dex< Cpd2 in RAW264.7). It is also clear that transcriptional induction of real target genes may not be predictable from responses to stereotypical GRE-driven reporters. For example, although potency of the different GR ligands correlated for *DUSP1* induction in A549 cells and GRE-dependent reporter expression (dex < Cpd1 < Cpd2), A_{max} values in the same cell type were not comparable, with different values and rank orders. Together with the difficulties faced to demonstrate transrepression using an NF-κB reporter, these findings call into question whether simple reporter constructs are of use when investigating properties and mechanisms of action of novel glucocorticoid receptor ligands.

These observations support other studies discussing the role for cellular environment and co-regulator recruitment in GR-dependent gene expression regulation, whether it is by classical GR ligands or SGRMs. This position was acknowledged some years ago by Nissen who wrote in 2000 that "transcriptional regulators such as GR are likely to exploit a diversity of mechanisms across different cellular and promoter contexts" (Nissen and Yamamoto 2000). This was more recently supported by Ronacher and colleague who published in 2009 a study investigating the relationship between the potency and efficacy of transcriptional activity, the relative binding affinity for GR and the apparent affinity for cofactor interaction (Ronacher, Hadley et al. 2009). The study involved several GR ligands, dex and prednisone but also some of the SGRMs described in the past years, including AL-438 and CpdA. The GR ligands were challenged in common *in vitro* transactivation (GRE-dependent reporter) and transrepression (NF-κB- and AP-1-dependent reporters) models and two-hybrid assays for co-regulators interaction. Clearly, all GR ligands had different patterns of activity, with CpdA being considered as "dissociated" whilst AL-438 was referred to as a "partial [GR] agonist", strongly corroborating results reported in the present chapter, on the transcriptional regulation abilities of the experimental SGRMs studied. The authors conclude that their study supports the idea that differential interaction of GR with co-regulators is ligand-selective and that it could be the biochemical basis for ligand- and promoter-specific differences in potency and efficacy of transactivation, as well as transrepression via AP-1 and NF-κB tethering mechanisms. In the case of AL-438 and CpdA, this concept is illustrated by the striking difference in GRIP-1 interaction: AL-438 recruited GRIP-1 to only 25% of that of dex, whilst CpdA had no effect (Ronacher, Hadley et al. 2009). It would be interesting to see if similar pattern of co-factor interaction can also be found in the case of Cpd1, Cpd2 or CpdA and if this correlates with a certain potency and efficacy, compared to those of typical GR ligands such as dex.

Chapter 5

DUSP1 DEPENDENCY OF THE ANTI-INFLAMMATORY EFFECTS OF SGRMS

5. DUSP1 DEPENDENCY OF THE ANTI-INFLAMMATORY EFFECTS OF SGRMS

In this chapter, I wanted to investigate (i) the effects of SGRMs on the expression of inflammatory mediators in the context of an inflammatory response and (ii) the dependency on DUSP1 of these anti-inflammatory effects.

To address the first point, expression of DUSP1 and pro-inflammatory mediators was measured in different cell types, in response to treatment with SGRMs and stimulation with pro-inflammatory stimuli. A first set of experiments was conducted in the A549 cell line, in order to correlate results with the effects of the compounds on simple GRE-dependent and NF-κB-dependent reporter constructs. IL-1β was used as an inflammatory stimulus, since it strongly induces expression of COX-2 and other inflammatory mediators in these cells. Other cell types include HeLa, RAW cells and BMDMs.

The second part of this chapter aimed to investigate the dependency on DUSP1 of the anti-inflammatory effects of the different SGRMs tested. This was performed using BMDMs from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice, pre-treated with different GR ligands and stimulated with LPS. Expression of several inflammatory mediators was quantified and compared between wild type and *Dusp1* deficient cells.

5.1. Anti-inflammatory effects of CpdA

In the last section of the previous chapter, DUSP1 mRNA was shown to be transiently induced by CpdA in cell lines from both human and murine origin. Here we want to know how CpdA regulates *DUSP1*, *COX-2* and *IL-6* mRNA expression in an inflammatory context. A549 cells were treated with vehicle (0.1% DMSO), increasing concentrations of dex or CpdA (1 nM to 10 µM) for 2 h then stimulated with 1 ng/ml of IL-1β for 4 h. Cells were then harvested, mRNA extracted and *DUSP1*, *COX-2* and *IL-6* mRNA expression quantified by real-time PCR. Results were normalised against the expression of the house keeping gene *GAPDH* and shown as a percentage of the response to treatment with IL-1β alone (set as 100%) (Figure 5.1).

As expected, *DUSP1* appeared to be strongly induced by dex, in a dose responsive manner. CpdA, however, did not appear to up-regulate *DUSP1* at all, with its levels being constant throughout the dose response experiment (Figure 5.1 a).

As previously described (Newton, Seybold et al. 1998; Tsatsanis, Androulidaki et al. 2006; Quante, Ng et al. 2008; Weber, Wasiliew et al. 2010) *COX-2* (Figure 5.1 b) and *IL-6* (Figure 5.1 c) mRNAs were strongly up-regulated by IL-1β and inhibited in a dose dependent manner by dex, returning near their basal levels of expression when treated with 10 nM or more of the classical GR ligand. On the other hand, CpdA was not able to inhibit the expression of these two pro-inflammatory mediators, regardless of the dose used. Even when the cells were treated with 10 µM of CpdA, no anti-inflammatory effect could be seen, when anti-inflammatory effects of this dose had previously been reported, particularly on IL-6 expression (De Bosscher, Berghe et al. 2005; De Bosscher, Haegeman et al. 2010; van Loo, Sze et al. 2010).

In our experimental set up, CpdA therefore did not appear to have any anti-inflammatory properties.

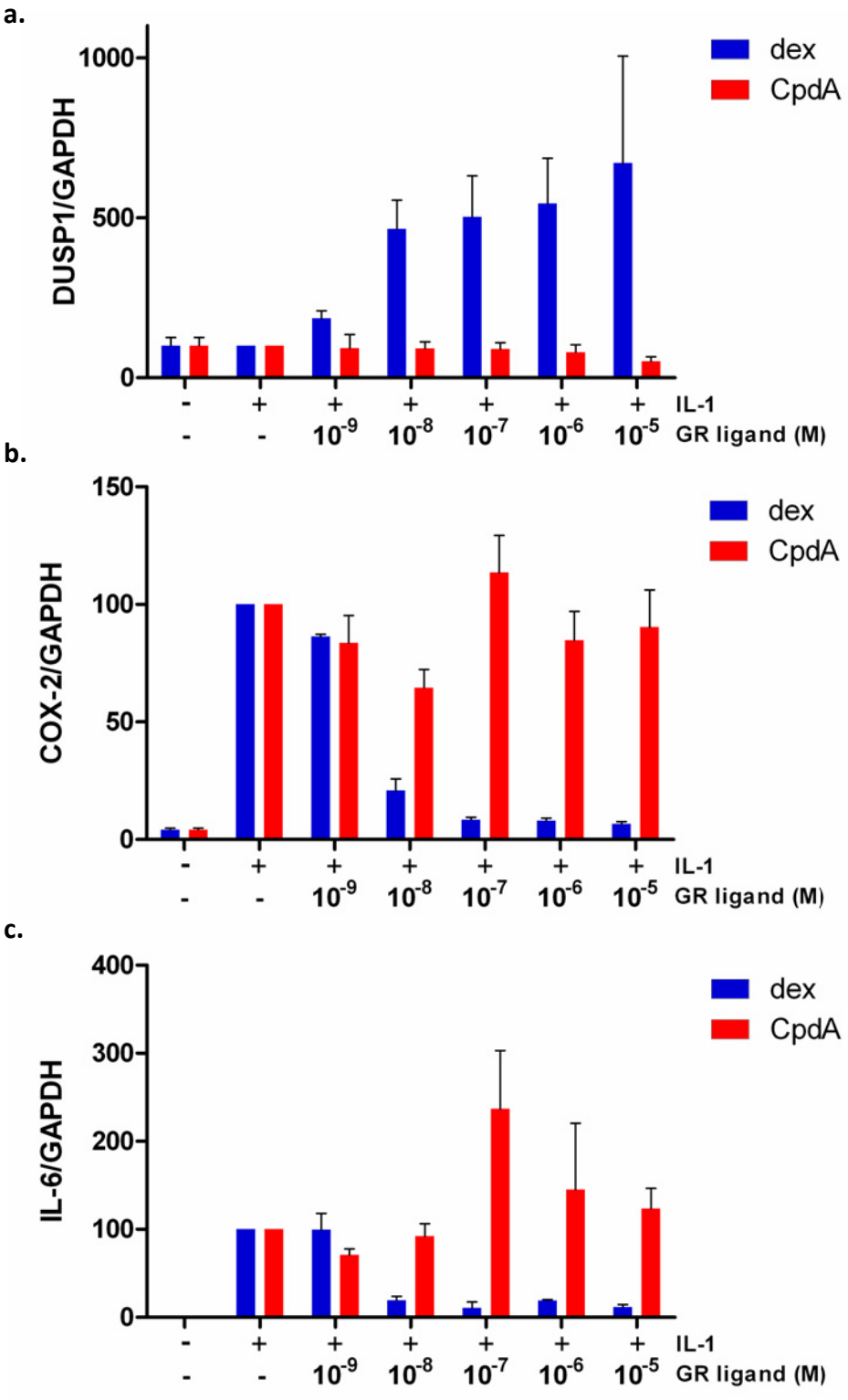


Figure 5.1. Effects of CpdA on DUSP1, COX-2 and IL-6 mRNA expression in A549 cells.
Cells were pre-treated with vehicle (0.1% DMSO) or increasing concentrations (1 nM to 10 µM) of dex or CpdA for 2 h then stimulated with 1 ng/ml of IL-1β for 4 h. mRNA was harvested and ***(a)*** *DUSP1 (n=4),* ***(b)*** *COX-2 (n=4) and* ***(c)*** *IL-6 (n=2) mRNA quantified by real-time PCR. Graphs represent mean mRNA expression ± SEM, normalised against that in cells treated with IL-1β alone (set as 100%).*

5.2. Effects of dex, Cpd1 and Cpd2 on DUSP1 and COX-2 expression

Having established that both Cpd1 and Cpd2 were able to induce the expression of DUSP1 in cell types from both human and murine origin (Figure 4.7 to 4.10), we investigated the relationship between DUSP1 up-regulation and the anti-inflammatory effects of SGRMs in the same cell types (Figures 5.3 to 5.6). Suppression of COX-2 was selected as a read-out of anti-inflammatory efficacy, because this gene is up-regulated by different pro-inflammatory stimuli in many cell types, is a well characterised GC target and its regulation involves the NF-κB pathway. *COX-2* promoter contains one (murine) or two (human) NF-κB sites, participating to the gene's tight transcriptional regulation (Newton, Kuitert et al. 1997; Kang, Mbonye et al. 2007; Cho and Kim 2009).

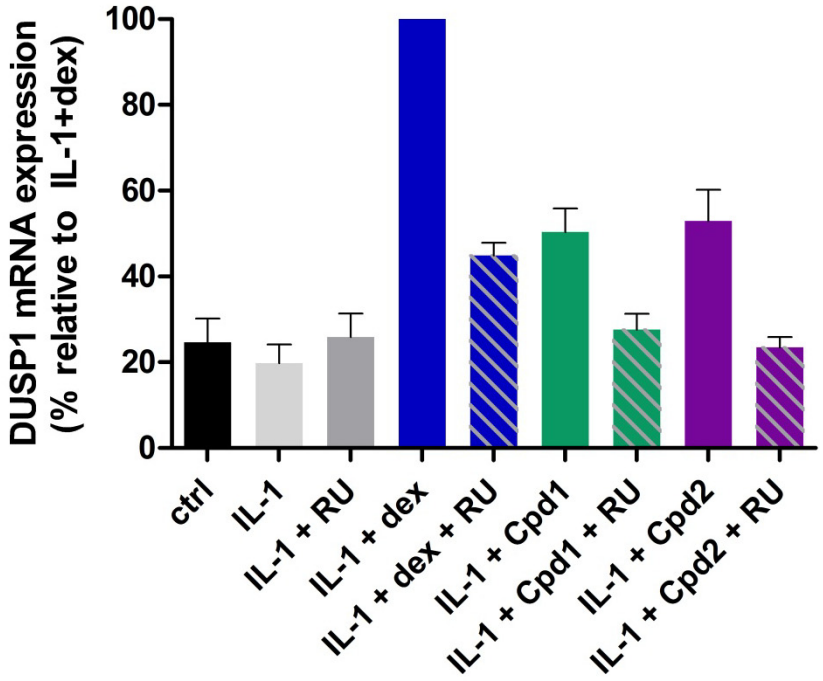
But before investigating these anti-inflammatory effects in more depth, I wanted to determine whether the observed effects of Cpd1 and Cpd2 were the result of the compounds interaction with GR. To address this question, we used the GR antagonist RU486 (RU) to co-treat A549 cells. If the effects of Cpd1 or Cpd2 were indeed mediated by GR, then co-treatment with RU would abrogate whatever effect is seen with the compound on its own. On the other hand, if the SGRMs effects were to be the result of any other type of interaction ligand-receptor (e.g. MR), then addition of RU would not make a difference in the outcome of the experiment, compared to the results obtained with Cpd1 or Cpd2 on their own.

Here (Figure 5.2), confluent A549 cells were treated with a combination of vehicle (0.1% DMSO), IL-1β and/or dex, Cpd1 or Cpd2 (1 µM each), according to the figure legend. Briefly, cells were pre-treated for 2 h with vehicle, RU486, dex, Cpd1 or Cpd2 alone or in combination, then stimulated with 1ng/ml of IL-1β for 4 h. At the end of the incubation period, cells were lysed, mRNA extracted and quantified by RT-PCR. *DUSP1* mRNA expression was plotted relative to that induced by IL-1β + dex (Figure 5.2 a). *COX-2* mRNA expression was plotted relative to that induced by IL-1β alone (Figure 5.2 b).

First of all, it is worth describing the results of dex, Cpd1 and Cpd2 on *DUSP1* and *COX-2* mRNA expression in an inflammatory context. In agreement with results described in Figure

5.1, dex was able to strongly induce *DUSP1* (Figure 5.2 a) and inhibit *COX-2* mRNA expressions when cells where stimulated by a pro-inflammatory stimulus. Interestingly, Cpd1 and Cpd2 were also capable of inducing *DUSP1* and reducing *COX-2* mRNA expression, although to a lesser extent than dex. As it can be observed in Figure 5.2, addition of RU486 to GR-ligand treated cells incompletely blocked the upregulation of *DUSP1* mRNA by dex (panel a), and incompletely rescued *COX-2* from dex-mediated suppression (panel b). RU486 completely prevented the upregulation of *DUSP1* mRNA by Cpd1 or Cpd2, and effectively rescued *COX-2* mRNA from inhibition by either of the SGRMs. Like dex, Cpd1 and Cpd2 therefore appear to be exerting anti-inflammatory effects via GR and not via an off-target mechanism.

a.



b.

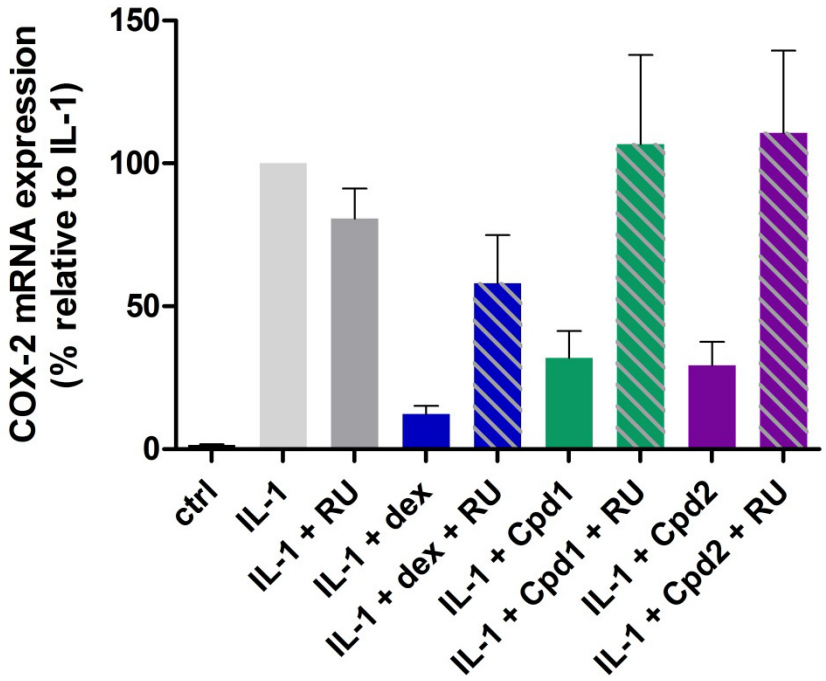


Figure 5.2. Effect of RU486 on dex-, Cpd1- and Cpd2-regulated DUSP1 and COX-2 mRNA expression.

A549 cells were treated with vehicle (0.1% DMSO), IL-1β (1 ng/ml), RU486 (RU), dex, Cpd1 or Cpd2 (each 1 μM) in the combinations indicated. mRNA was harvested and DUSP1 ***(a)*** *and COX-2* ***(b)*** *mRNAs quantified by real-time PCR and expressed with respect to cells treated with either IL-1β alone (Cox-2) or IL-1β + dex (Dusp1). Graphs represent averages ± SEM from four independent experiments, realised with the technical support of D. Qize.*

Once established that the SGRMs used here were exerting anti-inflammatory effects *via* GR, the next step was to look into these anti-inflammatory effects in more detail. For this purpose, murine and human cells were used and DUSP1 and COX-2 expression measured, both at the mRNA and protein levels.

Confluent cells were pre-treated for 2 h with increasing doses of dex, Cpd1 or Cpd2 (1 nM to 1 µM) then stimulated for 4 h with the appropriate pro-inflammatory stimulus (1 ng/ml IL1β for A549 and HeLa cells; 10 ng/ml LPS for RAW cells and BMDMs). *DUSP1* (continuous line) and *COX-2* (dashed line) mRNA were quantified by RT-PCR, whereas protein expression was assessed by Western blotting. For each cell type, panel (a) shows *DUSP1* and *COX-2* mRNA expression as a percentage of the maximal response to each treatment (dex in blue, Cpd1 in green and Cpd2 in purple), the corresponding proteins expression being shown in panel (b). Tubulin protein expression was used as loading control.

In A549 cells, at the mRNA level and in all cases (Figure 5.3 a) *COX-2* expression was strongly increased by stimulation with IL-1β whereas *DUSP1* did not seem to be much affected by the addition of the pro-inflammatory stimulus. However this observation could be explained by the fact that IL-1β-induced expression of *DUSP1* in A549 cells peaks at 1 h (unpublished observations), whereas the time point used here is much later. In this inflammatory context, the addition of increasing doses of dex, Cpd1 and Cpd2 led to dose dependent induction of *DUSP1* and inhibition of *COX-2*. An interesting thing to note here is that the induction of *DUSP1* seems to mirror the inhibition of *COX-2* expression, as symmetry can be observed between the responses to the different doses of GR ligands. Comparing the graphs, both responses appeared stronger with dex than with Cpd1 and Cpd2. This was confirmed by the calculation of EC_{50} for *DUSP1* induction and EC_{50} values for *COX-2* inhibition (Table 5.1). For both *DUSP1* induction and *COX-2* inhibition dex < Cpd1 < Cpd2, confirming dex as the most potent drug in this assay. Moreover, EC_{50} values were very closely related, for each treatment, reinforcing the apparent correlation between *DUSP1* induction and *COX-2* inhibition.

At the protein level (Figure 5.3 b) COX-2, but also DUSP1 were induced by IL-1β stimulation. Similarly as for mRNA expression, DUSP1 was increased and COX-2 decreased in a dose dependent manner in response to the increasing doses of dex, Cpd1 and Cpd2. Again, a "mirror effect" could be observed when putting in parallel the two responses. This was further confirmed by quantification of COX-2 protein expression and calculation of EC_{50} for each treatment (Table 5.1). As for mRNA, dex < Cpd1 < Cpd2 and the values strongly agreed with those obtained for *COX-2* inhibition and *DUSP1* induction at the mRNA level. EC_{50} values for induction of DUSP1 protein could not be determined because of non-specific background in the western blots.

