An investigation of the role of phosphorylation at Ser211 of the glucocorticoid receptor in ligand-specific transcriptional regulation.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work (unless acknowledged otherwise) and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Date

Abstract

Endogenous glucocorticoids (GCs) modulate many physiological functions in the human body and synthetic GCs are the most effective therapy in the treatment of inflammation, autoimmune and endocrine disorders. However, the long-term usage of synthetic GCs is associated with severe side-effects. GCs mediate their effects through the ligand-dependent transcription factor, the glucocorticoid receptor (GR), either by causing an increase (transactivation) or a decrease (transrepression) in gene transcription. The bioactivity of a ligand in GR-mediated transcriptional regulation is established by a transcriptional dose-response curve, where the potency (EC_{50} value) and the efficacy (maximal response) of the ligand are determined. A central question is how different GR ligands elicit their differential physiological responses for the same gene in the same cell. The main aim of this thesis is to investigate if the phosphorylation of GR at serine 211 (Ser211) correlates with the potency and/or efficacy of a particular ligand in transactivation and transrepression of gene expression. Firstly, the potency and efficacy elicited by twelve different test compounds (agonists, partial agonists, antagonists and SEGRAs) with the same synthetic promoter reporter construct were determined in two different cell systems, transiently transfected COS-1 cells and stably transfected U2OS-hGR cells. Secondly, the extent of phosphorylation of GR at Ser211 induced by the twelve test compounds was determined at both subsaturating (100 nM) and saturating (10 μM) concentrations of ligands in both cell systems. The data presented show a strong correlation between potency and efficacy for transactivation and the extent of GR phosphorylation at Ser211 induced by a ligand at saturating concentrations independent of the cell system investigated. However, the correlation analyses are weaker at subsaturating concentrations in the COS-1 cells, probably due to deviations caused by the partial agonists. This study also indicates that there might be a correlation between phosphorylation at Ser211 and the efficacy and potency in transrepression. Furthermore, it was shown that after ligand stimulation a phosphorylation deficient mutant (S211A) displays different efficacy, but not potency, in transactivation as compared to wildtype receptor. Thus, phosphorylation at Ser211 is not required for the differential efficacies elicited by different GR ligands. One of the ligands investigated is Compound A (CpdA), a non-steroidal plant derivative that dissociates between transactivation and transrepression (a SEGRA). The binding properties of CpdA to GR were further investigated, as CpdA displays unusual GR-binding characteristics. The results show that CpdA does not differentiate between the A- and B- isoform of GR or the

various subpopulations of phosphorylated GR. Understanding the mechanisms of ligand-selectivity of GR-mediated transcriptional regulation could be useful in the design of new drugs that have better therapeutic and side-effect profiles.

Opsomming

Endogene glukokortikoïede (GKe) moduleer 'n verskeidenheid fisiologiese funksies in die menslike liggaam, en sintetiese GKe is die mees effektiewe behandeling vir inflammasie, auto-immuun- en endokriene versteurings. Die langtermyn gebruik van sintetiese GKe word egter geassosieer met ernstige newe-effekte. Die effekte van GKe word bemiddel deur die glukokortikoïed-reseptor (GR), 'n ligand-afhanklike transkripsiefaktor, en behels óf 'n toename (transaktivering) óf 'n afname (transonderdrukking) in geentranskripsie. Die bio-aktiwiteit van 'n ligand in GR-bemiddelde transkripsionele regulering word bepaal deur middel van 'n transkripsionele dosis-respons grafiek, waardeur die sterkte (EC_{50}) en die doeltreffendheid (die maksimum respons) bereken kan word. 'n Kernvraag is hoe verskillende GR ligande verskillende fisiologiese response vanaf een geen in een sel-tipe kan ontlok. Die hoofdoel van hierdie tesis is om te bepaal of die fosforileringstatus van die GR op serien 211 (Ser211) ooreenstem met die sterkte en/of die doeltreffendheid waarmee 'n spesifieke ligand geenuitdrukking transaktiveer en transonderdruk. Die eerste stap was om die sterkte en doeltreffendheid van twaalf verskillende toetsverbindings (agoniste, gedeeltelike agoniste, antagoniste en selektiewe GR agoniste (SEGRAs)) vir die aktivering van dieselfde sintetiese promoter-rapporteerderkonstruk te bepaal. Die bepaling is gedoen in twee verskillende selsisteme, naamlik tydelik-getransfekteerde COS-1 selle en stabiel-getransfekteerde U2OS-hGR selle. Tweedens is bepaal tot watter mate fosforilering van die GR op Ser211 deur die twaalf toetsverbindings, by beide onversadigende (100 nM) en versadigende (10 µM) konsentrasies