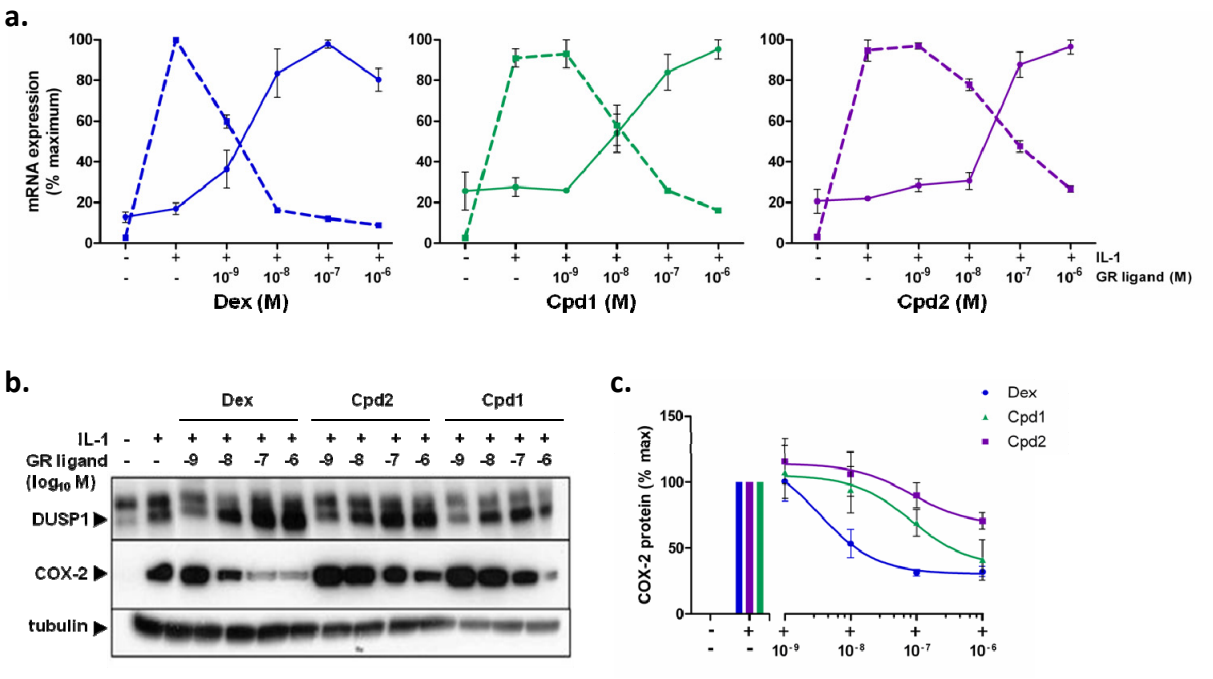


Figure 5.3. Effects of dex, Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in A549 cells.
Cells were pre-treated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (1 nM to 1 μM) of dex, Cpd1 or Cpd2; then challenged for 4h with 1 ng/ml of IL-1 β. ***(a)*** *Expression of DUSP1(—) and COX-2(---) mRNAs.* ***(b)*** *Representative western blot of DUSP1 and COX-2 protein expression.* ***(c)*** *COX-2 protein quantification from three independent experiments. Proteins were detected by western blotting and quantified by scanning densitometry. mRNAs were quantified by RT-PCR. Results are presented as percentages of the maximal response. Graphs indicate means ± SEM from three independent experiments.*

The same type of experiment was carried out in HeLa (Figure 5.4), RAW (Figure 5.5) cells and BMDMs (Figure 5.6). Globally the same "mirror effect" was observed. In all three cell types *DUSP1* mRNA was dose dependently increased by dex, Cpd1 and Cpd2, while *COX-2* expression was dose dependently decreased; with the exception of the response to Cpd2 in HeLa cells (Figure 5.4 a), where high experimental variation made the effect less clear. In that same cell type, dex appeared to be again the most potent GR ligand (Table 5.1; dex < Cpd1 < Cpd2), with the same rank order of EC_{50} of *DUSP1* induction and EC_{50} of *COX-2* inhibition, for which potency of dex and Cpd1 were equal (EC_{50} = $1.9x10^{-8}$ M) . In the macrophage-like RAW264.7 cell line (Figure 5.5 a), dex, Cpd1 and Cpd2 had almost identical EC_{50} values for induction of *Dusp1* and very close EC_{50} values of *Cox-2* inhibition, indicating a strong similarity in potency for dex and the two SGRMs (Table 5.1). In bone marrow derived macrophages (Figure 5.6 a) the same symmetrical induction of *Dusp1/*inhibition of *Cox-2* was observed, with *Cox-2* mRNA levels being very strongly repressed, especially in response to the treatment with dex. The potent suppression of *Cox-2* mRNA by dex and Cpd1 rendered the calculation of EC_{50} difficult with the software and were therefore verified manually. However, EC_{50} values of induction and EC_{50} values of inhibition were still in very close agreement (Table 5.1).

DUSP1 and COX-2 proteins were detected in all three cell types. Again, both proteins were induced by IL-1β or LPS. DUSP1 was dose dependently upregulated by dex, Cpd1 and Cpd2, while COX-2 expression was dose dependently inhibited by the three GR ligands. As in A549 cells, for each GR-ligand, COX-2 levels were the opposite of DUSP1 protein levels: the stronger the expression of DUSP1 was, the weaker was COX-2's. The potency of the different GR ligands described earlier at mRNA level also matched at the protein level. For example, in BMDMs (Figure 5.6 b), dex was the compound able to induce the strongest expression of DUSP1, when the inhibition of COX-2 was complete, from as little as 1 nM of the drug. Cpd1 was still very powerful, followed by Cpd2 which induced DUSP1 in a weaker manner than its counterparts. As a consequence COX-2 expression was totally inhibited only from the 100 nM dose. These observations are in agreement with those made at the mRNA level, when comparing the graphs of the responses to dex, Cpd1 and Cpd2.

All together this data obtained in different cell types, support the idea that a strong correlation exists between the induction of DUSP1 and the inhibition of COX-2 by the different GR ligands.

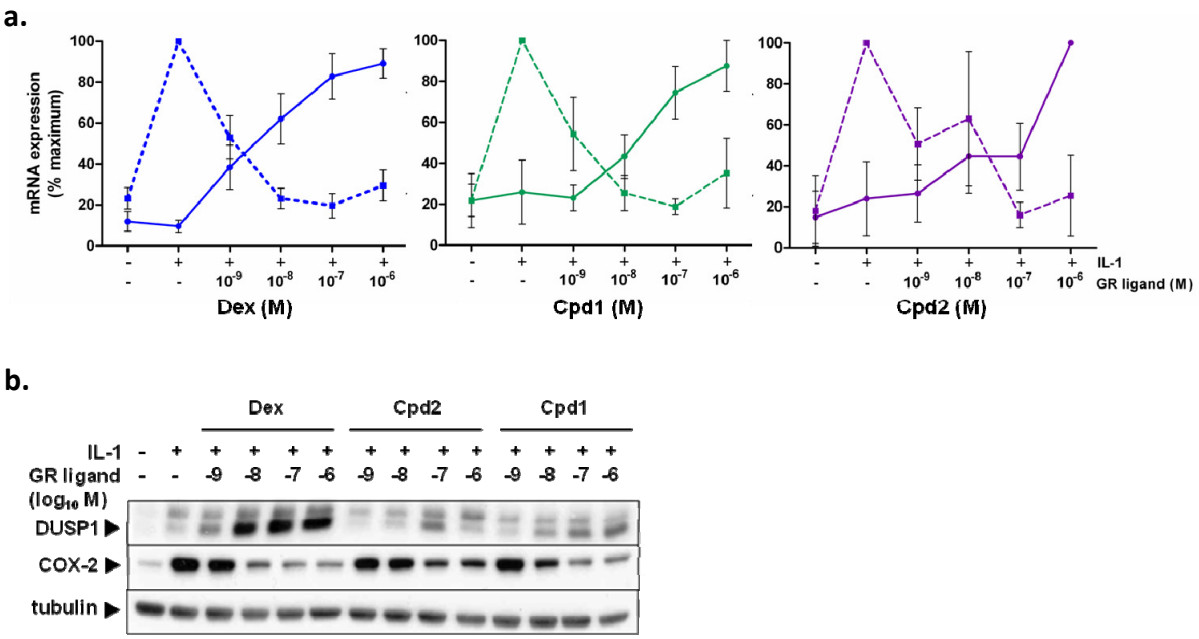


Figure 5.4. Effects of dex, Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in HeLa cells.
*Cells were pre-treated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (1 nM to 1 µM) of dex, Cpd1 or Cpd2; then challenged for 4h with 1 ng/ml of IL-1 β. **(a)** Expression of DUSP1(—) and COX-2(---)mRNAs. **(b)** Representative western blot of DUSP1 and COX-2 protein expression. mRNAs were quantified by RT-PCR. Results are presented as percentages of the maximal response. Graphs indicate means ± SEM from at least three independent experiments.*

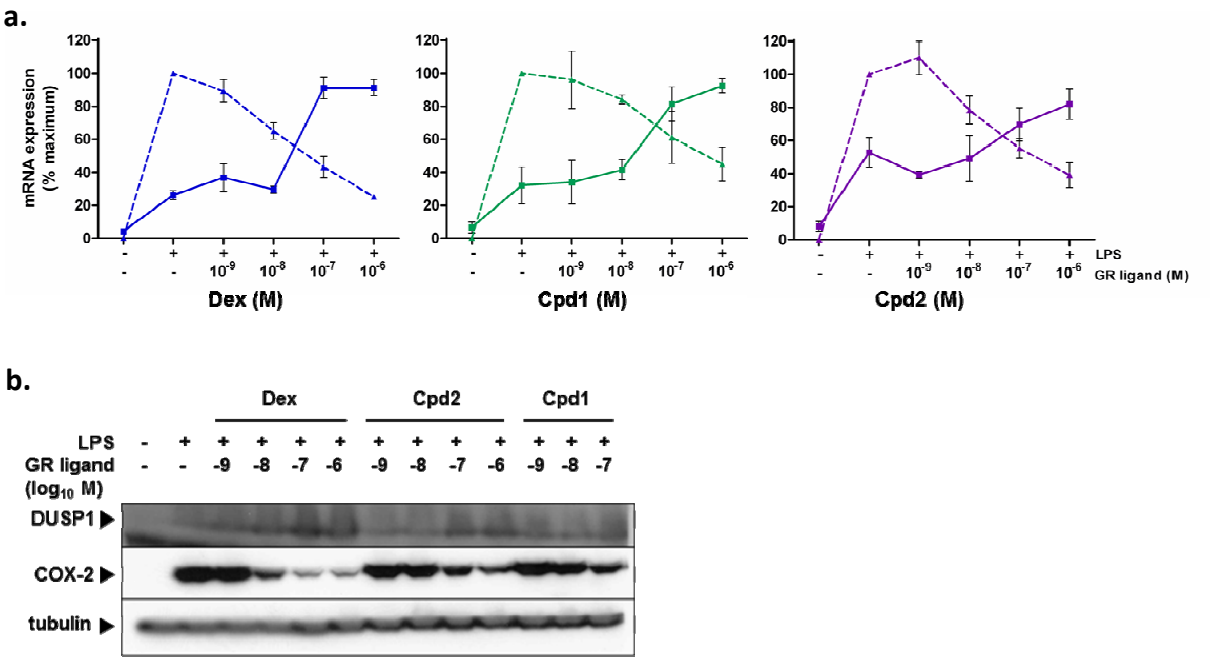


Figure 5.5. Effects of Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in RAW cells.
Cells were pre-treated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (1 nM to 1 µM) of dex, Cpd1 or Cpd2; then challenged for 4h with 10 ng/ml of LPS. ***(a)*** *Expression of DUSP1(—) and COX-2(---) mRNAs.* ***(b)*** *Representative western blot of DUSP1 and COX-2 protein expression. mRNAs were quantified by RT-PCR. Results are presented as percentages of the maximal response. Graphs indicate means ± SEM from three independent experiments.*

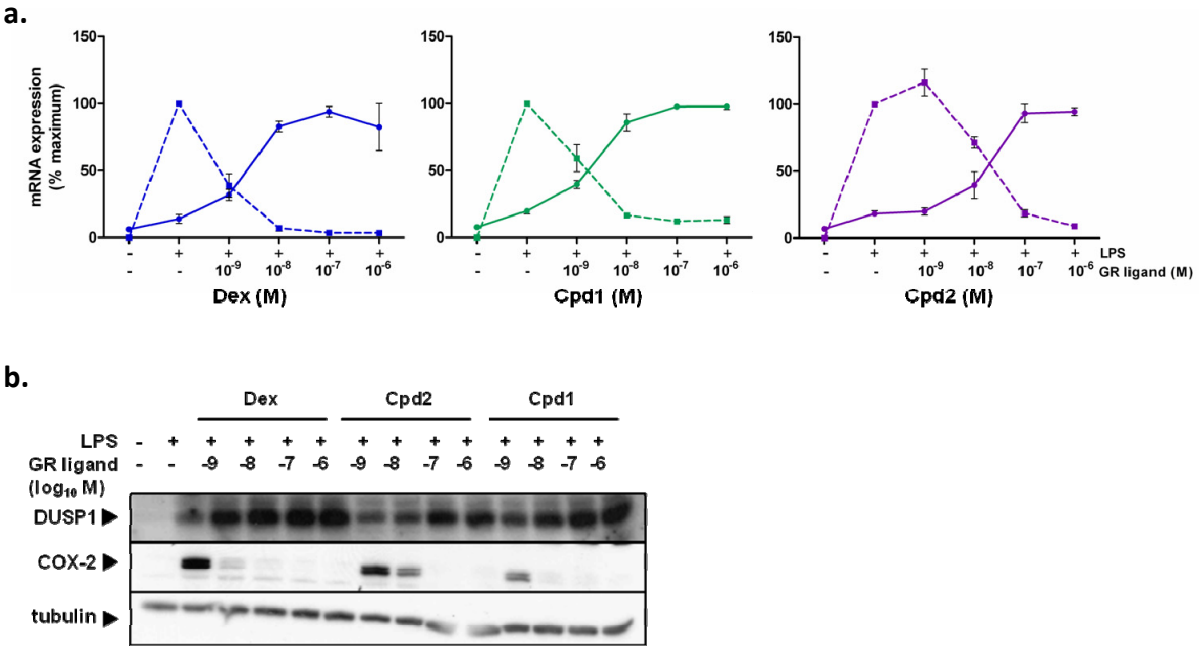


Figure 5.6. Effects of Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in BMDMs.
Cells were pre-treated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (1 nM to 1 µM) of dex, Cpd1 or Cpd2; then challenged for 4h with 10 ng/ml of LPS. ***(a)*** *Expression of DUSP1(—) and COX-2(---) mRNAs.* ***(b)*** *Representative western blot of DUSP1 and COX-2 protein expression. mRNAs were quantified by RT-PCR. Results are presented as percentages of the maximal response. Graphs indicate means ± SEM from three independent experiments.*

	EC_{60} DUSP1 induction			EC_{60} COX-2 inhibition		
	Dex	**Cpd1**	**Cpd2**	**Dex**	**Cpd1**	**Cpd2**
A549 mRNA	(1) $7.0x10^{-9}$	(2) $1.5x10^{-8}$	(3) $3.8x10^{-8}$	(1) $6.3x10^{-9}$	(2) $1.0x10^{-8}$	(3) $3.2x10^{-8}$
protein				**(1) $5.2x10^{-9}$**	**(2) $3.4x10^{-8}$**	**(3) $8.5x10^{-8}$**
HeLa	(1) $1.2x10^{-8}$	(2) $2.5x10^{-8}$	(3) $4.0x10^{-7}$	(1) $1.9x10^{-9}$	(1) $1.9x10^{-9}$	(2) $3.2x10^{-8}$
RAW	**(2) $3.7x10^{-8}$**	**(1) $3.6x10^{-8}$**	**(3) $3.9x10^{-8}$**	**(1) $1.7x10^{-8}$**	**(3) $4.9x10^{-8}$**	**(2) $2.0x10^{-8}$**
BMDMs	(1) $2.0x10^{-9}$	(2) $3.9x10^{-9}$	(3) $1.2x10^{-8}$	(1) $2.1x10^{-9}$	(1) $2.1x10^{-9}$	(2) $1.3x10^{-8}$

Table 5.1. Comparison of the EC_{50} of DUSP1 induction and COX-2 inhibition in the different cell types studied.

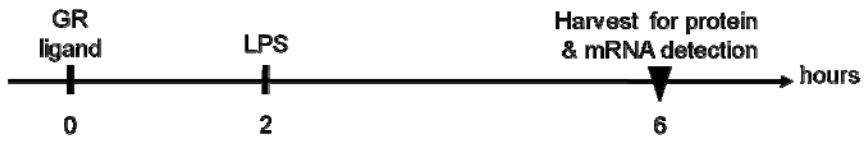
EC_{50} (M) were calculated for the dex, Cpd1 and Cpd2 dose responses in A549, HeLa, RAW cells and BMDMs. Values were obtained by non-linear regression analysis, with the help of the Prism 5.0 software. Numbers in brackets indicate the rank order of the efficacy of the different compounds.

5.3. Anti-inflammatory effects and DUSP1 dependency

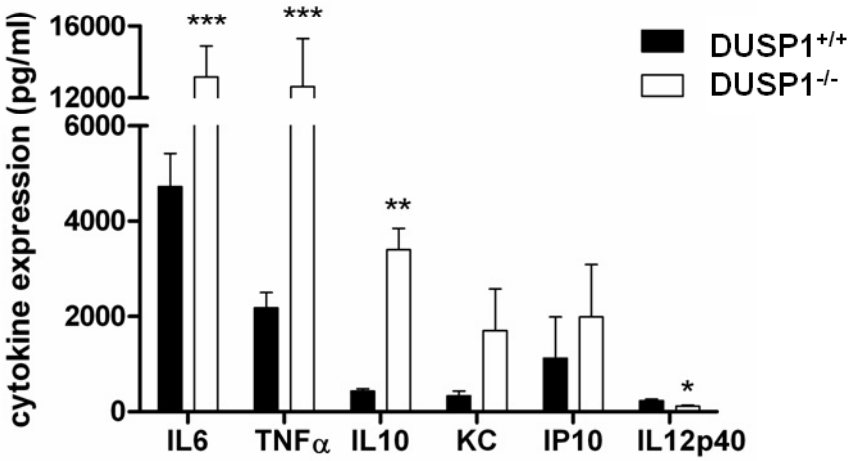
The results described in the previous section are consistent with, but do not prove, an important role for DUSP1 in the anti-inflammatory effects of both dex and SGRMs. To investigate this further, we examined the effects of GR ligands on the expression of several inflammatory genes in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ murine bone marrow derived macrophages. Cells were pre-treated with 10 nM or 1 µM of dex, Cpd1 or Cpd2 for 2 h then stimulated with 10 ng/ml of LPS for another 4 h. At the end of the incubation period, supernatants were harvested for cytokine detection while cell lysates were kept for later mRNA extraction and quantification (Figures 5.8 to 5.17). IL-6, TNFα, IL-10, KC and IP10 protein expression were measured by Luminex, IL-12p40 by ELISA. *Cox-2, IL-1α, TTP* and *iNos* mRNA were quantified by real time PCR. Figure 5.7 shows the basal levels of the different inflammatory mediators quantified. Results are presented as cytokine and mRNA expression after LPS treatment, in both *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages. Figures 5.8 to 5.17 represent the expression of each inflammatory mediator as a percentage of their expression in response to LPS alone (set as 100%) in wild type and *Dusp1* deficient BMDMs. Significance was determined by comparison of each dose of dex or SGRMs to the corresponding LPS-induced expression (#), as well as for the comparison of their effects in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages (*).

After LPS stimulation, IL-6, TNFα and IL-10 protein levels appeared to be significantly higher in the cells lacking *Dusp1* (Figure 5.7). Although not statistically significant, KC protein*, Cox-2, IL-1α* and *iNos* mRNA expression levels also appeared to be higher in response to the pro-inflammatory stimulus in the *Dusp1* deficient cells. This over expression of inflammatory mediators was consistent with similar observation by our group (Abraham, Lawrence et al. 2006) and studies reporting hyper sensitivity to pro-inflammatory challenges in *Dusp1* knock-out mice (Chi, Barry et al. 2005; Hammer, Mages et al. 2006; Wang, Zhao et al. 2009; Yu, Li et al. 2011). IP10 expression did not seem to differ between WT and KO macrophages, whereas IL-12p40 levels were significantly lower in the *Dusp1*$^{-/-}$ BMDMs. *TTP* mRNA expression also seemed to be weaker in the KO cells, despite not coming out as a significant difference.

a.



b.



c.

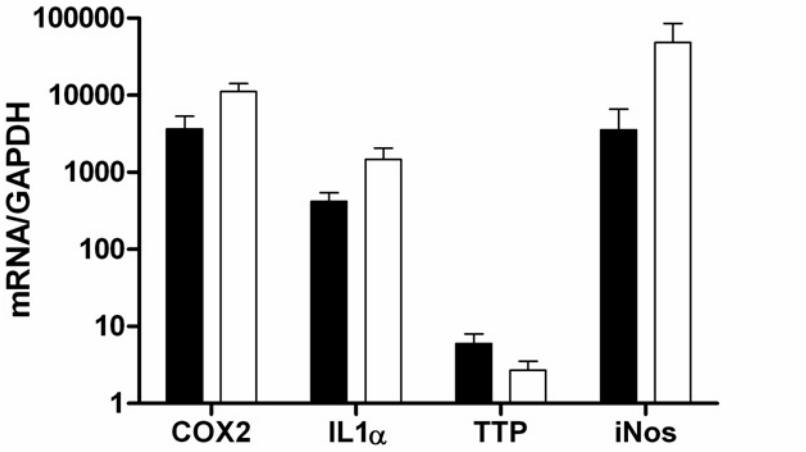


Figure 5.7. Expression of inflammatory mediators after LPS stimulation of Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macropages.

(a) *BMDMs from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice were pre-treated with vehicle (0.1% DMSO), 10 nM or 1 µM of dex, Cpd1 or Cpd2, then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, supernatants were harvested for cytokine detection and cells were lysed for mRNA extraction.*

Inflammatory mediators were quantified at time 0, after treatment with LPS alone. ***(b)*** *IL-6, TNFα, IL-10, KC and IP-10 proteins expression were detected by Luminex, IL-12p40 by ELISA.* ***(c)*** *COX-2, IL-1α, TTP and iNOS mRNAs were detected by real-time PCR. Graphs indicate means ± SEM from at least three independent experiments. ***, p < 0.001; **, p < 0.01; *, p < 0.05 relative to the difference between the mean expression in cells from DUSP1$^{+/+}$ and Dusp1$^{-/-}$.*

From the data used in Figure 5.6, and with the same experiment design, I calculated A_{max} of DUSP1 induction with dex, Cpd1 and Cpd2, in LPS-stimulated macrophages. Dex up-regulated DUSP1 with an A_{max} of 7.1, whilst Cpd1 and Cpd2 had very similar potencies (5 and 5.4, respectively). As described previously, efficacy was also calculated (Table 5.1), with EC_{50} for dex coming up at $1.32x10^{-11}$ M, Cpd1 at $1.42x10^{-9}$ M and Cpd2 at $1.92x10^{-8}$ M. These observations suggest that DUSP1 expression was maximal or near maximal at both 10 nM (10^{-8} M) and 1 µM (10^{-6} M) for dex and Cpd1. On the other hand, Cpd2 induced DUSP1 with a considerably higher EC_{50}, hence DUSP1 expression was still increasing in the range of 10 nM to 1 µM. As the aim of this section is to investigate the dependency on DUSP1 of the anti-inflammatory effects of SGRMs, these observations provide context for the analysis of the responses to SGRMs in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages.

We (Abraham, Lawrence et al. 2006), and others (Quante, Ng et al. 2008; Turpeinen, Nieminen et al. 2010), previously reported that the dex-mediated inhibition of IL-6 mRNA and protein expression was largely dependent on DUSP1. In agreement with these previous observations we show here a strong repression of IL-6 protein expression by both doses of dex in the *Dusp1*$^{+/+}$ cells ($p < 0.01$ and 0.001), but no effect of the GC in *Dusp1*$^{-/-}$ cells (Figure 5.8 a). Cpd1 was also able to very strongly inhibit IL-6 expresion, to about the same extent as dex in the wild type cells (Figure 5.8 b), whereas Cpd2 reached these levels of efficacy at only the higher dose (Figure 5.8 c). Again, almost no anti-inflammatory effect could be seen in response to either SGRM- in *Dusp1*$^{-/-}$ cells, suggesting entire dependence on DUSP1. This latter observation is supported by highly significant differences between wild type and Dusp1 deficient cells, in response to both doses of compounds.

I also investigated the effect of all three GR ligands on TNFα protein expression (Figure 5.9). 10 nM and 1 µM of dex strongly decreased the expression of TNFα protein in *Dusp1*$^{+/+}$ macrophages but had relatively little effect in the *Dusp1* deficient cells (Figure 5.9 a), confirming the dependency on DUSP1 previously observed at both the mRNA and protein level (Abraham, Lawrence et al. 2006). Likewise, Cpd1 significantly inhibited TNFα protein expression at both doses (Figure 5.9 b), whereas Cpd2 was effective only at the higher dose (Figure 5.9 c). Cpd1-mediated inhibition of TNFα expression was also clearly dependent on

DUSP1, although in this case the difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages became significant only at 1 µM. No difference in TNFα expression could be seen at the 10 nM dose of Cpd2, but macrophages treated with that same compound had significantly different levels of TNFα mRNA between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells at the higher doses tested in this experiment, suggesting at least some partial dependency on DUSP1.

As presented on Figure 5.10, IP-10 protein expression was clearly repressed by dex at both 10 nM and 1 µM in wild type macrophages, whereas no inhibitory effect could be seen in the cells from *Dusp1* knock-out animals. A significant difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells was found for 1 µM of dex, supporting some dependency on DUSP1 (Figure 5.10 a). Although not significant, Cpd1 (Figure 5.10 b) and Cpd2 (Figure 5.10 c) seemed to be able to negatively regulate IP-10 protein expression, to a maximum of 37% and 34% repression, respectively, at 1 µM. Differences between wild type and *Dusp1* deficient cells could also be noticed. However, it was significant only for 10 nM of Cpd2.

Suppression of IP-10 by the classical GC dex appeared relatively weak but apparently dependent on DUSP1. The SGRMs being weaker inhibitors, dependence on DUSP1 was therefore difficult to demonstrate here.

In this series of experiments, we also looked at KC protein expression. Previous work in our lab (Abraham, Lawrence et al. 2006) reported quite strong inhibition of KC mRNA and protein expression in response to dex in *Dusp1*$^{+/+}$ cells, whereas dex increased the expression of KC mRNA and protein in *Dusp1*$^{-/-}$ cells, therefore identifying KC as one of the DUSP1 dependent targets of dex. Here (Figure 5.11), we found KC protein expression to be repressed to 26% and 39% in response by 10 nM and 1 µM of dex in *Dusp1*$^{+/+}$ cells, whereas expression was increased by dex in the *Dusp1* deficient cells (Figure 5.11 a). At the higher dose of dex, the difference in KC expression between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$was highly significant ($p < 0.01$), confirming the DUSP1 dependent regulation of KC reported by Abraham and colleague a few years ago. In the wild type cells, neither Cpd1 (Figure 5.11 b) nor Cpd2 (Figure 5.11 c) inhibited KC expression, but both compounds increased expression of KC protein in cells lacking *Dusp1.* Although the difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$

was significant at only one dose of Cpd1 or Cpd2 (1 μM and 10 nM, respectively), these observations still suggest a role for DUSP1 in the regulation of KC by the SGRMs.

Another protein whose expression was investigated here is the anti-inflammatory cytokine IL-10. In agreement with previous work from our group (Abraham, Lawrence et al. 2006), IL-10 protein (Figure 5.12 a) expression was not modified by the different doses of dex in the wild type cells but was upregulated by the same treatment in the *Dusp1* deficient macrophages. The difference between wild type and *Dusp1* deficient cells became significant at the higher dose of dex. Similarly, IL-10 expression was not disrupted by the SGRMs in *Dusp1*$^{+/+}$ cells. Again, in the cells lacking *Dusp1*, IL-10 expression appeared to be increased in response to both Cpd1 and Cpd2, the difference reaching significance at the higher dose of Cpd1 ($p < 0.001$) (Figure 5.12 b) and both doses of Cpd2 (Figure 5.12 c).

The last protein whose expression we studied here is IL-12p40. In their study, Abraham and colleagues found dex-mediated overall inhibition of IL-12p40 mRNA expression to be independent of DUSP1. However it could be argued that this was not the case for the 10 nM dose of dex, for which the author reported more inhibition of IL-12p40 mRNA expression in the *Dusp1*$^{-/-}$ cells ($p=0.034$). Even so, this difference was not seen at higher doses investigated and overall dex-mediated inhibition of IL-12p40 was concluded to be independent of DUSP1 (Abraham, Lawrence et al. 2006). In agreement with these findings, we show in the present report that dex was strongly able to repress IL-12p40 protein expression, but that no difference could be found between the responses in the *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages (Figure 5.13 a). Although a little less potent at repressing IL-12p40 than dex, Cpd1 (Figure 5.13 b) and Cpd2 (Figure 5.13 c) both appeared to regulate IL-12p40 expression independently of DUSP1.

Next, I looked at *Cox-2* mRNA expression regulation. As expected, *Cox-2* expression was strikingly reduced in response to both doses of dex in the *Dusp1*$^{+/+}$ macrophages, up to 93.5% inhibition. In agreement with many other studies (Riina, Riku et al. ; Abraham, Lawrence et al. 2006; Cho and Kim 2009; Turpeinen, Nieminen et al. 2010), we found this response to be impaired in *Dusp1*$^{-/-}$ BMDMs (Figure 5.14 a), confirming once again the

partial dependency on DUSP1 of dex-mediated COX-2 inhibition. Cpd1 was also able to strongly repress *Cox-2* mRNA expression in the wild type cells. So was Cpd2 but again was less potent at the lower dose. In the *Dusp1* deficient BMDMs, the response to Cpd1 (Figure 5.14 b) and Cpd2 (Figure 5.14 c) could be considered as partially impaired although the difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells did not prove statistically significant. This would once more suggest a role for DUSP1 in the regulation of COX-2 expression by SGRMs.

Another mRNA expression investigated here is IL-1α. Once again in agreement with previous findings (Abraham, Lawrence et al. 2006), dex was able to inhibit IL-1α mRNA expression in wild type BMDMs, up to 78%. This response was found to be partially impaired in the cells lacking *Dusp1*, with a non-significant inhibition, barely reaching 50% (Figure 5.15 a) The difference in dex response of *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ cells was statistically significant at 10 nM dex. As seen for other inflammatory mediators described earlier, Cpd1 (Figure 5.15 b) and Cpd2 (Figure 5.15 c) were also able to strongly reduce IL-1α mRNA expression in the wild type cells, with Cpd2 being less potent at 10 nM. As in the response to dex, responses to both compounds were found to be significantly impaired in the *Dusp1*$^{-/-}$ macrophages, demonstrating again a DUSP1 dependent regulation of an inflammatory mediator by Cpd1 and Cpd2.

As previously discussed in chapter 3 (Figure 3.4.b), *Zfp36* mRNA (coding for TTP protein) expression was found to be strongly decreased by dex in *Dusp1*$^{+/+}$ BMDMs, while it appeared upregulated in *Dusp1*$^{-/-}$ cells (Figure 5.16 a). Although not reaching significance, the difference in mRNA expression between wild type and *Dusp1* deficient cells clearly suggested a role for DUSP1 in the regulation of *Zfp36* by dex. Here, Cpd1 (Figure 5.16 b) and Cpd2 (Figure 5.16 c) were both able to repress *Zfp36* mRNA expression in the wild type cells, significantly at the 1 µM dose. Similarly to dex, Cpd1 and Cpd2 not only were not able to repress *Zfp36* in *Dusp1* deficient BMDMs but they even appeared to increase its expression. The difference between wild type and *Dusp1* deficient BMDMs was found to reach significance at the highest dose for both compounds, confirming the idea of a role for DUSP1 in the regulation of *Zfp36* expression by SGRMs.

The last inflammatory mediator to be studied here is *iNos***.** Its mRNA expression is presented in Figure 5.17. Accordingly to previous finding by our group (Abraham, Lawrence et al. 2006), dex was strongly repressing *iNos* mRNA expression, up to 76%, in wild type cells. An analogous response was observed in *Dusp1*$^{-/-}$ BMDMs, confirming that *iNos* was regulated by dex in an entirely DUSP1 independent manner (Figure 5.17 a). Both SGRMs (Figures 5.17 b and c) were weaker than dex, but still able to repress *iNos* to similar extents in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMDMs.

From these experiments, we confirmed the DUSP1 dependency pattern of dex regulated inflammatory mediators and were able to draw some conclusion regarding the effects of SGRMs on the expression of those same mediators and their dependence on DUSP1. Regarding the anti-inflammatory effect, Cpd1 generally behaved like a version of dex with lower efficacy but similar potency, whereas the anti-inflammatory effects of Cpd2 would invariably be less effective than Cpd1 at 10 nM. Regarding DUSP1 dependency, the emerging model of involvement of DUSP1 in the SGRMs-mediated regulation of inflammatory mediators would be very similar to the one observed with dex: regulation by SGRMs could be entirely dependent (IL-6), partially dependent (COX-2, TNFα or IL-1α) or independent (IL-12p40, iNos) on dual-specificity phosphatase 1.

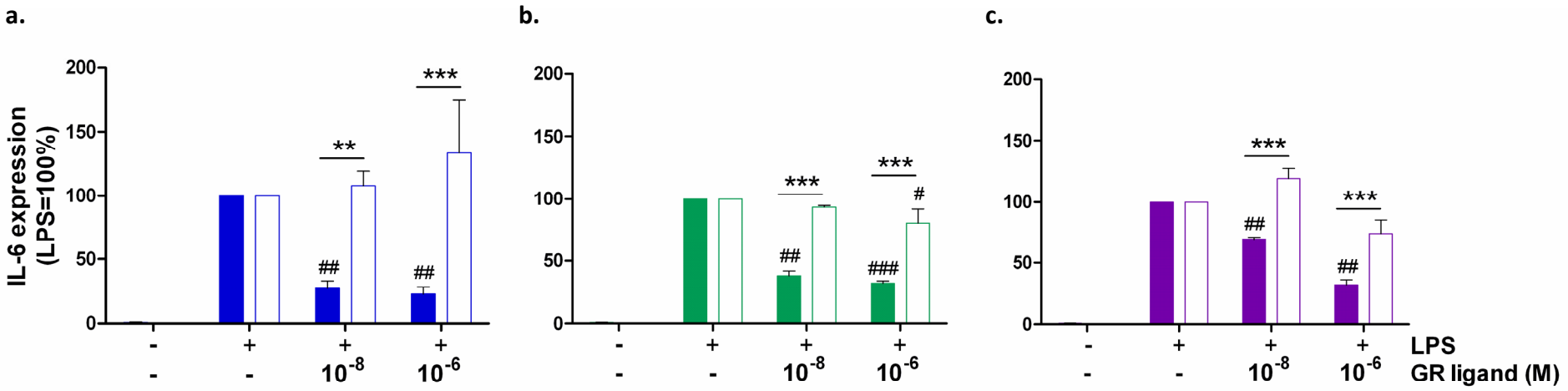


Figure 5.8. Effects of Cpd1 and Cpd2 on IL-6 protein expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, supernatants were harvested for protein detection by Luminex. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%); n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

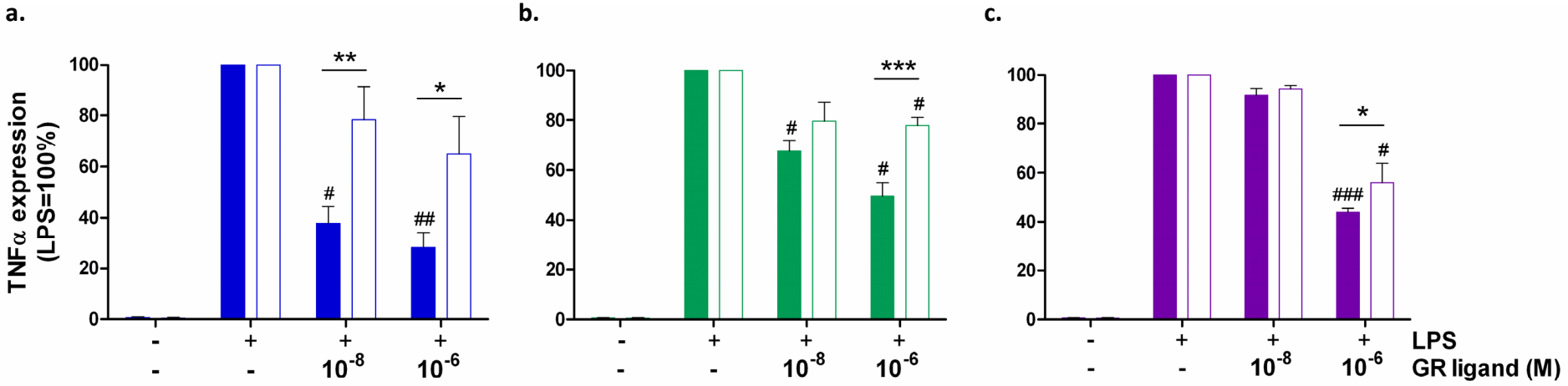


Figure 5.9. Effects of Cpd1 and Cpd2 on TNFα protein expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, supernatants were harvested for protein detection by Luminex. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%); n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

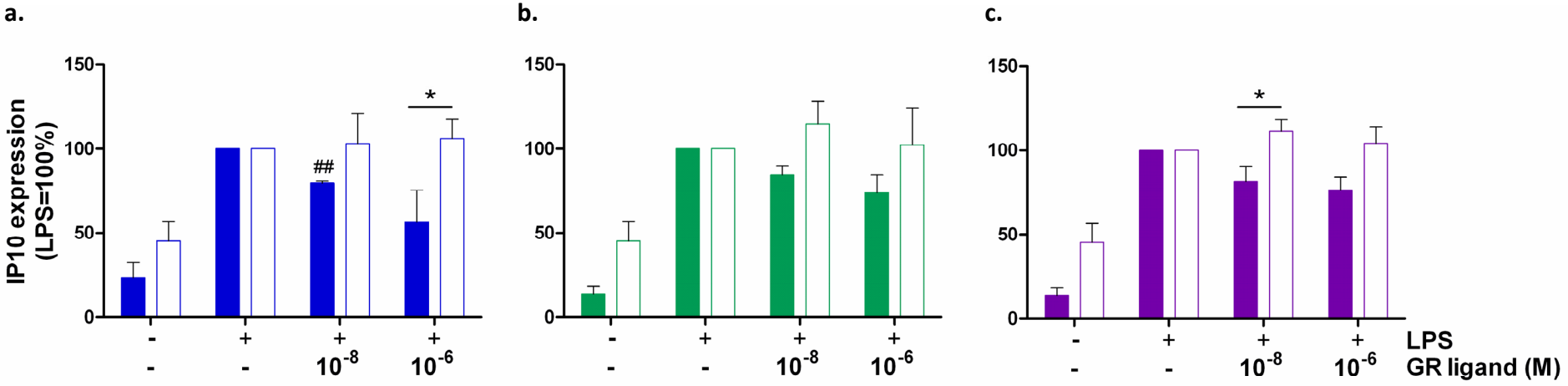


Figure 5.10. Effects of Cpd1 and Cpd2 on IP-10 protein expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, supernatants were harvested for protein detection by Luminex. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%); n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

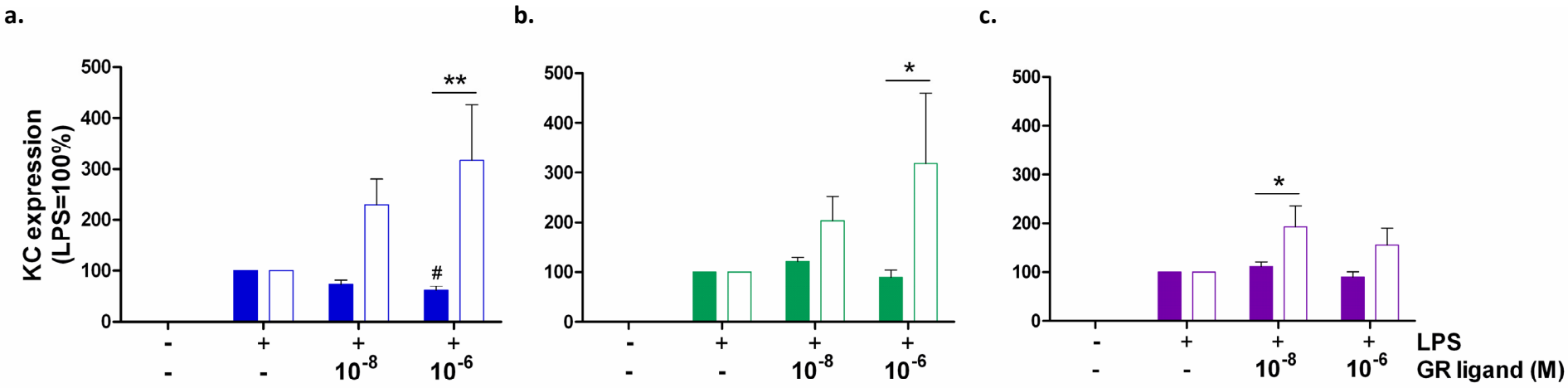


Figure 5.11. Effects of Cpd1 and Cpd2 on KC protein expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, supernatants were harvested for protein detection by Luminex. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%). n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

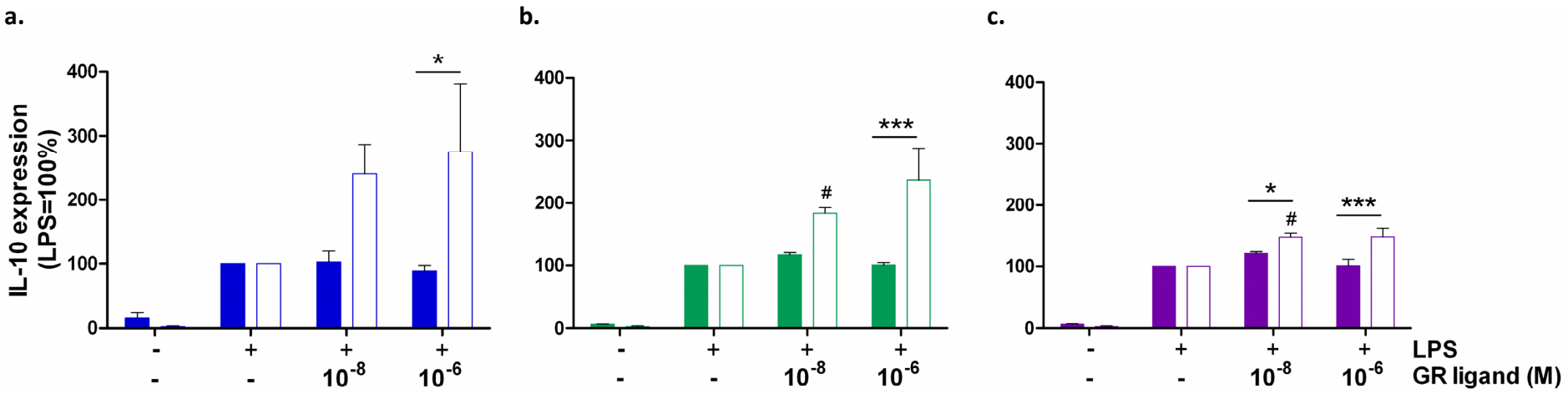


Figure 5.12. Effects of Cpd1 and Cpd2 on IL-10 protein expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, supernatants were harvested for protein detection by Luminex. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%). n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

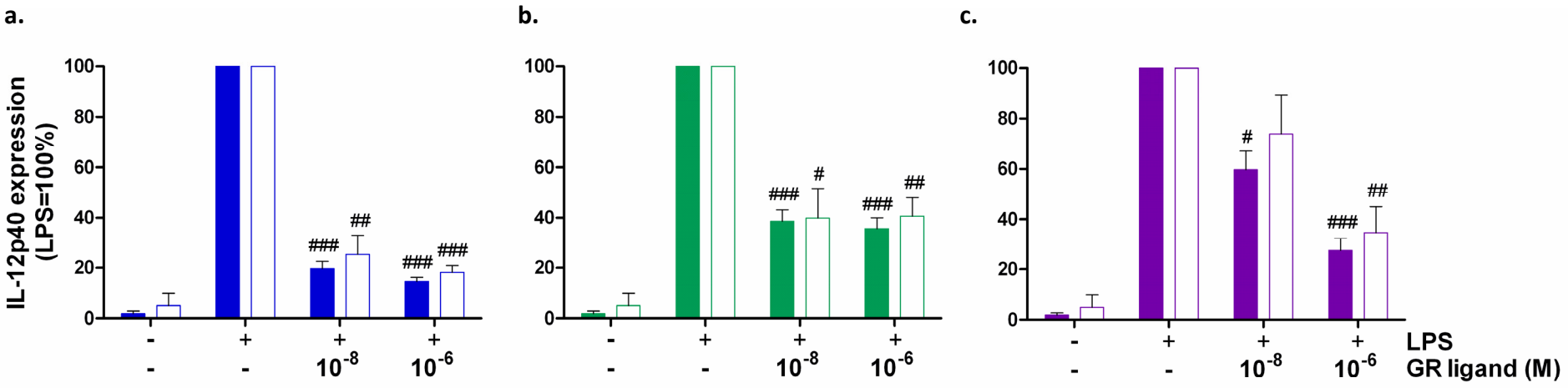


Figure 5.13. Effects of Cpd1 and Cpd2 on IL-12p40 protein expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, supernatants were harvested for protein detection by ELISA. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%). n=4. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */#, p < 0.05; **/##, p < 0.01and ***/###, p < 0.001.*

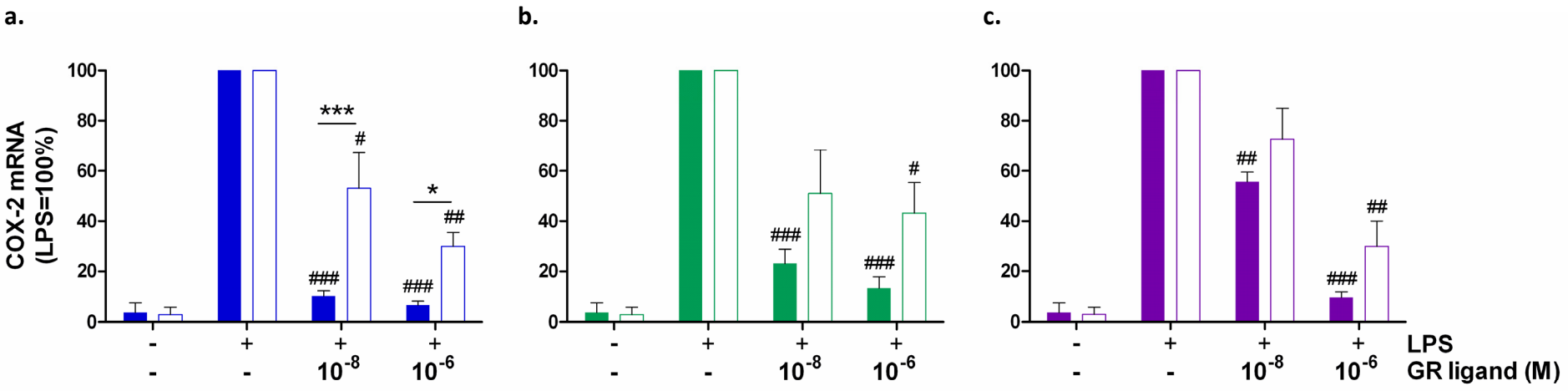


Figure 5.14. Effects of Cpd1 and Cpd2 on Cox-2 mRNA expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, cells were harvested for mRNA quantification by real-time PCR. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%). n=4. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

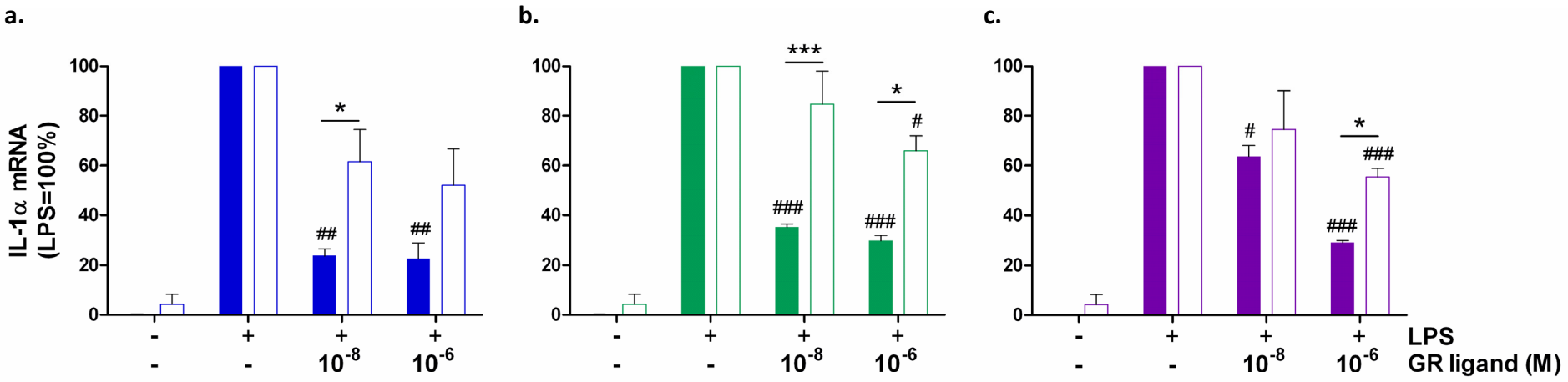


Figure 5.15. Effects of Cpd1 and Cpd2 on IL-1α expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, cells were harvested for mRNA quantification by real-time PCR. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%). n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

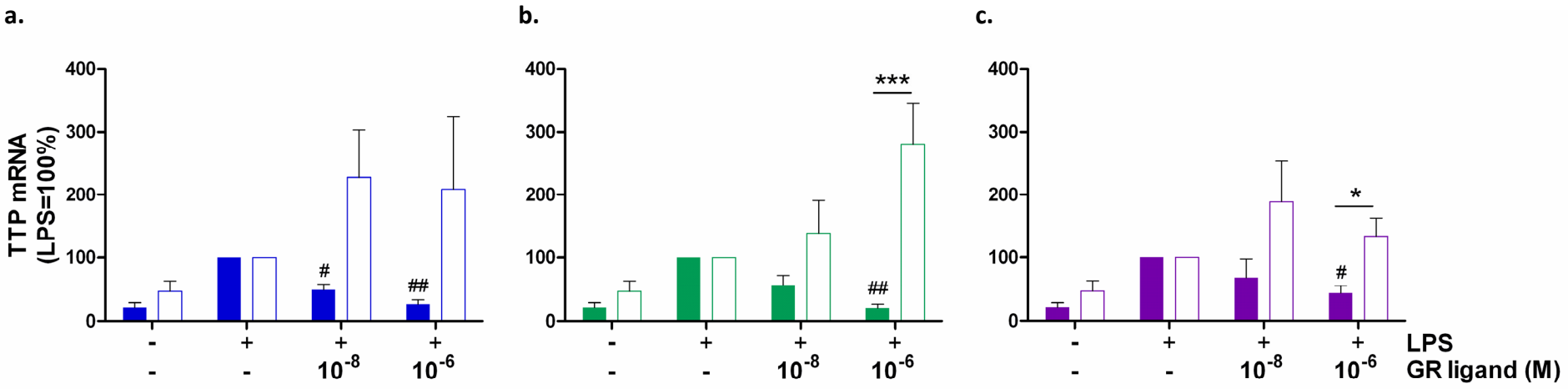


Figure 5.16. Effects of Cpd1 and Cpd2 on TTP mRNA expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, cells were harvested for mRNA quantification by real-time PCR. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%). n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, p < 0.05; **/$^{\#\#}$, p < 0.01and ***/$^{\#\#\#}$, p < 0.001.*

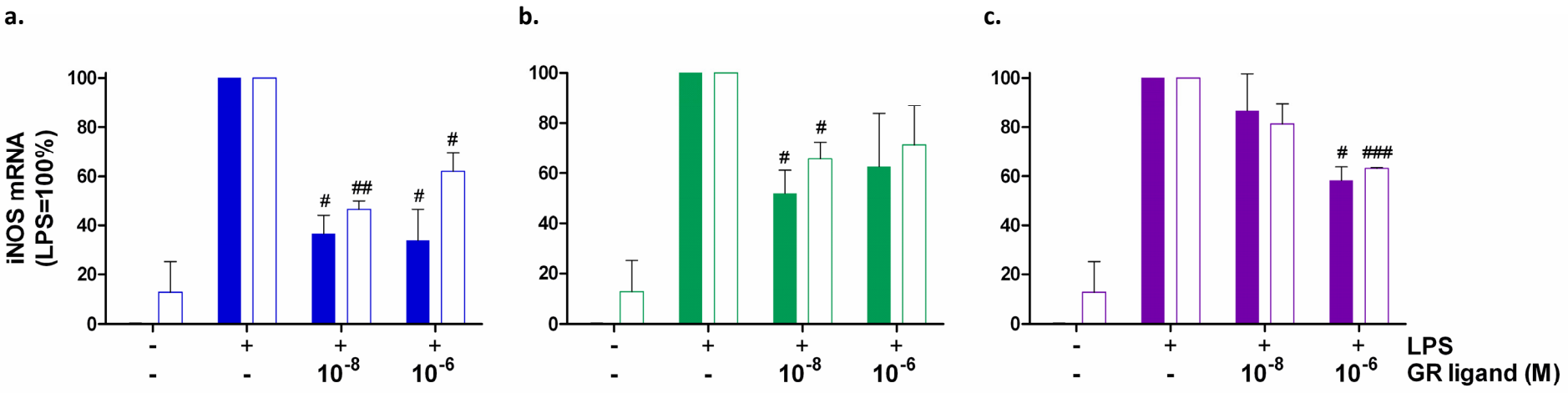


Figure 5.17. Effects of Cpd1 and Cpd2 on iNOS mRNA expression in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ macrophages.

*BMDMs from Dusp1$^{+/+}$ (filled bars) and Dusp1$^{-/-}$ mice (open bars) were pre-treated for 2 h with vehicle (0.1% DMSO), 10 nM or 1 µM of dex (blue), Cpd1(green) or Cpd2 (purple), then challenged with 10 ng/ml LPS for another 4 h. At the end of the incubation period, cells were harvested for mRNA quantification by real-time PCR. Graphs indicate mean expression ± SEM, normalised against that in cells treated with LPS alone (set as 100%). n=3. Statistical analysis was performed using one sample t-test to assess the effects of the different compounds against LPS alone (#) and two-way ANOVA to assess the difference of expression in cells from Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mice (*). Significance was taken as follow: */$^{\#}$, $p < 0.05$; **/$^{\#\#}$, $p < 0.01$and ***/$^{\#\#\#}$, $p < 0.001$.*

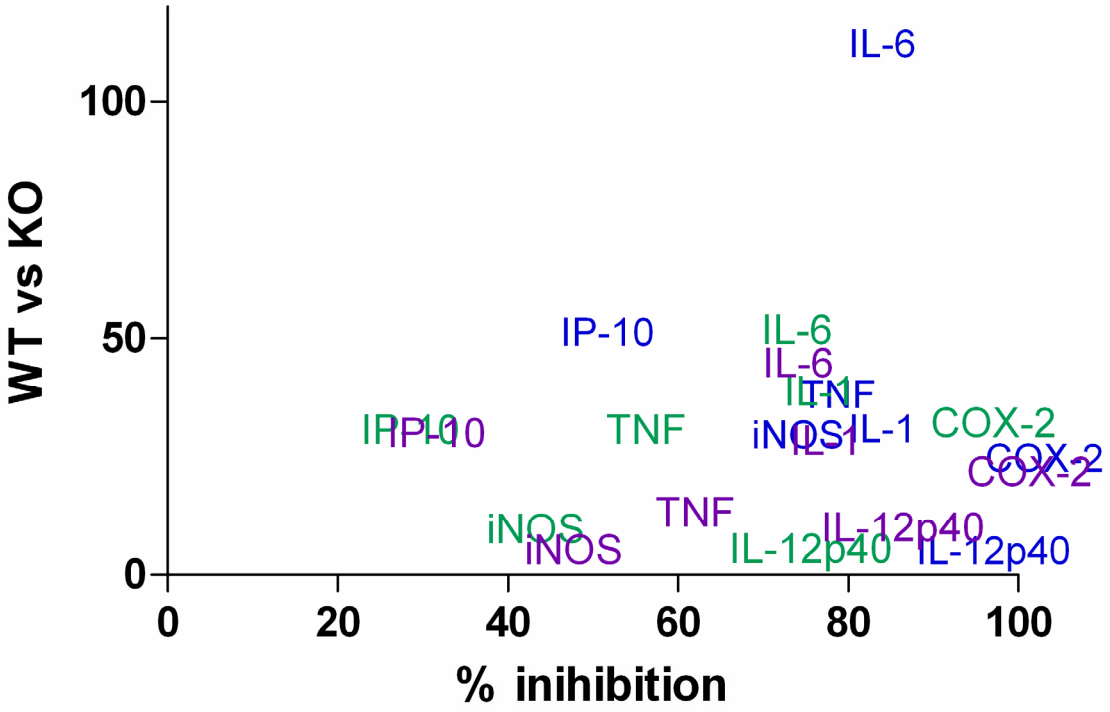


Figure 5.18. Visual summary of the effects of dex, Cpd1 and Cpd2 on inflammatory mediators expression.

Inflammatory mediators were positioned on the graph according to the percentage inhibition by the compound of interest (dex, Cpd1, Cpd2) on the x axis, against the difference in expression between Dusp1$^{+/+}$ (WT) and Dusp1$^{-/-}$ (KO), on the y axis. Difference in the mediators expressions between WT and Dusp1 deficient cells were obtained by simply subtracting WT value to that of KO, for each mediator, for each treatment. This representation is intended as giving the reader a quick overview of the data showed in figures 8 to 17. It does not take into account SEMs and is only representative of the effect of the GR ligands at the dose of 1 µM.

5.4. **Discussion**

In the preceding chapter, it was shown that CpdA was totally dissociated, in agreement with previous studies (De Bosscher, Berghe et al. 2005). Indeed, the compound was not able to induce the expression of a GRE-dependent reporter whilst it was able to repress IL-1β-induced 6κB-luc expression by 39%. However, this only occurred at the highest dose of 10μM, dose that is acknowledged by many (De Bosscher, Berghe et al. 2005; Dewint, Gossye et al. 2008; Gossye, Elewaut et al. 2009; Wüst, Tischner et al. 2009; Robertson, Allie-Reid et al. 2010) as being the effective dose of CpdA in *in vitro* assays. It is to be noted here that in *in vivo* work as well, the effective dose is much higher than the one of classical GR ligands, up to more than 10-fold that of dex (Gossye, Elewaut et al. 2009). Although these high doses of CpdA have been shown to display some therapeutic effects, they also have been linked to cytotoxicity (Wüst, Tischner et al. 2009; Rauner, Goettsch et al. 2011). At the end of chapter 4, I showed that CpdA was only able to transiently induce *DUSP1* mRNA in murine and human cells, at 30 min. In our stereotypical assay of regulation of gene expression by GCs (2 h of GC followed by 4 h pro-inflammatory stimulus), CpdA did not induce *DUSP1* expression at all. Interestingly, in the same cell type, CpdA, even at the 10μM dose, did not exert any anti-inflammatory effects on either *COX-2* nor *IL-6* mRNA expression, whilst dex strongly repressed their expression. The total absence of suppression of pro-inflammatory mediator expression, especially *IL-6*, was surprising, as this cytokine was previously shown to be inhibited by CpdA *in vitro* in various cell types (De Bosscher, Berghe et al. 2005; Rauch, Gossye et al. 2011; Rauner, Goettsch et al. 2011) as well as *in vivo* (van Loo, Sze et al. 2010). Moreover, an IL-6 promoter construct was used to demonstrate CpdA transrepression properties and the compound was proven very effective at inhibiting its expression (De Bosscher, Berghe et al. 2005). Here, lack of response could, of course, be attributed to an inability of CpdA to exert any anti-inflammatory effect in the context of this work. However, given the track record of published anti-inflammatory effects of CpdA and some occurrences of pro-inflammatory mediator repression in my model (anecdotal, data not shown), a total lack of answer seems unlikely. Another possibility to explain the results discussed in this chapter could be either a question of timing of the experiment or the instability of the compound. The former could be easily challenged by a time course experiment of the effect of the highest dose of CpdA on pro-inflammatory gene expression. The second explanatory

possibility is, despite all efforts made to care for the compounds in the best conditions possible, quite plausible. Indeed, narrow therapeutic window and toxic instability of the compound has previously been discussed (Wüst, Tischner et al. 2009).The study presented here could only come to reinforce that argument.

In line with the 4th chapter of this report, in which it was investigated whether transactivation properties of Cpd1 and Cpd2 were the result of an interaction with GR, ability of these compounds to regulate *DUSP1* and *COX-2* mRNA expression was challenged with the addition of the GR antagonist RU 486 to the experimental mix. As expected, all effects of dex were counteracted by RU and both Cpd1 and Cpd2 were able to induce *DUSP1* and repress *COX-2* mRNA expression in A549 cells. RU completely blocked these effects, bringing mRNA levels back to those achieved before treatment with the SGRMs. This demonstrated that once again, Cpd1 and Cpd2 effects are due to GR agonism; not only those on a GRE-dependent reporter but also on endogenous target genes. It is worth mentioning that data obtained here with dex was in agreement with previously reported effect of RU on dex-regulated gene expression, notably *DUSP1* (Lasa, Abraham et al. 2002; Toh, Yang et al. 2004) and *COX-2* (Chivers, Cambridge et al. 2004).

I previously discussed the fact that although Cpd1 and Cpd2 were able to induce DUSP1 expression, this appeared to be cell type- and context-dependent. In this section this idea was taken further, with the aim to compare DUSP1 induction and COX-2 inhibition in different cell types. From the observations made, it can be said that, globally, the anti-inflammatory effects of the GR ligands appeared quite closely related to their ability to induce DUSP1. In all cell types studied here, rank orders and EC_{50} values were in close agreement for DUSP1 induction and COX-2 inhibition (dex < Cpd1 < Cpd2 in A549; dex ≈ Cpd1 < Cpd2 in HeLa and BMDMs; dex ≈ Cpd1 ≈ Cpd2 in RAW), supporting the “mirror” – or symmetry – effect graphically observed at both the mRNA and protein level. Altogether this is in favour of a correlation between DUSP1 induction and COX-2 inhibition. Correlation that was later proven when comparing *COX-2* regulation by GR ligands in *Dusp1* wild type and knock-out mice. Going back to the “mirror effect”, there is a certain number of observations still to be discussed. First, it has to be noted that the potency of *DUSP1* induction by dex and the SGRMs in the different cell types was different in an inflammatory context, from what

was observed with the GR ligands on their own. This agrees with the context-dependency argument discussed previously. Second, the symmetry between *DUSP1* induction and *COX-2* inhibition is less clear in HeLa cells, especially in the case of Cpd2. This was due to high experimental variations that are hard to explain. Indeed, for each cell type, the three GR ligands were added to the same batch of cells, concomitantly. mRNA samples were also handled side by side. Variation may also come from an imprecise dosing of Cpd2 or a faulty aliquot of the compound. This could only be challenged by repeating the experiment. Finally, ability of dex to induce DUSP1 and repress COX-2 mRNA and protein expression was in concordance with what had already been shown in A549 (Newton, Seybold et al. 1998), HeLa (Lasa, Abraham et al. 2002) and RAW cells (R. Perelli, unpublished observations), as well as BMDMs (Abraham, Lawrence et al. 2006).

Although these experiments suggest a strong correlation between DUSP1 induction and COX-2 repression by SGRMs, it was necessary to prove this potential dependence. For this, primary macrophages from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice were used and regulation of inflammatory gene expression by Cpd1 and Cpd2 investigated, compared to that of dex. Also, if transrepression accounted for the majority of the anti-inflammatory effects of the SGRMs, their capacity to inhibit inflammatory gene expression should be unaffected by the DUSP1 deletion.

After LPS stimulation alone, many pro-inflammatory mediators were overexpressed in *Dusp1*$^{-/-}$ BMDMs, in agreement with previous observations reported by us (Abraham, Lawrence et al. 2006) and many others (Chi, Barry et al. 2005; **Hammer**, **Mages** et al. 2006; Wang, Zhao et al. 2009; Yu, Li et al. 2011). We also report here that IP-10 expression did not seem to differ between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$, which was again in agreement with observations from Hammer and colleagues, that "IP-10 was not affected by the absence of DUSP1" (**Hammer**, **Mages** et al. 2006). TTP, was subject to more controversy. In BMDMs, LPS-induced *Zfp36* (coding for TTP) mRNA expression seemed lower in *Dusp1* deficient cells. This contradicts other observations made by our group and what is consistently seen at the protein level (strong overexpression of TTP in *Dusp1*$^{-/-}$ BMDMs). However, what is seen here at the mRNA level is not significant and therefore no definite conclusion can be drawn before similar experiments have been performed.

The set of experiments reported here showed that, as for classical GCs, DUSP1 was required for effects of SGRMs on some key inflammatory mediators. As previously reported by our group (Abraham, Lawrence et al. 2006), DUSP1 dependency of dex action could be classified in three groups, namely 'dependent', 'independent' and 'partly dependent' on DUSP1 expression. Here, I show that this is the case for SGRMs too. A summary of the anti-inflammatory effects of dex (blue), Cpd1 (green) and Cpd2 (purple) on the inflammatory mediators expression studied in this report is illustrated by Figure 5.18. On the x axis, the larger the percentage, the greater is the repression of inflammatory mediator. On the y axis, the higher the number, the more dependent on DUSP1 is the regulation by a given compound. Briefly, based on this representation and on the data from figures 8 to 17, and in an attempt to classify the DUSP1 dependency of the SGRMs anti-inflammatory effects, were considered:

- Independent of DUSP1: IL12p40 and iNos
- Partly dependent on DUSP1: Tnfα, IL-1α and Cox-2; but also IP-10 and TTP
- Strongly dependent on DUSP1: IL-6

This dependency pattern observed with SGRMs was very similar to the one observed, in the same experiments, with dex treatment and confirmed what had already been published on the DUSP1 dependency of classical GCs, by us (Abraham, Lawrence et al. 2006) or others .
Also, it has to be noted that IL-10, TTP and KC were excluded from this representation. For IL-10 and KC, the reason was that the expression of these compounds in *Dusp1*$^{+/+}$ cells was generally very weakly or not affected by dex or SGRMs and therefore it could not be talked about a DUSP1-dependent effect of GR ligands. On the other hand, TTP expression regulation was not displayed on Figure 5.18 for a visual reason only, difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ reaching more than 200 and therefore difficult to visualise alongside the other mediators investigated here.

IL-6, TNFα, Cox-2 and IL-1α were the genes the most strongly inhibited by the SGRMs and this inhibition was shown to be fully (IL-6) or partly (TNFα, COX-2, IL-1α) dependent on DUSP1. Neither dex nor Cpd1 or Cpd2 were capable of decreasing expression of IL-6 protein in *Dusp1*$^{-/-}$ BMDMs. The same GR ligands were repressing Cox-2, TNFα and IL-1α expression to a much lesser extent in *Dusp1* deficient cells than in the *Dusp1* wild type ones. This not

only underlines the importance of DUSP1 in the effect of the glucocorticoid receptor, it advocates in favour of a role for GR-induced genes in the regulation of key inflammatory mediators by novel GR ligands.

Although previously reported as being repressed by glucocorticoids (Man, Xuekui Zhang et al. 2009), it is the first time that regulation of IP-10 expression by GR ligands is studied in the context of presence or absence of DUSP1. Data collected here show that dex significantly inhibit IP-10 and that this inhibition is dependent on DUSP1, at least at the highest dose. SGRMs seem to only weakly downregulate IP-10 in *Dusp1*$^{+/+}$ cells and this seemed to be impaired in *Dusp1* deficient BMDMs. Despite being non-significant these results point in the direction of some DUSP1-dependency for SGRMs-regulated IP-10 expression. This would need to be confirmed by further work but could highlight a role for DUSP1 in IP-10 regulation, reinforcing its possible importance in chronic inflammatory disorders pathogenesis.

Agreeing with results previously published by our group (Abraham, Lawrence et al. 2006), DUSP1 was shown to be important in the effects of dex on KC and IL-10. Observations made here also suggest a role for the phosphatase in the regulation of expression of these proteins by Cpd1 and Cpd2. Similarly to findings reported by Abraham and colleagues, strong overexpression of both proteins was observed here, in response to dex in *Dusp1*$^{-/-}$ macrophages. This was also the case in response to Cpd1, and to a lesser extent to Cpd2. As discussed in chapter 3, TTP expression regulation by dex was subject to controversy. However, experiment performed here show significant differences in *Zfp36* mRNA expression in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$, in response to dex, but also Cpd1 and Cpd2. This suggests a role for DUSP1 in the effect of GR on TTP regulation. Another interesting observation was that TTP expression appeared to be increased in *Dusp1* deficient cells in response to dex and SGRMs.

So, for all three inflammatory genes discussed in this section, much higher levels of expression were observed in the *Dusp1*$^{-/-}$ macrophages. This could be explained by several mechanisms.

First, absence of DUSP1 was shown to result in increased and prolonged activity of p38 MAPK in response to dex. As discussed in chapter 3, this may result in more TTP being phosphorylated, leading to stabilisation of TTP target mRNA, therefore impacting protein expression measured. IL-10 and KC were shown to be regulated by TTP (Datta, Biswas et al. 2008; Tudor, Marchese et al. 2009). Understanding the role of GR ligands in this context is difficult but need to be studied, in order to better understand the compensatory mechanisms leading to overexpression of certain inflammatory mediators in *Dusp1* deficient cells.

Second, it is well acknowledged that IL-10 is an anti-inflammatory mediator. Hence, strong upregulation in the absence of another anti-inflammatory mediator such as DUSP1 would most likely be the result of rearrangement of the endogenous "anti-inflammatory balance", to avoid excessive inflammatory response. Such dynamic regulation could explain some of the inhibition of genes such as IL-1α and TNFα, sensitive to IL-10 regulation (Schaljo, Kratochvill et al. 2009) and which expression was still downregulated to some extent here, in the *Dusp1*$^{-/-}$ cells by dex but also Cpd1 and Cpd2. Fine tuning regulation between DUSP1, IL-10 and TTP is currently being investigated in our group and will most certainly shed some light on understanding this type of interaction and regulation.

As mentioned earlier in this chapter, DUSP1 expression in BMDMs was maximal at both doses of dex and Cpd1 used here, whilst Cpd2-induced DUSP1 was at its maximum only at the highest dose tested in the present set of experiments discussed (1 μM). Cpd1 and Cpd2 potency and efficacy were lower than dex (dex > Cpd1 > Cpd2). This could explain why, in the experimental context described here, Cpd1 would generally behave like a less powerful version of dex, and why Cpd2 was a relative poor inhibitor of pro-inflammatory gene expression: because both compounds exerted anti-inflammatory effects in proportion to their ability to upregulate DUSP1. In this report Cpd2 is reported as being the most "dissociated", with a strong preference for transrepression over transactivation. It is indeed, the less able to induce DUSP1 expression but also the less able to retain classical GCs anti-inflammatory properties, at least in the experimental set up presented here. Moreover, anti-inflammatory properties of both compounds were shown to be both following a similar pattern of DUSP1-dependency to dex. Once again, this questions whether transactivation

and transrepression assays are the ideal manner to screen for a GR ligand which would be a strong repressor of the anti-inflammatory response but have less unwanted side effects.

Now that DUSP1 dependency was established for some of the effects of the experimental SGRMs discussed here, it would be interesting to know the effects of these compounds on genes classically considered as being involved in the unwanted side effects of GCs. Regulation of genes involved in glucose or bone metabolism for example could be studied, as it has already been the case for some of the most recent novel GR ligands (Coghlan, Jacobson et al. 2003; Schacke, Schottelius et al. 2004; Lopez, Ardecky et al. 2008; Schäcke, Zollner et al. 2009).

Also, the next step in this study would be to investigate the effects of these compounds in *in vivo* models, in two ways. First, it would be interesting to see if the effects on pro-inflammatory mediators can be reproduced *in vivo* in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice. Second, use of disease models in these wild type and *Dusp1* deficient animals would bring into more perspective the importance of DUSP1 in the effects of novel GR ligands, as it has recently been demonstrated in the case of the effect of dex in a *Dusp1* deficient arthritis mice model. In this study, *Dusp1*$^{-/-}$ mice were shown to be insensitive to the therapeutic effects of dex in collagen-induced arthritis (CIA), an experimental model of rheumatoid arthritis (Y. Vattakuzhi, unpublished observations). It would be interesting to study whether the SGRMs have therapeutic effects in CIA, and whether any such effects depend on DUSP1.

Chapter 6

FINAL DISCUSSION

6. FINAL DISCUSSION

As largely described and discussed throughout this report, DUSP1 involvement in the anti-inflammatory effects of classic and novel GR ligands was the cornerstone of the investigations reported here. I was first particularly interested in understanding the role of DUSP1 in the anti-inflammatory effects of GCs, especially its role in post-transcriptional regulation of pro-inflammatory gene expression. Later on, I went on to investigate whether DUSP1 was involved in the anti-inflammatory effects of SGRMs and what was the impact of such findings on the 'dissociated glucocorticoid' concept.

In this report, findings confirmed that regulation of certain inflammatory gene expression by GCs was DUSP1-dependent and suggested that post-transcriptional regulation of Cox-2 and IL-1α gene expression by GCs was also dependent, to some degree, on DUSP1. Moreover, as novel GR ligands, selected upon the sacrosanct principle of a marked preference for transrepression, were also able to induce DUSP1 expression and that DUSP1 appeared to be necessary for some of their anti-inflammatory effects, many questions remains as for the relevance of such a concept in a search of a GR ligand with improved benefit/risk ratio.

In chapter three I showed that the classical GR ligand dex was able to upregulate DUSP1 in mouse macrophages, and to inhibit the expression of COX-2 in a manner that was partially dependent on DUSP1, in the same cell type. Time courses experiments revealed that induction of DUSP1 expression was prolonged by the cooperation of dex and LPS in these cells. This also appeared to be the case when comparing data of *DUSP1* expression in A549 cells, in response to increasing concentrations of dex in the presence or absence of IL-1β. In the presence of IL-1β and dex the dose response curve appeared shifted to the left, indicating that cooperation between pro-inflammatory stimulus and dex was more potent in inducing *DUSP1* gene expression than the GC on its own (Figure 6.1), clearly putting forward the importance of such synergy. But how might this cooperativity occur? A few years ago, Da Silva and her colleagues showed that similar cooperativity between a GC, budesonide, and IL-1β, resulted in increased expression of stem cell factor (SCF) in human lung fibroblasts. In the same study, the authors demonstrated that co-treatment with GC and

pro-inflammatory stimulus potentiated the promoter activity and that this cooperation required adjacent GRE and κB sites (Da Silva, Heilbock et al. 2003). Along the same lines, another group showed that the cooperativity of TNF and dex observed on a synthetic promoter's transcriptional activation not only necessitated GRE and κB sites but that it strictly depended on the spacing between these GREs and κB sites (Hofmann and Schmitz, 2002). Another possibility to explain cooperativity between dex and pro-inflammatory stimulus would be that NF-κB could alter chromatin structure in such a way that GR recruitment is enhanced and stabilised. This concept was suggested very recently by Rao and colleagues, who showed that co-activation of GR and p65 altered the repertoire of regulated genes and resulted in their association with novel sites in a mutually dependent manner. They also demonstrated that the co-activation of GR and NF-κB altered signalling pathways that were regulated by each factor separately (Rao, McCalman et al. 2011). It would be interesting to test these concepts, applied to the *DUSP1* gene, to investigate whether cooperation requires GRE and κB sites. The cooperation between dex and pro-inflammatory stimuli described in this report was observed as a result of total mRNA measurement. It cannot be excluded that GCs and pro-inflammatory stimuli function at different stages of gene expression, for example one at the level of pol II recruitment, the other at the level of transcriptional elongation. It would also be interesting to investigate these possibilities, looking at the influence of dex and/or pro-inflammatory stimuli on pol II recruitment, as well as on that of other members of the elongation complex. Identifing the mechanistics behind such cooperation would most certainly help understand better the feedback processes controling inflammation, where endogenous GCs play an important role and lack of DUSP1 was shown to result in an exagerated inflammatory response (Abraham and Clark 2006).

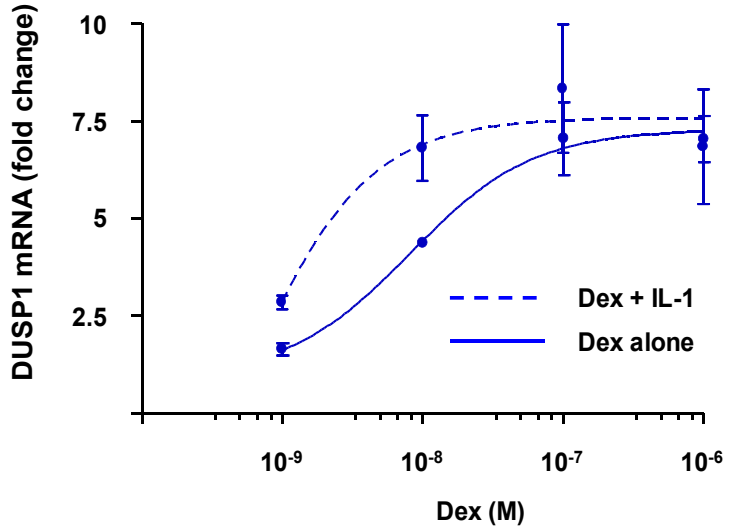


Figure 6.1. Cooperative regulation of DUSP1 by dex and IL-1 in A549 cells.

This figure was made by combining data of DUSP1 gene expression in response to dex and dex + IL-1 in A549 cells. DUSP1 mRNA levels in response to dex were derived from the experiment presented in figure 4.9, whilst that of DUSP1 in response to dex + IL-1 came from experiments discussed in chapter 5.

In this report, it was also confirmed that DUSP1 expression was required for certain anti-inflammatory effects of GCs. Indeed, using primary macrophages from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice, it was showed that DUSP1 was required, to various degrees, for regulation of COX-2, IL-6, TNF, IL-10, KC, IL-1α and iNOS expression. From the experiments reported here, those from our group (Abraham, Lawrence et al. 2006) and from others (Dickinson and Keyse 2006; Beck, Vanden Berghe et al. 2009), it appears that anti-inflammatory effects of GCs are selectively impaired in *Dusp1*$^{-/-}$. The fact that some responses to dex were lost in the absence of *Dusp1* whilst some were retained is incompatible with the idea that pro-inflammatory cytokines could reduce response to GR, *via* a MAPK-mediated phosphorylation of GR and its subsequent inactivation (Galliher-Beckley and Cidlowski 2009; Pace, Hu et al. 2007). To counter this argument, it would be interesting to show that GC-responsive expression of DUSP1 is necessary for suppression of inflammatory responses. This should be possible by using an hypothetical genetically modified mouse strain in which GC-responsive regions of the *Dusp1* gene would be deleted.

As previously mentioned, GCs have long been shown to be able to destabilise several pro-inflammatory mRNAs, including *COX-2*, *IL-6*, *IL-1* and *iNOS* (Newton and Holden 2007). One explanation for this phenomenon would be that, according to some, GCs are able to upregulate TTP expression (Smoak and Cidlowski 2006). This in contradiction with data from our group, showing that dex had minimal effect on its own in BMDMs (H. Cunliffe, unpublished observations). Data from this report rather show some inhibition of LPS-induced TTP expression by dex in cells from wild type animals and this was in agreement with findings from another group (Jalonen, Lahti et al. 2005). Another possible explanation for destabilisation of pro-inflammatory gene expression by GCs would be that GR could act as an mRNA binding and destabilising protein on its own, without promoting mRNA decay through an intermediate. This concept was reported a few months ago in the Journal of Immunology, where the authors (Ishmael, Fang et al. 2011) showed that transcripts associated with GR contained G-C rich motif, capable of binding to GR *in vitro*, therefore suggesting a potential new post-transcriptional role for GR as an mRNA-binding protein. Finally, the last mechanism that could be suggested to explain the effects of dex on mRNA stability would be the fact that GCs work by controlling the p38 MAPK pathway, which in turns regulated mRNA stability. Many genes, including *COX-2*, *GM-CSF* and *IL-1* were

described as being post-transcriptionally regulated by both GCs and p38 MAPK (Clark, Dean et al. 2003).

This would fit with the idea of DUSP1 being involved in post-transcriptional regulation of inflammatory gene expression by dex and was the hypothesis that I tried to demonstrate using ActD chases in cells from *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mice. As previously discussed, these experiments turned out to be quite challenging and although some destabilisation of *Cox-2* and *IL-1α* mRNAs was described at one hour and appeared to require DUSP1, timing of the experiment was an issue and it was difficult to fully demonstrate the involvement of DUSP1. Since I left the lab, however, alternative protocols have been tested and results from one of these experimental designs are illustrated in Figure 6.2**.** Adding ActD and/or dex to LPS-treated BMDMs finally prove what was hinted in my experiment, *i.e.* destabilisation over time of *Cox-2* mRNA by dex in wild type cells and abrogation of such effect in cells lacking *Dusp1*. Loss of dex-induced destabilisation of pro-inflammatory mRNA in *Dusp1*$^{-/-}$ cells indicated that this destabilising effect was likely to be dependent on dex-induced DUSP1 expression. At this stage it is worth mentioning that the figure discussed here is representative of two independent experiments but that further repeats are ongoing and planned to confirm post-transcriptional regulation mechanisms of *Cox-2*, as well as others, mRNAs. Also, going back to the possibility that GR would act directly as an mRNA binding protein, our latest results described in this section (Figure 6.2) argue against such mechanism. Indeed, addition of dex together with ActD resulted in the abolition of the destabilising activity of dex, suggesting that *de novo* transcription is required for the effects of dex. So, in regard to this data, post-transcriptional regulation of pro-inflammatory gene expression by dex could now be explained by GCs inducing DUSP1 expression, which would in turn, deactivate p38 MAPK, therefore preventing phosphorylation of TTP. Hence, GCs would likely work by modulating the balance between unphosphorylated and phosphorylated TTP. Continuing experiments in the lab were design to address this hypothesis, assuming that GCs exert post-transcriptional effects by targeting serine 52 and 178 (S52 and S178). These residues have indeed been shown as being those phosphorylated by the p38-MK2 complex, which phosphorylation would prevent recruitment of deadenylase complex, resulting in greater stability of target mRNAs (Marchese, Aubareda et al. 2010; Clement, Scheckel et al. 2011). Using a knock-in mouse strain in which S52 and S178 are

both substituted by non-phosphorylatable alanine residues (TTPaa), it was predicted that BMDMs from such mice would under-express pro-inflammatory mediators and be partially insensitive to dex-mediated inhibition. In Figure 6.3, TNF protein expression is represented in response to LPS and/or dex in BMDMs from wild type of TTPaa mice. As expected, TNF protein levels were much lower in TTPaa cells, in response to dex (Figure 6.3 a) and inhibition by dex was much less in macrophages from knock-in animals, compared to that in the wild types (Figure 6.3 b). It is also to be noted here that IL-6, KC and IL-10 protein expressions are also under investigations, in the same conditions. Furthermore, the stability of the corresponding mRNAs has been predicted to be low and unresponsive to dex in cells from TTPaa mice. This is, to date, ongoing work in the lab but preliminary data suggest that such prediction has been proved correct (unpublished observations). Altogether, these preliminary findings support a mechanism by which GCs regulate pro-inflammatory gene expression at the post-transcriptional level, involving DUSP1 and TTP phosphorylation by the p38/MK2 complex. However, several aspects of such a mechanism still need to be addressed. First, using specific phospho antibodies, we could show whether dex decreases phosphorylation of both S52 and S178, in cells from wild type mice. Using cycloheximide, an inhibitor of protein synthesis, we could also look at TTP protein stability and how it is impacted by changes in phosphorylation status. Additionally, TTPaa mice could also be used to identify targets of TTP, as an mRNA that is targeted for degradation by TTP would be under-expressed in cells from knock-in animals. Lastly, it would be interested to study *in vivo* responses to pro-inflammatory stimuli. In this context, TTPaa mice are expected to be hypo-responsive to pro-inflammatory challenges and be protected against endotoxic shock. Global response ought to be somehow similar to that of GC-treated wild type mice, after a pro-inflammatory challenge. Besides, unphosphorylatable TTP could therefore be considered as some sort of anti-inflammatory mediator and finding a way to protect unphosphorylated TTP from degradation could become a target for treatment of inflammatory conditions. These ideas of future work described above are a few possibilities, among many others. Although my original experiments on post-transcriptional mechanisms of GCs were clearly frustrating at the time, they since have led into a productive area of research and hopefully, ongoing and future work by our group will contribute to building our understanding of the precise mechanisms supporting post-transcriptional regulation of inflammatory gene expression by GCs.

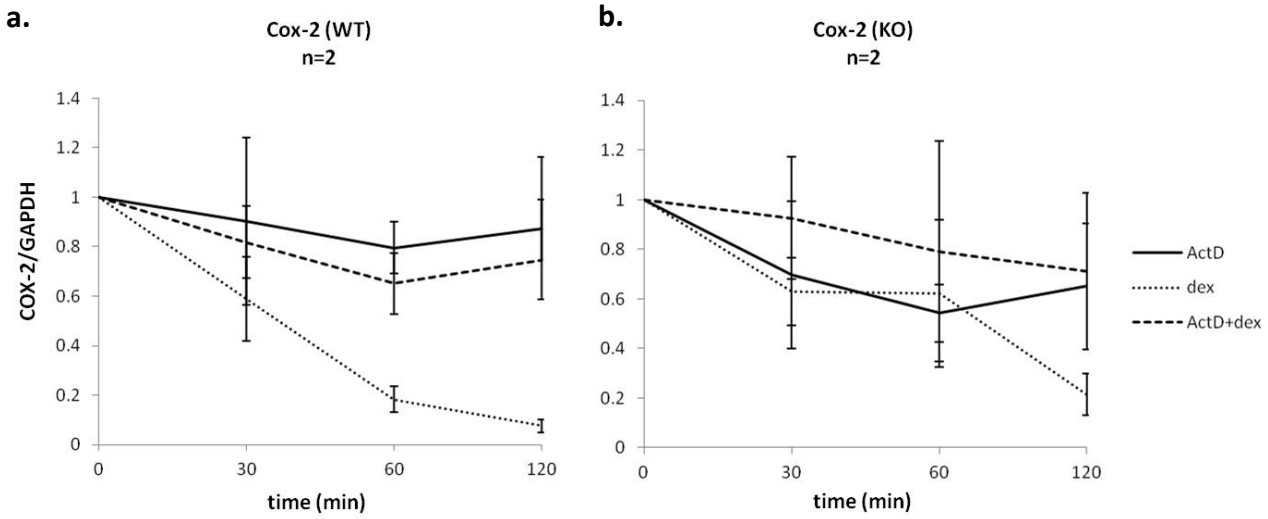


Figure 6.2. Effects of dex on Cox-2 mRNA stability in Dusp1$^{+/+}$ and Dusp1$^{-/-}$ mouse macrophages.
Primary macrophages from Dusp1$^{+/+}$ (WT) (a) and Dusp1$^{-/-}$ (KO) (b) mice were treated with LPS (10 ng/ml) for 4 h then dex (100 nM) and/or ActD (10 µg/ml) were added. mRNA was harvested at time 0, 30 min, 1 h and 2 h. Cox-2 mRNA was then quantified by real-time PCR. mRNA quantity was normalised against the house keeping gene GAPDH and expressed as a ratio of the quantity at time 0. For each time point, graph indicate mean ±SEM (error bars) from n=2 independent experiments. Each experiment consists of two replicates. Data shown was the result of experiments performed by Dr. A. Clark.

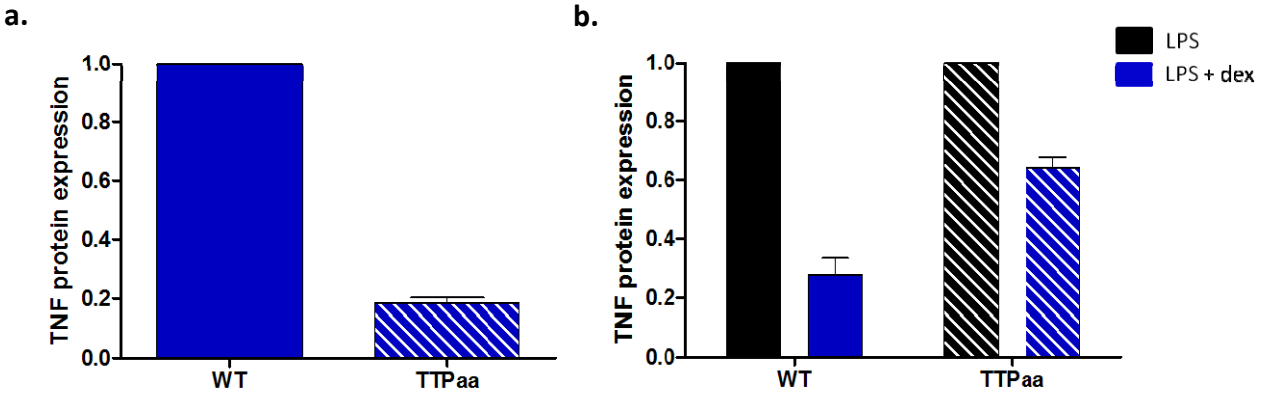


Figure 6.3. TNF protein expression in wild type and TTPaa mouse macrophages.
*Cells from wild type (WT) and TTP knock-in (TTPaa) mice were treated with LPS and/or dex for 4 h. Supernatants were then harvested and TNF protein levels measured by ELISA. In **(a)**, TNF levels after LPS + dex treatment were shown as a ratio of those in WT cells. **(b)** TNF levels were shown as a ratio of those observed in response to LPS treatment alone (black bars, set as 1), in cells from WT (closed bars) and TTPaa (stripped bars) mice. Data shown was the result of experiments performed by Dr. A. Clark and Dr. T. Smallie.*

Used in the treatment of inflammatory diseases for more than half a century, GCs therapy is also associated with severe side effects. Over the past years, many research groups and many pharmaceutical companies have tried to identify a compound able to retain anti-inflammatory properties of classical GCs whilst having fewer deleterious effects. Because it has been believed for many years that tranrepression of gene expression would be responsible for GCs anti-inflammatory effects and transactivation of gene expression would account for unwanted effects, focus was aimed at screening chemical banks for GR ligands able to favour transrepression over transactivation. Classically, discrimination of such preference is derived from *in vitro* assays in which GR ligands ability to activate or repress transcription is assessed using simplified reporters.

Cpd1 and Cpd2, the two SGRMs used in this report, have emerged from deliberate efforts to selectively promote the transprepression rather than transactivation function of GR. They were characterised and shown to preferentially mediate transrepression rather than transactivation, as illustrated by dissociation indices superior to those of dex. However, they were capable of inducing the expression of DUSP1 in several different cellular systems, and both compounds exerted anti-inflammatory effects in proportion to their ability to upregulate DUSP1. In the experimental system used here, their capacity to inhibit the expression of COX-2 was correlated with DUSP1 induction and several of the anti-inflammatory effects of the SGRMs were found significantly impaired in mouse macrophages lacking DUSP1.

To test whether Cpd1 and Cpd2 really worked via GR, I challenged their effects using RU 486, a GR antagonist. Although this series of experiments proved that the experimental SGRMs exerted their action on κB-dependent and GRE-dependent reporters, as well as *DUSP1* and *COX-2* mRNAs, through GR, it would be interesting to complement this pharmacological approach with the use of a *GR* deficient model. For example, using siRNA, it should be possible to decrease expression of GR in one of our cellular model, namely BMDMs or A549 cells, and look at the effects of the SGRMs in this context. If the compounds truly act *via* GR, then the anti-inflammatory effects or the ability of Cpd1 and Cpd2 to induce DUSP1 expression should be abrogated or at least severely impaired.

In this study I show that Cpd1 and Cpd2 were capable of anti-inflammatory properties, on a selection of genes. An interesting follow-up to this would be to investigate further these anti-inflammatory properties and perform a genome-wide analysis. This could be achieved by using a micro-array approach where cells would be treated with dex, Cpd1 or Cpd2, in an inflammatory context, and messengers levels measured and compared in response to the different treatments. Profiles of gene expression would be obtained, showing genes repressed, or activated by the SGRMs. From the work discussed here, it could be predicted that Cpd1 would behave the more similarly to dex, whereas Cpd2 would be weaker at repressing inflammatory genes. It can also be anticipated that *Dusp1* expression would be induced by the SGRMs, along that of other genes considered as anti-inflammatory and classically activated by glucocorticoids. Some novel SGRMs have already been shown to be able to upregulate GR-regulated genes. RU24858 (Chivers, Gong et al. 2006; Janka-Junttila, Moilanen et al. 2006) was able to induce expression of DUSP1, GILZ and AMP N, whilst LGD-552 (Lopez, Ardecky et al. 2008) upregulated IL-10. On the basis of reporter assays, Cpd2 appeared particularly strongly "dissociated", but in BMDMs it was a relatively poor inhibitor of pro-inflammatory gene expression, reflecting its low potency of DUSP1 gene regulation. The importance of DUSP1 as a mediator of the effects of GR was particularly demonstrated in the case of IL-6. Neither dex, Cpd1 nor Cpd2 was capable of decreasing expression of IL-6 in *Dusp1*$^{-/-}$ macrophages. It is ironic that IL-6 is a well-characterised NF-κB target, whose inhibition by GCs has often been interpreted in terms of transrepression. Once again, this raises the question whether genuine and consistent separation between transactivation and transrepression properties of GR can be demonstrated using simple reporters, or even whether it can be achieved.

Here, I show directly (impaired anti-inflammatory effects in *Dusp1*$^{-/-}$ cells) and indirectly (correlation between DUSP1 induction and COX-2 inhibition) that Cpd1 and Cpd2 exert anti-inflammatory effects via DUSP1 induction. However conclusive, the approach used here to demonstrate this dependence has its limitations. It is an *in vitro* murine model. Several steps would be needed to be able to generalise the conclusions I make here: human cells and *in vivo* models. Part of the work performed here was in A549 cells, a model of human pulmonary epithelial cells, where expression of DUSP1 correlates with COX-2 inhibition in response to the SGRMs. We can imagine that knocking down DUSP1 in these cells and

looking at inflammatory gene expression profiles in response to Cpd1 and Cpd2 would be a good start towards understanding the role of DUSP1 in response to SGRMs in human cells. Also, use of *in vivo* disease models would be recommended. In the past years, DUSP1 has been suggested to play a role in human diseases. DUSP1 expression was reported to be elevated in patients with multiple sclerosis (Eljaschewitsch, Witting et al. 2006) and major depressive disorder (Duric, Banasr et al. 2010). *In vivo* anti-inflammatory effects of dex were dependent on DUSP1 in experimental models of acute inflammation (Abraham, Lawrence et al. 2006; Wang, Nelin et al. 2008), asthma (Li, Zhang et al. 2011) and rheumatoid arthritis (Y. Vattakuzhi, unpublished data). Indeed, in RA, work performed by our group showed that anti-inflammatory effects of dex in CIA model were impaired in the *Dusp1* deficient mouse. There are therefore problems with a paradigm that equates anti-inflammatory effects of GCs with transrepession and not transactivation and it would be of major interest to determine whether the same pattern of DUSP1 expression in these diseases models is shown in response to SGRMs. A key question here is do we really want a GR ligand that is unable to upregulate gene expression? In view of the different arguments detailed here, wouldn't such a compound be a poor anti-inflammatory drug?

Michael Berlin recently reviewed the development of those novel GR ligands, reflecting all patented SGRMs and the many number of pharmaceutical companies involved in the search of a SGRMs with an ever improved benefice/risk ratio (Berlin 2010). Of the several dozens of compounds listed, only few have thus far reached clinical trial development status and none have yet received the ever sought after approval.

MK-5932, from Merck, was described as potentially able to retain anti-inflammatory activity of GCs, with a reduced capacity for unwanted effects in a rat model of dermatitis (Bungard, Hartman et al. 2011). This compound is listed by the company as having completed phase I clinical investigation, for "neurologic and respiratory disease" but no updates have been made since February 2009. Cpd1 is closely related to ZK 245186 (otherwise known as BOL-303242-X or Mapracorat), which is being tested as a novel drug for dermatological or ophthalmological indications such as atopic dermatitis, dry-eye syndrome and postoperative eye inflammation (Schäcke, Zollner et al. 2009; Cavet, Harrington et al. 2010; Zhang, Cavet et al. 2009; Pfeffer, DeWitt et al. 2010; Shafiee, Bucolo et al. 2011). ZK 245186 is, to my

knowledge, the only SGRM involved in an active development program and is listed as being in phase II and III of clinical trial, by Baush & Lomb and Intendis. ZK 245186 was found to inhibit JNK and p38 MAPK phosphorylation in corneal epithelial cells subjected to hyperosmolar stress (Cavet, Harrington et al. 2010). Inhibition of the MAPKs is thought to contribute to the therapeutic effects of the SGRM, and may be mediated by upregulation of DUSP1. The classical GC dex induces expression of DUSP1 in primary human lens epithelium (Gupta, Galante et al. 2005). In the present study I was not able to address responses of ZK 245186 itself, and furthermore I acknowledge the limitations of extrapolating too far from *in vitro* studies. Nevertheless, I consider it quite possible that the anti-inflammatory efficacy and safety of SGRMs like ZK 245186 have little to do with whether or not these compounds are "dissociated". This raises, again, questions about how the properties of the current generation of SGRMs should be interpreted, and how the next generation of SGRMs might best be identified. Recently, another GR ligand, L5, have been identified (Roohk, Varady et al. ; Shah and Scanlan 2004) and although recognised as not being a good drug candidate, due to the lack of anti-inflammatory effects, the authors (Roohk, Varady et al.) recommend the use of *in vivo* pathways measurement for the evaluation of the selectivity of GR ligands. This may be another step in walking away from dichotomous reporter assays.

In conclusion, this report not only highlights the importance of DUSP1 in the anti-inflammatory effects of GR, it also demonstrate that some of the anti-inflammatory of SGRMs are dependent on DUSP1, consequently challenging an important concept of modern steroid receptor biology. Additionally, it hints at a role for the phosphatase in some of GCs post-transcriptional effects, hypothesis which is on the way to be confirmed by work currently undergone by some members of our group. Altogether these findings support, more than ever, a place for DUSP1 and other GR-induced anti-inflammatory genes in novel GR ligands screenings, as well as a potential target for treatment of rheumatic and other chronic inflammatory diseases.

Chapter 7

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7. REFERENCES

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Appendix

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RESEARCH PAPER

Anti-inflammatory effects of selective glucocorticoid receptor modulators are partially dependent on up-regulation of dual specificity phosphatase 1

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BACKGROUND AND PURPOSE

It is thought that the anti-inflammatory effects of glucocorticoids (GCs) are largely due to GC receptor (GR)-mediated transrepression of NF-κB and other transcription factors, whereas side effects are caused by activation of gene expression (transactivation). Selective GR modulators (SGRMs) that preferentially promote transrepression should retain anti-inflammatory properties whilst causing fewer side effects. Contradicting this model, we found that anti-inflammatory effects of the classical GC dexamethasone were partly dependent on transactivation of the dual specificity phosphatase 1 (DUSP1) gene. We wished to determine whether anti-inflammatory effects of SGRMs are also mediated by DUSP1.

EXPERIMENTAL APPROACH

Dissociated properties of two SGRMs were confirmed using GR- and NF-κB-dependent reporters, and capacity to activate GC-responsive elements of the DUSP1 gene was tested. Effects of SGRMs on the expression of DUSP1 and pro-inflammatory gene products were assessed in various cell lines and in primary murine *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$macrophages.

KEY RESULTS

The SGRMs were able to up-regulate DUSP1 in several cell types, and this response correlated with the ability of the compounds to suppress COX-2 expression. Several anti-inflammatory effects of SGRMs were ablated or significantly impaired in *Dusp1*$^{-/-}$ macrophages.

CONCLUSIONS AND IMPLICATIONS

Like dexamethasone, SGRMs appear to exert anti-inflammatory effects partly via the up-regulation of DUSP1. This finding has implications for how potentially therapeutic novel GR ligands are identified and assessed.

Abbreviations

AP-1, activating protein 1; BMM, bone marrow-derived macrophage; CXCL1, chemokine (CXC motif) ligand 1; dex, dexamethasone; DUSP1, dual specificity phosphatase 1; GC, glucocorticoid; GR, GC receptor; GRE, GC response element; GRR, GC responsive region; SEGRA, selective GR agonist; SGRM, selective GR modulator

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Introduction

For more than half a century, synthetic glucocorticoids (GCs) have been extensively used to treat chronic inflammatory diseases such as rheumatoid arthritis, asthma and inflammatory bowel diseases (Barnes, 2006; Hillier, 2007). The basis of their therapeutic action is the impairment, in most cell types, of the expression of pro-inflammatory genes. Various unpredictable and occasionally life-threatening side effects of GCs have been documented since their earliest clinical use (Schacke *et al.*, 2002). These include osteoporosis and diabetes mellitus, atrophy of skin and muscle, hypertension and increased susceptibility to infection. Nevertheless, GCs are still the cornerstone of treatment for many diseases. At the same time, major research initiatives attempt to separate the desired anti-inflammatory effects of GCs from their side effects (Schacke *et al.*, 2007; Hudson *et al.*, 2008; De Bosscher *et al.*, 2010).

GCs modulate gene expression via the GC receptor (GR), a transcription factor belonging to the nuclear hormone receptor superfamily (Newton, 2000; Tuckermann *et al.*, 2005). Lipophilic ligands such as the endogenous GC cortisol or the synthetic GC dexamethasone (dex) diffuse across the cell membrane and bind to GR in the cytoplasm. This promotes the release of GR from a large complex of chaperone proteins and its migration to the nucleus. In most but not all cases, transcriptional activation by GR (transactivation) is dependent on homodimerization, which is mediated by a short motif adjacent to the first of two zinc finger DNA-binding motifs. GR homodimers recognize sequences related to the idealized, palindromic consensus AGAACAnnnTGTTCT (GC response element or GRE).

A second physiologically important function of GR is to inhibit transcription via a mechanism known as transrepression (Kassel and Herrlich, 2007; De Bosscher *et al.*, 2010; Glass and Saijo, 2010). In this case, GR does not bind directly to DNA but instead is recruited to DNA via direct or indirect interactions with other transcription factors, notably members of the activating protein 1 (AP-1) and NF-κB families, both of which play important roles in the expression of pro-inflammatory genes. The presence of GR at AP-1 or NF-κB binding sites is thought to inhibit transcriptional activation by impairing recruitment of transcriptional co-activators, or by promoting recruitment of co-repressors.

It is often stated that the anti-inflammatory effects of GCs are largely mediated by transrepression, whereas side effects are largely mediated by transactivation. If this is correct, it may be possible to improve upon classical GCs by identifying novel ligands of GR that selectively promote its transrepressive function rather than its transactivating function (Schacke *et al.*, 2007; Berlin, 2010; De Bosscher *et al.*, 2010; Newton *et al.*, 2010). Such compounds are known as dissociated GR ligands, selective GR agonists (SEGRAs) or modulators (SGRMs). They are predicted to retain anti-inflammatory effects of classical GCs like dex but cause fewer or less severe side effects. Typically, SGRMs have been identified from drug libraries first on the basis of affinity for GR and second on the basis of effects on reporter constructs. Transactivation is tested against well-known GC target genes such as tyrosine aminotransferase (TAT), or against constructs that contain well-characterized GC responsive promoters or multimerized GR binding sites. Transrepression is tested using promoters that contain AP-1 and/or NF-κB binding sites and are activated by pro-inflammatory stimuli. Alternatively, reporters containing multimerized AP-1 or NF-κB binding sites may be used. As reviewed elsewhere (Schacke *et al.*, 2007; Berlin, 2010; De Bosscher *et al.*, 2010), a number of interesting compounds have been identified using this basic approach. Recently described examples include ZK216348 and LGD-552, which are non-steroidal GR ligands (Schacke *et al.*, 2004; Humphrey *et al.*, 2006; Miner *et al.*, 2007; Lopez *et al.*, 2008).

As well as directly inhibiting expression of pro-inflammatory genes by means of transrepression, GCs can exert indirect therapeutic effects, via the up-regulation of several anti-inflammatory genes (Clark, 2007; Newton and Holden, 2007). For example, many cell types respond to GCs by expressing dual specificity phosphatase (DUSP1), an enzyme that dephosphorylates and inactivates both p38 MAPK and JNK (Abraham and Clark, 2006; Owens and Keyse, 2007). The up-regulation of DUSP1 has been suggested to contribute to destabilization of pro-inflammatory mRNAs (Lasa *et al.*, 2001; 2002; Quante *et al.*, 2008) and to the inhibition of AP-1 and NF-κB function (Diefenbacher *et al.*, 2008; Bladh *et al.*, 2009; Cho and Kim, 2009; King *et al.*, 2009). Correspondingly, many of the anti-inflammatory effects of GCs are impaired in macrophages derived from *Dusp1*$^{-/-}$ mice, or cells in which DUSP1 has been down-regulated using RNA interference (Abraham *et al.*, 2006; Furst *et al.*, 2007; Issa *et al.*, 2007; Kang *et al.*, 2008; Quante *et al.*, 2008; King *et al.*, 2009). *In vivo* anti-inflammatory effects of dex were dependent on DUSP1 in experimental models of acute inflammation (Abraham *et al.* 2006; Wang *et al.*, 2008), asthma (Li *et al.*, 2010) and rheumatoid arthritis (our unpublished results). There are therefore problems with a paradigm that equates anti-inflammatory effects of GCs with transrepression and not transactivation. In fact, such a model is not strongly supported by experimental evidence. For example, there is no genetically modified mouse strain that clearly demonstrates a separation between side effects and transactivation on one hand, and transrepression and anti-inflammatory effects on the other. A knock-in mouse strain expressing a dimerization defective mutant of GR (known as GR^{dim}) was initially thought to provide evidence of just such a mechanistic separation between therapeutic and harmful effects (Tuckermann *et al.*, 1999), but emerging complexities in the phenotype of the GR^{dim} mouse now undermine rather than support the paradigm (Kleiman and Tuckermann, 2007; Frijters *et al.*, 2010; Rauch *et al.*, 2010). Recent results also show that, although SGRMs were identified on the basis of impaired transcriptional activation, they may be quite capable of inducing expression of DUSP1 and other genes with anti-inflammatory roles (Chivers *et al.*, 2006; Janka-Junttila *et al.*, 2006; Lopez *et al.*, 2008; Newton *et al.*, 2010). We therefore asked whether anti-inflammatory effects of SGRMs may actually be dependent on the induction of DUSP1. The answer to this question will have an important impact on how novel GR ligands with improved therapeutic indices are discovered, and how their properties are to be understood.

Methods

Mice and reagents

All animal procedures were performed under United Kingdom Home Office regulations and with local Ethical Review Committee approval. *Dusp1*$^{-/-}$ mice were generated as described previously (Dorfman *et al.*, 1996). They were back-crossed against C57BL/6 for 10 generations to generate a colony with almost pure (99.9%) C57BL/6 genetic background, genotype at the *Dusp1* locus being determined by a PCR assay. The same breeding programme was used to generate a *Dusp1*$^{+/+}$ colony with equivalent genetic background. Age- and sex-matched *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ animals were used to generate bone marrow-derived macrophages (BMMs) by differentiation from BM haematopoietic stem cells for 5 days in RPMI 1640 medium supplemented with 10% FCS, 100 U·mL^{-1} penicillin, 100 µg·mL^{-1} streptomycin (PAA) and 10 ng·mL^{-1} M-CSF (Peprotech, London, UK). This method routinely generates macrophages of at least 85% purity (Lari *et al.*, 2007). Dulbecco's modified Eagle's medium (DMEM, PAA) supplemented with 10% FCS was used for the culture of the HeLa, RAW264.7 and A549 cell lines. Stably transfected A549 cells were maintained with additional 0.2 mg·mL^{-1} of G-418 (Peprotech). All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and seeded to confluence (unless otherwise stated) in FCS supplemented medium the day before the experiment. Cells were treated with different doses of dex (Sigma, Dorset, UK), Cpd1 and Cpd2 (Roche, Palo Alto, CA, USA) or vehicle [dimethyl sulfoxide (DMSO) Sigma] and stimulated with 1 ng·mL^{-1} IL-1β (made in house) or 10 ng·mL^{-1} LPS (Alexis Biochemicals, Exeter, UK).

Plasmids, transfection and luciferase assays

The GRE A549 reporter line (pGL3.neo.TATA.2GRE) was a gift from R Newton (University of Calgary). The NF-κB-dependent reporter containing three κB binding sites linked to a TATA box and a firefly luciferase coding sequence (3κBtkluc) was also generously provided by R Newton. pGL3b-Hs.-4.8, pGL3p-Mm-29GRR, -Hs-1.3GRR and -Hs-4.6GRR were as previously described (Tchen *et al.*, 2010). Cells were seeded to approximately 50% confluence the day before the experiment then transiently transfected using Superfect (Qiagen, Crawley, UK) with 200 ng of *firefly* luciferase reporters as indicated plus 100 ng of *Renilla* luciferase expression vector and pBluescript (Agilent Technologies, Edinburgh, UK) as carrier to make the total quantity of DNA up to 1 µg. Following transfection, cells were treated with vehicle (0.1% DMSO), Dex, Cpd1 or Cpd2 as described. Cells were harvested, and luciferase activities were measured using the dual luciferase reporter assay kit (Promega, Southampton, UK) and Microbeta luminometer (PerkinElmer Life Sciences, Seer Green, UK). Firefly luciferase activities were normalized against *Renilla* luciferase activities.

Western blotting

Whole cell lysates were harvested in ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 1% Triton X-100, 10% glycerol, 0.5% NP40, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride, 3 µg·mL^{-1} aprotinin, 23 µM E64]. Lysates were clarified by centrifugation at 13 000× *g* for 1 min at 4°C, and protein concentrations were measured using Bradford assay. Equal amounts of total protein were loaded on SDS-PAGE gels. After electrophoresis samples were transferred to PVDF membranes (PerkinElmer Life Science), probed with anti-DUSP1, anti-COX-2 and anti-tubulin primary antibodies (Santa Cruz, Heidelberg, Germany; Cayman Chemical, Tallinn, Estonia and Sigma, respectively) then with appropriate horseradish peroxidase-coupled secondary antibodies (Dako, Cambridge, UK). Proteins were detected using the enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK). COX-2 protein expression was estimated by scanning densitometry of Western blots using a calibrated imaging densitomer (GS-710; Bio-Rad Laboratories, Hertfordshire, UK) and the Phoretix ID software.

Measurement of cytokine expression

Supernatants were collected and stored at –20°C until used for measurement of cytokine proteins. IL-12p40 protein was detected by ELISA, using an eBioscience kit, according to the manufacturer's instructions. IL-6, TNF-α and CXCL-1 proteins were detected simultaneously using the xMap Technology from Luminex® and 96-well filter plates (Millipore, Dundee, UK). Briefly, colour-coded Bio-plex beads (Bio-Rad Laboratories), or microspheres, were coupled to antibodies against IL-6, TNF-α and CXCL-1 (R&D Systems, Abingdon, UK) using an amine coupling kit from Biacore Life Sciences (Buckinghamshire, UK). Beads were coated with 50 µg·mL^{-1} of the primary antibodies; standards and samples were then added and left overnight at 4°C. After addition of 0.5 µg·mL^{-1} of the corresponding biotinylated secondary antibodies and streptavidin-PE (Peprotech), cytokines were detected by laser excitation of each internal dye identifying the different microspheres, using the Luminex® 100 Total System.

Measurement of mRNAs

Total cellular RNA was isolated using the RNAeasy® Mini Kit (QIAGEN, Crawley, UK), performing the recommended on-column DNase treatment step. mRNA levels were measured by quantitative real-time PCR using One-Step RT qPCR Mix from Eurogentec (Seraing, Belgium) and TaqMan probes purchased from Applied Biosystems (Paisley, UK) (mouse GAPDH Mm99999915_g1, DUSP1 Mm00457274_g1, COX2 Mm00478374_m1, human GAPDH Hs99999905_g1, DUSP1 Hs00610256_g1, COX-2 Hs00153133_m1). The PCRs were performed in a total volume of 10 µL. The amplification condition consists of an initial reverse transcriptase step of 30 min at 48°C, and 10 min denaturation at 95°C followed by 45 cycles of 3 s at 95°C and 30 s at 60°C. Rotor-Gene 3000 (Corbett Research Ltd, Crawley, UK) was used to quantify the mRNAs. Changes in abundance were assessed by the comparative threshold cycle (ΔCt) method and normalized against GAPDH (measured by the same method).

Statistics and calculation of dissociation indices

Statistical analysis was performed using one-sample *t*-test or ANOVA with the Bonferroni's post-test for multiple comparisons. All tests were performed using Prism software version 5 (GraphPad, La Jolla, CA, USA). A P-value <0.05 was considered significant.

Dissociation indices were calculated for Cpd1 and Cpd2, based on their potencies, in other words, the EC_{50} values for activation of a GRE-dependent reporter and inhibition of an NF-κB-dependent reporter, relative to the corresponding values for dex.

$$\text{Dissociation index} = [EC_{50}{}^{\text{transactivation}}\ \text{Cpd}/EC_{50}{}^{\text{transrepression}}\ \text{Cpd}] \times [EC_{50}{}^{\text{transrepression}}\ \text{dex}/EC_{50}{}^{\text{transactivation}}\ \text{dex}].$$

Dissociation indices were also calculated on the basis of the efficacies of Cpd1 and Cpd2 for activation of the GRE-dependent reporter and repression of the NF-κB-dependent reporter, relative to the corresponding values for dex.

$$\text{Dissociation index} = [\text{Fold activation}^{\text{GRE}}\ \text{Cpd}/\text{Fold inhibition}^{\text{NF-κB}}\ \text{Cpd}] \times [\text{Fold inhibition}^{\text{NF-κB}}\ \text{dex}/\text{Fold activation}^{\text{GRE}}\ \text{dex}].$$

Results

Compounds

The two compounds used in this study were selected from patents registered by two companies that have led research into SGRMs. Compound 1 (Jaroch *et al.*, 2002) is closely related to ZK 216348 (Schacke *et al.*, 2004) and ZK 245186 (Schacke *et al.*, 2009) (Figure 1). In THP-1 cells stimulated with LPS, Cpd1 inhibited the expression of IL-8 with 77% efficacy and EC_{50} of 4.3×10^{-9} M. For comparison, prednisolone (pred) inhibited expression of IL-8 with 95% efficacy and EC_{50} of 2.4×10^{-8} M. In the croton oil ear oedema model, pred and Cpd1 were similarly effective at a dose of 30 mg·kg^{-1}, inhibiting inflammation by 81% and 84%. In the same model, pred induced liver tyrsoine aminotransferase (a surrogate marker of metabolic side effects) by 8-fold, Cpd1 by 3.7-fold (Jaroch *et al.*, 2002). Cpd2 (Coghlan *et al.*, 1999; Kym *et al.*, 2003) is very closely related to LGD-5552 (Lopez *et al.*, 2008) (Figure 1). It is highly selective for GR over progesterone receptor (PR), the respective K_i values being 1.5×10^{-9} M and 1.434×10^{-6} M.

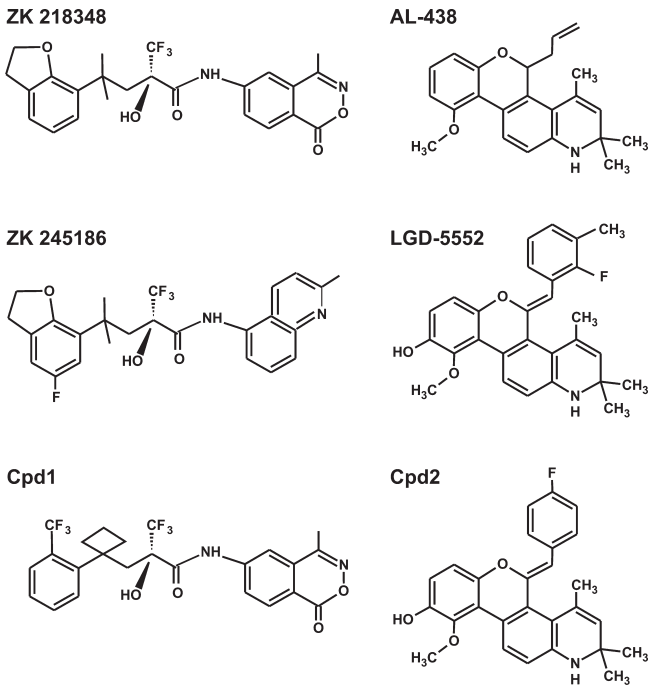


Figure 1
Molecular structures of Cpd1, Cpd2 and near relatives.

Dissociated properties of Cpd1 and Cpd2

Dissociated properties of Cpd1 and Cpd2 were determined using reporters with multimerized binding sites for GR and NF-κB. Similar methods have previously been used to identify SGRMs (Vayssiere *et al.*, 1997) and continue to be used as screening tools in the pharmaceutical industry. The transactivation functions of Cpd1 and Cpd2 were assessed using an A549 pulmonary epithelial cell line stably transfected with a luciferase reporter containing two tandem GREs derived from the rat TAT gene upstream of a minimal TATA box from the rabbit β-globin gene (Figure 2A) (Chivers *et al.*, 2006). Dex activated this construct with A_{max} of 16.4 and EC_{50} of 4.1×10^{-9} M. The novel GR ligands up-regulated luciferase expression with A_{max} and EC_{50} values of 8.4 and 2.9×10^{-8} M (Cpd1); 5.9 and 1.30×10^{-7} M (Cpd2), respectively. Both Cpd1 and Cpd2 therefore have relatively low efficacy and potency in this assay of transcriptional activation. Activation of the GRE reporter by dex, Cpd1 or Cpd2 was effectively blocked by an equivalent concentration of the GR antagonist RU486 (mifepristone), confirming that the SGRMs regulate transcription via GR (Figure 2C). Since Cpd1 and Cpd2 appeared to function as partial agonists of GR-mediated transcription, we considered whether they might impair transcriptional activation by a full agonist such as dex. To address this question, the GRE reporter was activated by 10^{-8} M dex in the presence of increasing concentrations of Cpd1 or Cpd2, from 10^{-9} to 10^{-7} M (Figure 2D). Statistically significant impairment of dex-induced transcription was observed only in the presence of a 10-fold molar excess of Cpd2 over dex.

Transrepression was then tested following transient transfection of A549 cells with a luciferase reporter construct containing three NF-κB binding sites from the human COX-2 gene upstream of a minimal TATA box (Figure 2B) (Holden *et al.*, 2007). This construct was strongly activated by IL-1β and dose-dependently inhibited by dex with an EC_{50} of 3.4×10^{-9} M. However, the extent of inhibition did not exceed 67%. Cpd1 and Cpd2 impaired the activation of the NF-κB reporter with similar efficacy (53% and 54%) and potency (EC_{50} values of 7.8×10^{-9} M and 3.5×10^{-9} M). Dissociation indices were calculated for Cpd1 and Cpd2, based on their potency and efficacy of transactivation and transrepression, relative to the corresponding values for the reference compound dex (see Methods). In either case, a dissociation index greater than 1 indicates selective capacity for transrepression over transactivation. By potency, Cpd1 and Cpd2 had dissociation indices of 3.04 and 30.8, respectively. By efficacy, their dissociation indices were 2.53 and 4.32, respectively. Both compounds therefore appeared to conform to the description of selective GR modulators.

The extent of inhibition of a NF-κB reporter by both dex and the SGRMs was less than anticipated; for example, it was

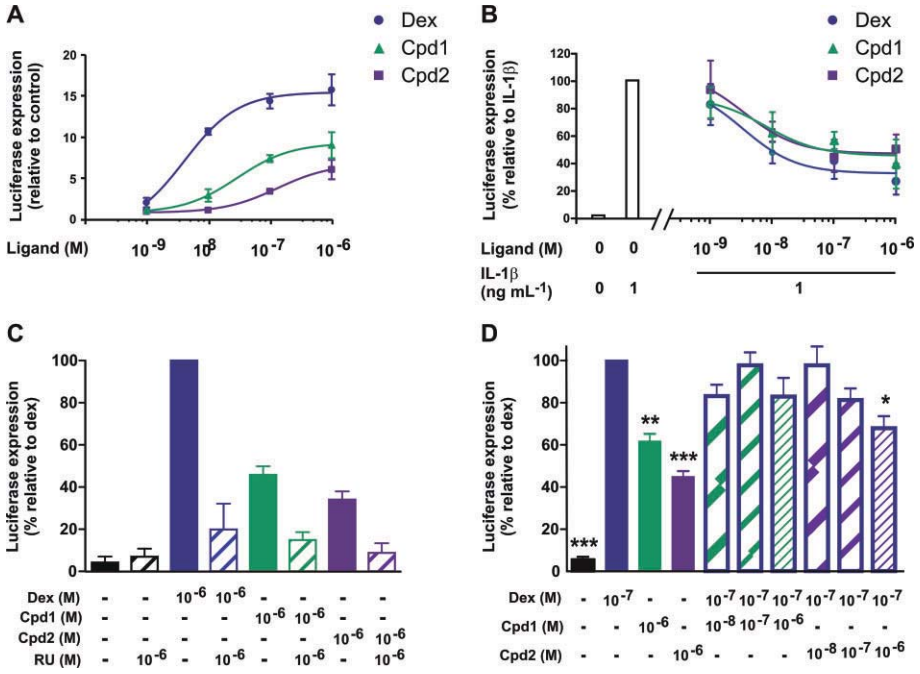


Figure 2

Dissociation parameters and GR dependence of Cpd1 and Cpd2. (A) A549 cells stably transfected with a GRE-dependent reporter were incubated for 6 h with vehicle (0.1% DMSO) or increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 and Cpd2. Cell lysates were prepared, and luciferase activities were measured. Graph indicates mean ± SEM luciferase activities relative to vehicle treated control cells from four independent experiments. (B) A549 cells were transiently transfected with a reporter plasmid expressing firefly luciferase under the control of three tandem NF-κB sites, and with a control plasmid expressing *Renilla* luciferase. The following day, cells were pretreated for 2 h with increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 and Cpd2, then stimulated with 1 ng·mL^{-1} of IL-1β for 6 h. Cell lysates were harvested, and firefly and *Renilla* luciferase activities were measured. Firefly luciferase activities were normalized against *Renilla* luciferase activities to correct for variations in transfection efficiency. The graph represents % of the activity of the IL-1β-stimulated cells ± SEM from four independent experiments. (C) The A549 GRE reporter cell line was incubated for 6 h with vehicle (0.1% DMSO) or 10^{-6} M dex, Cpd1 or Cpd2 in the absence or presence of 10^{-6} M RU486 (RU). Luciferase activities are expressed relative to that of cells treated with dex alone. The graph shows averages from two independent experiments ± SD. (D) The A549 GRE reporter cell line was incubated for 6 h with vehicle, dex and/or SGRMs at the concentrations indicated. Luciferase activities are expressed relative to that of cells treated with 10^{-7} M dex alone. The graph shows averages from three independent experiments ± SEM. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$ relative to cells treated with 10^{-7} M dex alone.

invariably less than the extent of down-regulation of COX-2 mRNA in the same cells (see later, Figure 5). We tested responses of the same NF-κB reporter in a stably transfected A549 cell line; used an alternative reporter based on NF-κB binding sites from the HIV long terminal repeat; or varied concentrations of IL-1β between 0.2 and 10 ng·mL^{-1}. Under no condition was reporter gene expression inhibited by more than 70% (data not shown).

SGRMs are able to induce expression of DUSP1

We and others previously identified two GC responsive regions (GRRs) within the human DUSP1 5′ region, at –1.3 and –4.6 kb with respect to the transcription start site (Johansson-Haque *et al.*, 2008; Shipp *et al.*, 2010; Tchen *et al.*, 2010). Activation of GRR-4.6 by dex was impaired by mutation of the dimerization domain of GR, whereas activation of GRR-1.3 by dex was insensitive to this mutation (Tchen *et al.*, 2010), suggesting that GR may interact differently with the two sites. Having established that SGRMs were capable of transcriptional activation of a stereotypical GRE reporter, we asked whether they could also regulate transcription via GRR-1.3 and -4.6 (Figure 3). The reporter constructs pGL3p-GRR-1.3 and pGL3p-GRR-4.6 (Tchen *et al.*, 2010) are based on the pGL3p vector (Promega), in which firefly luciferase is expressed under the control of an SV40 early promoter. Upstream of the SV40 promoter, pGL3p-GRR-1.3 contains the region of the human Dusp1 locus from –1366 to –1237 with respect to the transcription start site. pGL3p-GRR-4.6 contains the region –4834 to –4369. Cpd1 and Cpd2 significantly activated transcription via both GRR-1.3 and GRR-4.6. The proximal element (GRR-1.3) was slightly less responsive to the two SGRMs than to dex. The distal element (GRR-4.6) was approximately five-fold more responsive to dex than to either of the SGRMs. It was also established by chromatin IP (ChIP), that all three ligands could promote recruitment of GR to both GRR-1.3 and GRR-4.6 in HeLa cells (not shown).

The ability of GR ligands to induce expression of DUSP1 mRNA and protein was next tested in A549, HeLa (human epithelial carcinoma cell line), RAW264.7 (mouse macrophage-like cell line) and BMM (primary mouse bone marrow-derived macrophages) (Figure 4). Note that BMMs displayed anomalous responses to the highest doses of GR

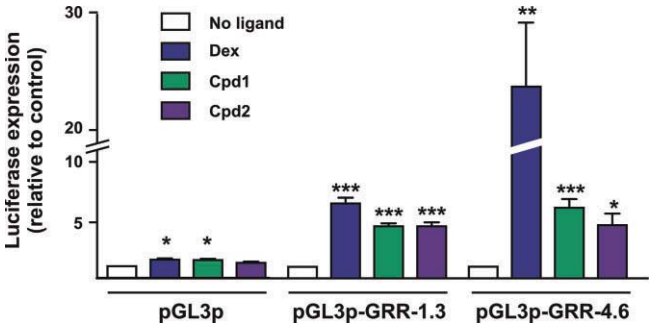


Figure 3

Cpd1 and Cpd2 activate transcription via GC-responsive regions of the human DUSP1 gene. HeLa cells were transiently transfected with pGL3p or derivatives that contain GRR-1.3 or GRR-4.6 from the human DUSP1 gene. A plasmid expressing *Renilla* luciferase was co-transfected as a control for transfection efficiency. Cells were treated with vehicle (0.1% DMSO) or 10^{-7} M GR ligands as indicated for 6 h, then lysates were prepared, firefly and *Renilla* luciferase activities were measured and fold responses to ligand were calculated. The graph represents mean ± SEM from three (pGL3p), six (pGL3p-GRR-1.3) or seven (pGL3p-GRR-4.6) independent experiments. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$ relative to vehicle-treated cells.

ligands (Figure 4D), possibly due to effects on cell viability or proliferation. This phenomenon was not investigated further. The pattern of response of the DUSP1 gene was highly variable between cell types and was not predictable from the response of the GRE reporter, even within a single cell type. For example, in A549 cells Cpd2 was a poor activator of the GRE reporter but activated the endogenous DUSP1 gene with similar efficacy to dex (although with a rather higher EC_{50}). In HeLa cells, the endogenous DUSP1 gene responded similarly to dex and Cpd2. Cpd1 reached the same efficacy (fold activation) but had a higher EC_{50} (lower potency) than the other two compounds. Compared to dex, both SGRMs had low efficacy and potency in RAW264.7 cells and in primary murine macrophages. Rank orders of efficacy and potency of the three compounds for activation of DUSP1 gene expression were not the same for any two cell lines, illustrating the extreme variability of response.

Anti-inflammatory effects of SGRMs are partially dependent on DUSP1

Having shown that SGRMs are able to induce expression of DUSP1, we investigated the relationship between DUSP1 up-regulation and anti-inflammatory effects of SGRMs in A549 cells (Figure 5) RAW264.7 cells and mouse macrophages (Figure 6). Suppression of COX-2 was selected as a read-out of anti-inflammatory efficacy, because this gene is up-regulated by different pro-inflammatory stimuli in many cell types, and is a well characterized GC target with NF-κB sites in its promoter. In A549 cells, the down-regulation of Cox-2 mRNA and protein mirrored the up-regulation of Dusp1 mRNA and protein (Figure 5A–C). Inhibition of COX-2 protein expression was estimated by scanning densitometry of Western blots. EC_{50} values for induction of Dusp1 mRNA and inhibition of COX-2 mRNA and COX-2 protein were calculated. EC_{50} values

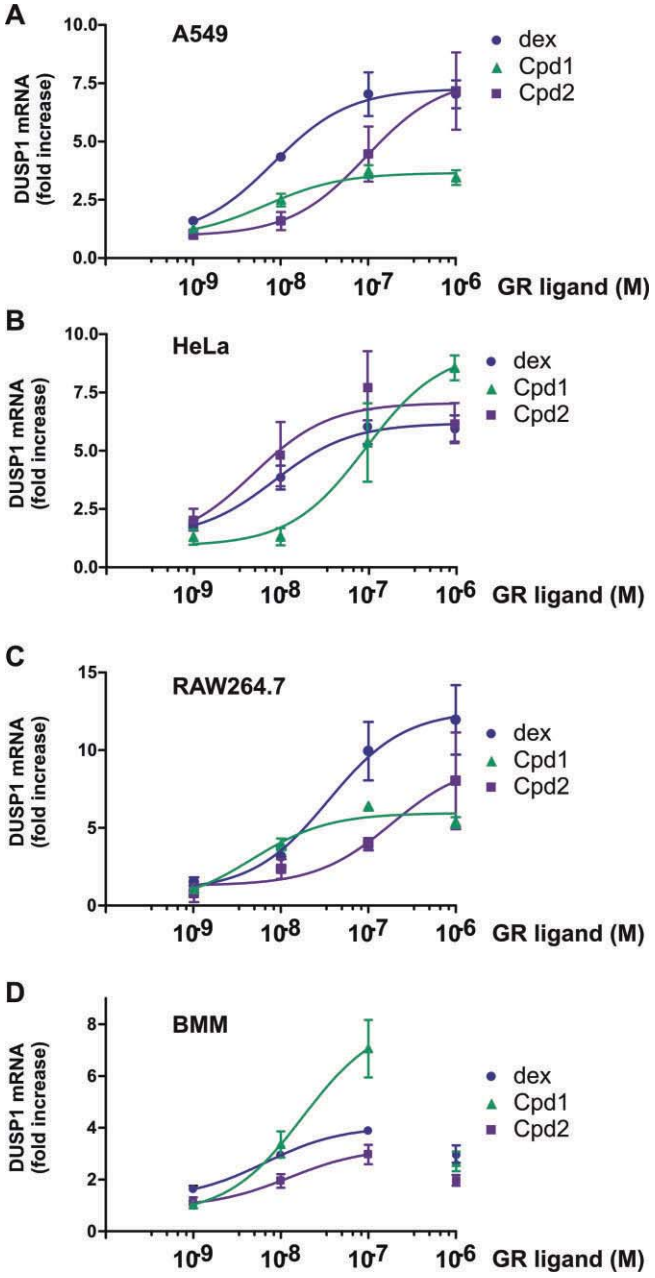


Figure 4

Induction of DUSP1 mRNA by Cpd1 and Cpd2. (A) A549 (B) HeLa (C) RAW cells and (D) BMM were treated with increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 and Cpd2 for 1 h. mRNA was harvested and DUSP1 expression quantified using RT-PCR. For each cell type, results were presented as fold induction relative to cells treated with vehicle alone (0.1% DMSO). Graphs indicate means ± SEM from at least three independent experiments.

for induction of DUSP1 protein could not be determined because of non-specific background in the Western blots. For each GR ligand, the calculated EC_{50} values for DUSP1 induction and COX-2 inhibition were close to one another (Table 1). At the mRNA level, the correspondence between Dusp1 up-regulation and Cox-2 inhibition can be seen in the symmetry of the dose-response curves (Figure 5C). RU486 incom-

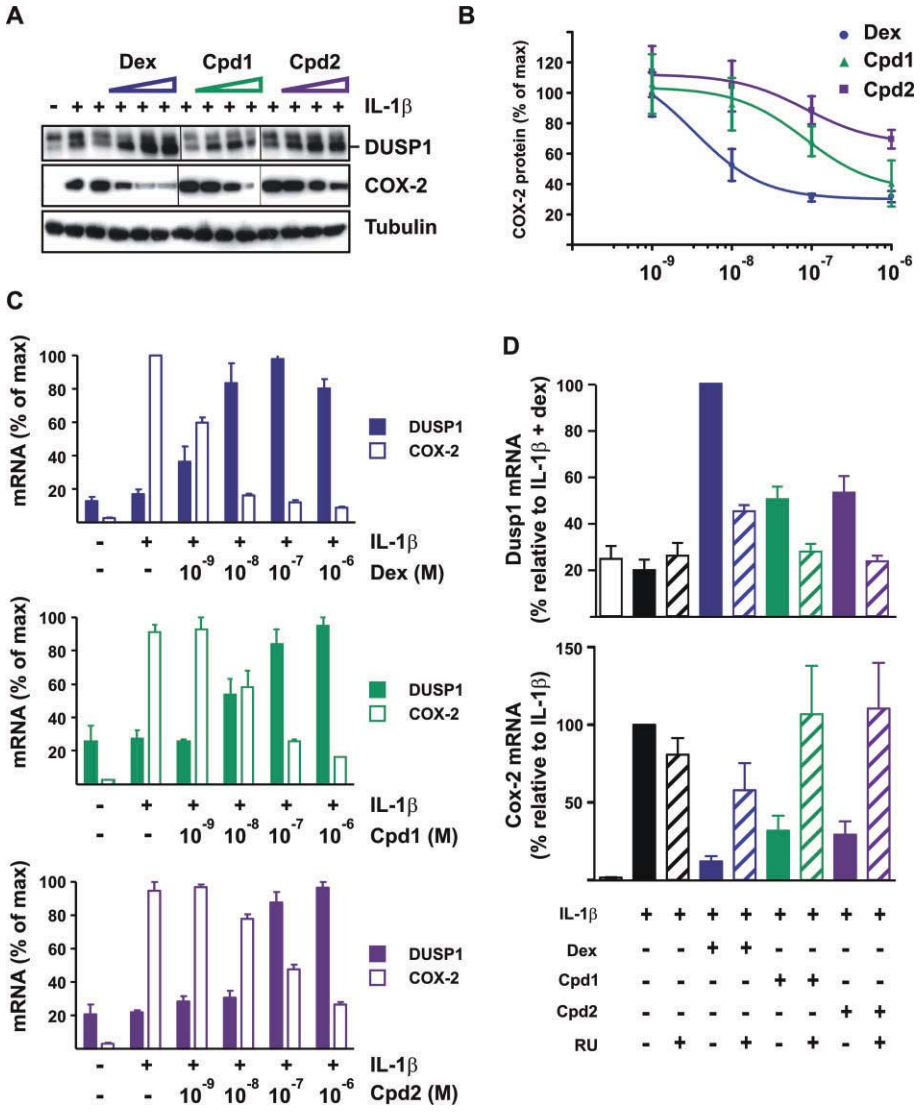


Figure 5

Effects of Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in A549 cells. (A, B, C) A549 cells were pretreated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 or Cpd2 then challenged for 4 h with 1 ng·mL^{-1} of IL-1β. (A) Representative Western blot of DUSP1 and COX-2 protein expression. (B) COX-2 protein expression was quantified by scanning densitometry in three independent experiments. (C) Dusp1 and Cox-2 mRNA were measured by real-time PCR and plotted relative to maximum expression. Graphs show averages ± SEM from three independent experiments. (D) A549 cells were treated with IL-1β (1 ng·mL^{-1}), RU486 (RU), dex, Cpd1, Cpd2 (each 10^{-6} M) in the combinations indicated. Dusp1 and Cox-2 mRNAs were measured and expressed with respect to cells treated with either IL-1β alone (COX-2) or IL-1β + dex (Dusp1). Graphs represent averages ± SEM from four independent experiments.

pletely blocked the up-regulation of Dusp1 mRNA by dex and incompletely rescued COX-2 from dex-mediated suppression (Figure 5D). RU486 completely prevented the up-regulation of Dusp1 mRNA by Cpd1 or Cpd2 and effectively rescued COX-2 mRNA from inhibition by either of the SGRMs. Like dex, Cpd1 and Cpd2 therefore exert anti-inflammatory effects via GR and not via an off-target mechanism.

In RAW264.7 cells (Figure 6A) or primary mouse macrophages (Figure 6B) stimulated with LPS, the up-regulation of *Dusp1* and down-regulation of *COX-2* mRNAs by GR ligands displayed similar dose-dependence, again demonstrated by the symmetry of the dose-response curves. In BMM, Cpd1 and Cpd2 up-regulated *Dusp1* expression with similar A_{max} (5 and 5.4, respectively), slightly less than the A_{max} value of 7.1 for induction of *Dusp1* by dex. Cpd1 induced *Dusp1* with EC_{50} of 1.4×10^{-9} M; therefore, *Dusp1* expression was near maximal at both 10^{-8} and 10^{-6} M. Cpd2 induced *Dusp1* with a considerably higher EC_{50} of 1.9×10^{-8} M; therefore, expression was still increasing in the range 10^{-8} and 10^{-6} M. These observations provide context for the following analysis of responses to SGRMs in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages.

The above results are consistent with, but do not prove, an important role for DUSP1 in the anti-inflammatory effects

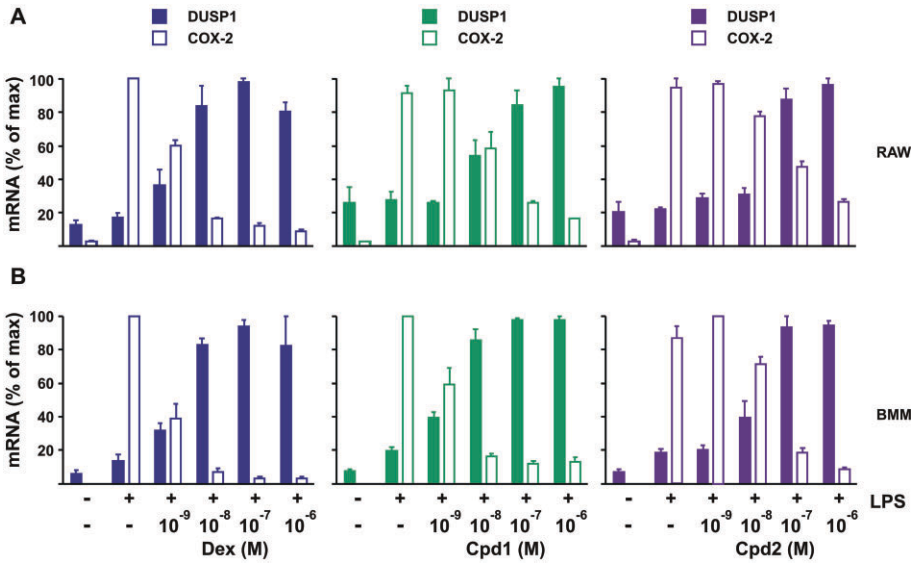


Figure 6

Effects of Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in RAW264.7 cells and BMMs. RAW264.7 cells (A) or BMMs (B) were pretreated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 or Cpd2 then challenged for 4 h with 10 ng·mL^{-1} of LPS. *COX-2* and *Dusp1* mRNAs were measured and plotted as in Figure 5D.

Table 1

EC_{50} values for induction of DUSP1 mRNA, inhibition of COX-2 mRNA and protein expression in A549 cells

	EC_{50} (M) DUSP1 mRNA	COX-2 mRNA	COX-2 protein
Dex	7.0×10^{-9}	6.3×10^{-9}	5.2×10^{-9}
Cpd 1	1.52×10^{-8}	1.01×10^{-8}	3.35×10^{-8}
Cpd 2	3.83×10^{-8}	3.19×10^{-8}	8.52×10^{-8}

Values were calculated on the basis of the data illustrated in Figure 5B and C.

of both dex and SGRMs. To investigate this further, we tested effects of GR ligands on the expression of several pro-inflammatory genes in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mouse BMMs. As previously reported (Chivers *et al.*, 2006; Hammer *et al.*, 2006), *Dusp1*$^{-/-}$ macrophages overexpressed IL-6 and TNFα proteins when stimulated with LPS, whilst the expression of IL-12p40 was significantly less than in *Dusp1*$^{+/+}$ macrophages (Figure 7). Increased expression of *COX-2* mRNA in the knockout macrophages did not reach statistical significance. In agreement with previous observations (Abraham *et al.*, 2006), 10^{-8} or 10^{-6} M dex strongly decreased the expression of TNF protein in *Dusp1*$^{+/+}$ macrophages but had relatively little effect in *Dusp1*$^{-/-}$ macrophages (Figure 8). We previously reported that dex down-regulated *Il-6* mRNA in a manner largely dependent on DUSP1. Here it is shown that inhibition of IL-6 expression at the protein level is entirely dependent on DUSP1. The same experiments confirm that dex inhibits expression of IL-12p40 protein independently of DUSP1, emphasizing that both DUSP1-dependent and -independent mechanisms contribute to the anti-inflammatory action of GCs (Abraham *et al.*, 2006). Finally, inhibitory effects of dex on *COX-2* mRNA were largely DUSP1-dependent as previously shown.

In terms of its anti-inflammatory effects, Cpd1 generally behaved like a version of dex with lower efficacy but similar potency. It inhibited IL-6 less effectively than dex but again in an entirely DUSP1-dependent manner. Its inhibitory effects on *COX-2* mRNA were less strong than those of dex but also largely dependent on DUSP1. In all of these cases, inhibitory effects were similar at 10^{-8} and 10^{-6} M. Cpd1-mediated inhibition of TNF expression was also clearly dependent on DUSP1, although in this case the difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages became significant only at 10^{-6} M. IL-12p40 expression was equally inhibited by Cpd1 in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages. These observations suggest that, like dex, Cpd1 exerts anti-inflammatory effects in part via DUSP1.

Cpd1 and Cpd2 had similar inhibitory effects on pro-inflammatory genes at 10^{-6} M concentration in wild-type macrophages. At 10^{-8} M concentration, Cpd2 was invariably less effective than Cpd1. Nevertheless, some of the anti-inflammatory effects of Cpd2 were clearly dependent on DUSP1. For example, expression of IL-6 was significantly decreased by either 10^{-8} or 10^{-6} M Cpd2 in *Dusp1*$^{+/+}$ macrophages but not in *Dusp1*$^{-/-}$ macrophages. In the cases of TNF protein or COX-2 mRNA, responses to Cpd2 significantly differed between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages at only one of the two concentrations tested. Inhibition of IL-12p40 expression was independent of DUSP1 as expected. The overall picture is of a compound that depends upon DUSP1 for some of its anti-inflammatory effects but induces DUSP1 expression with relatively low potency.

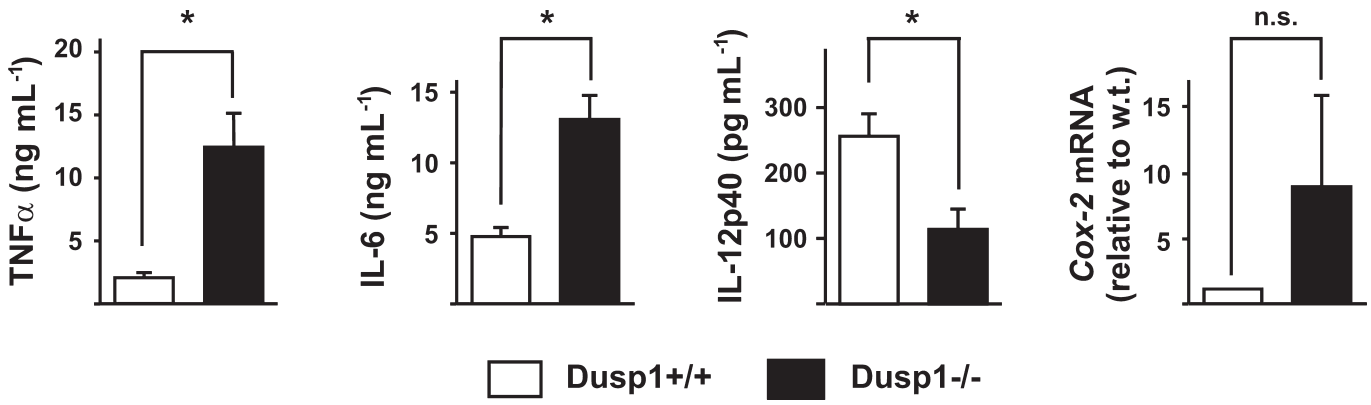


Figure 7

Altered expression of inflammatory mediators in *Dusp1*$^{-/-}$ macrophages. *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMM were stimulated with 10 ng·mL^{-1} LPS for 4 h, supernatants were harvested and mRNA was isolated. TNFα and IL-6 proteins were quantified by Luminex, IL-12p40 by ELISA, and *COX-2* mRNA by quantitative PCR. Graphs represent averages from four independent experiments ± SEM. * indicates statistically significant difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMM ($P < 0.05$). n.s., not statistically significant.

Discussion

Novel compounds that preferentially mediate transrepression have been predicted to cause fewer side effects than classical GCs (Newton and Holden, 2007; Schacke *et al.*, 2007; Berlin, 2010; De Bosscher *et al.*, 2010). The notional mechanistic uncoupling of therapeutic and harmful consequences of GR activation suggested a straightforward course of action. Safer GR ligands might be discovered through screening strategies based on constructs that contain multimerized binding sites for GR itself (as a reporter for transactivating function) or for NF-κB or AP-1 (as reporters for transrepressing function). However, this idea has its roots in relatively simplistic and now outdated conceptions of how GR controls gene expression. Transcriptional activation by GR is now known to be a remarkably diverse process. Binding sites for GR can extensively vary from the idealized consensus AGAACAnnnTGTTCT, only five or six positions within this sequence being strongly constrained (So *et al.*, 2007; Reddy *et al.*, 2009; John *et al.*, 2011). Single nucleotide variations in binding site sequence can have profound effects on the conformation adopted by GR and the downstream consequences (Meijsing *et al.*, 2009). Only about 0.4% of possible binding sites are recognized by GR in one cell type, this repertoire being dictated by cell type-specific modulation of chromatin accessibility (John *et al.*, 2011). GR also co-operates with a large number of other transcription factors to control transcription (Clark, 2007; Kassel and Herrlich, 2007). Some of these accessory factors are likely to be required for the establishment of domains of open chromatin structure within which GR can bind to DNA. At individual GC-regulated genes, and probably at individual *cis*-acting elements of one gene, GR displays different requirements for transcriptional cofactors (Chen *et al.*, 2006; Galliher-Beckley *et al.*, 2008; John *et al.*, 2011). Finally, GR is extensively post-translationally modified, and GC-responsive elements may display differential requirements for different GR modifications (Beck *et al.*, 2009; Galliher-Beckley and Cidlowski, 2009).

To stand as representative of all GR-mediated transcriptional activation events is therefore an unreasonably large burden for one highly simplified reporter, or even for one or two endogenous genes (Clark, 2007). Some of the problems of extrapolating from simple reporters are well illustrated by the present study. Even within one cell type (A549), the dose-dependence of induction of Dusp1 gene expression by the two SGRMs did not resemble the dose dependence of activation of the GRE reporter (compare Figures 2A and 4A). The predictive value of the reporter became even poorer when other cell types were considered (Figure 4B–D). Individual response elements of the Dusp1 locus were not necessarily better predictors of the behaviour of the endogenous gene. For example, a previous study identified a powerfully GC-responsive region located 4.6 kb upstream of the Dusp1 transcription start site (Tchen *et al.*, 2010). In HeLa cells, this element was quite weakly activated by Cpd1 and Cpd2 compared with dex (Figure 3), whereas the endogenous gene was similarly activated by all three GR ligands at the relevant dose of 10^{-8} M (Figure 4B). At least some of this variation in response of reporter constructs and endogenous GC-regulated genes is probably explained by differential cofactor requirements and variable expression of cofactors in different cell types.

It is hard to escape the conclusion that idealized reporters containing tandem GR binding sites are of little practical help when trying to determine the transactivating properties (and hence dissociated nature) of GR ligands. RU24858, an earlier example of a supposedly dissociated GR ligand, was later found to be capable of up-regulating a subset of GR-regulated genes (Chivers *et al.*, 2006; Janka-Junttila *et al.*, 2006). LGD-5552, a near-relative of Cpd2, was also found to be capable of transcriptional activation but differed from the classical GC prednisolone in the profile of genes activated (Lopez *et al.*, 2008). When a compound is described as being dissociated or as having poor capacity to activate transcription, it should therefore be asked exactly what this means and how it has been demonstrated. It is unclear whether genuine and consistent separation between transactivation and transrepres-

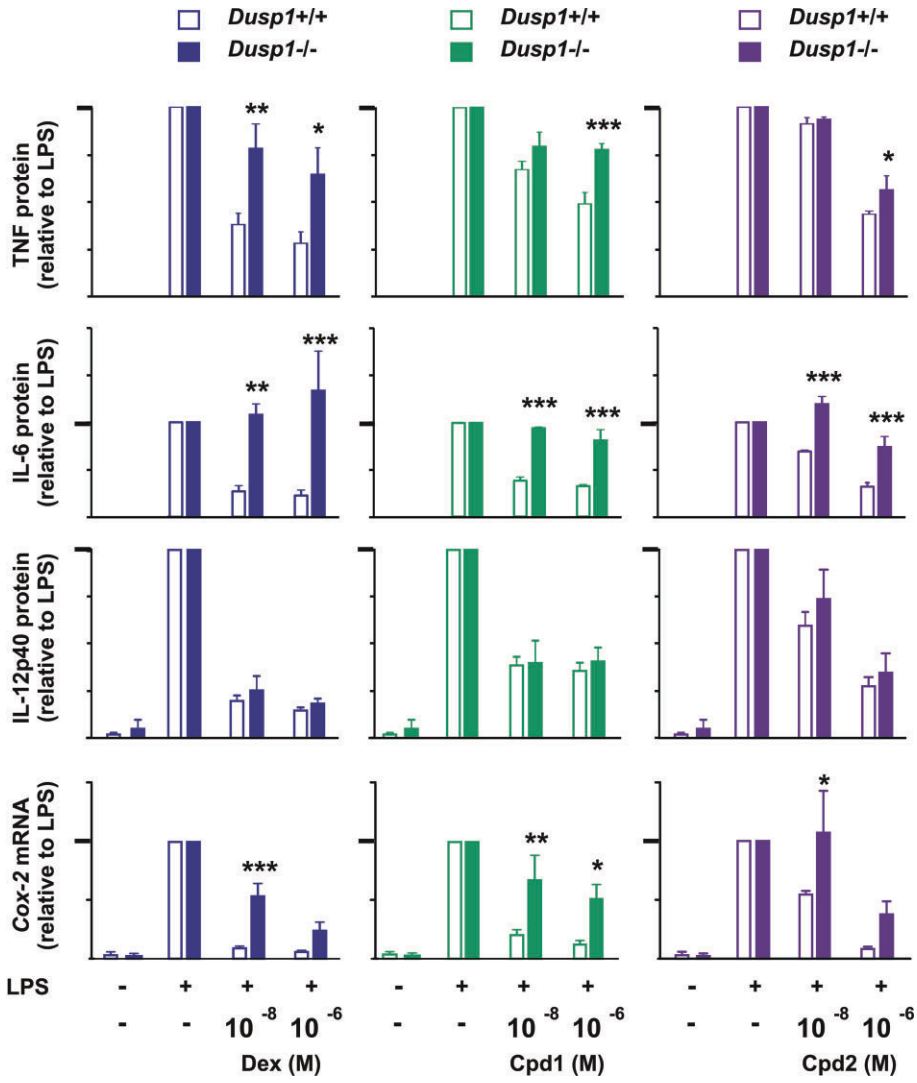


Figure 8

Effects of Cpd1 and Cpd2 on expression of pro-inflammatory mediators in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages. BMM from *Dusp1*$^{+/+}$ mice and *Dusp1*$^{-/-}$ mice were pretreated for 2 h with vehicle (0.1% DMSO), 10^{-8} or 10^{-6} M dex, Cpd1 or Cpd2, then challenged with 10 ng·mL^{-1} of LPS for another 4 h. TNF, IL-6 and IL-12p40 protein expression were measured by Luminex or ELISA and *COX-2* mRNA by real-time PCR. Results are presented as percentages of the response in cells treated with LPS and vehicle. The *y*-axes differ in scale. In each case, the 100% level is represented by a heavy tick mark. Graphs indicate mean ± SEM from at least three independent experiments. Statistical analysis is shown for *Dusp1*$^{-/-}$ versus *Dusp1*$^{+/+}$ under the same conditions. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$.

sion properties of GR can be demonstrated using simple reporters, or whether it can be achieved. We and other investigators have questioned whether such separation is even desirable, given that GCs up-regulate a number of anti-inflammatory factors, and depend on these factors for at least some of their anti-inflammatory effects (Smoak and Cidlowski, 2006; Clark, 2007; Newton and Holden, 2007).

Both Cpd1 and Cpd2 have emerged from deliberate efforts to selectively promote the transrepression rather than transactivation function of GR. With respect to transcriptional activation of GRE reporters and endogenous Dusp1 genes, both compounds behaved like partial agonists of GR. However, Cpd1 did not significantly block transcriptional activation by the full agonist dex (Figure 2D). This surprising finding is reminiscent of the first generation SGRM RU24858, which was also found not to inhibit transcriptional activation by the full agonist dex (Vayssiere *et al.*, 1997). A possible conclusion is that dex and Cpd1 recognize different surfaces of GR and do not bind the receptor in a mutually exclusive manner. This appears unlikely because of the ability of RU486 to antagonize transcriptional activation by either compound (Figure 2C). A second possibility is that Cpd1 has relatively low affinity for GR in intact cells and is therefore not an effective competitor for binding. It should also be pointed out that demonstration of the expected partial antagonism is quite challenging in the case of Cpd1. At the respective concentrations of 10^{-7} and 10^{-6} M, dex and Cpd1 differ by only about 40% in transactivation of the GRE reporter (Figure 2D). This is a relatively small window in which to

demonstrate competitive inhibition by Cpd1 of the response to dex. We cannot conclude that partial antagonism does not occur, only that we have been unable to demonstrate it. Cpd2 being a weaker activator of transcription, partial antagonistic behaviour was more straightforward to demonstrate (Figure 2D). To fully understand the partial agonist/antagonist properties of the two SGRMs requires biochemical and crystallographic studies that are beyond the scope of the present study. In any case, this issue does not effect our major conclusions.

Most importantly, the anti-inflammatory effects of the SGRMs were in direct proportion to their capacity to induce DUSP1 expression in a number of cell types and demonstrably dependent on DUSP1 in mouse macrophages. Cpd2, which had the higher dissociation indices, was the weaker inducer of DUSP1 and the poorer anti-inflammatory agent in all experimental settings used. The importance of DUSP1 as a mediator effect of GR was particularly clearly demonstrated in the case of IL-6. Neither dex, Cpd1 nor Cpd2 was capable of decreasing expression of IL-6 protein in *Dusp1*$^{-/-}$ macrophages. It is ironic that IL-6 is a well-characterized NF-κB target, whose inhibition by GCs has usually been interpreted in terms of transrepression.

Cpd1 is closely related to ZK 245186 (otherwise known as BOL-303242-X or Mapracorat), which is being tested as a novel drug for dermatological or opthalmological indications such as atopic dermatitis, dry-eye syndrome and postoperative eye inflammation (Schacke *et al.*, 2009; Zhang *et al.*, 2009; Cavet *et al.*, 2010; Pfeffer *et al.*, 2010; Shafiee *et al.*, 2010). ZK 245186 was found to inhibit JNK and p38 MAPK phosphorylation in corneal epithelial cells subjected to hyperosmolar stress (Cavet *et al.*, 2010). Inhibition of the MAPKs is thought to contribute to the therapeutic effect of the SGRM and may be mediated by up-regulation of DUSP1. The classical GC dex induces expression of Dusp1 in primary human lens epithelium (Gupta *et al.*, 2005). In the present study, we were not able to assess responses to ZK 245186 itself, and we acknowledge the danger of extrapolating too far from *in vitro* studies. Nevertheless, we consider it quite possible that the anti-inflammatory efficacy and safety of SGRMs like ZK 245186 have little to do with whether or not these compounds are 'dissociated'. This raises important questions about how the properties of the current generation of SGRMs should be interpreted and how the next generation of SGRMs might best be identified.

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Conflicts of interest

The authors have no conflict of interest.

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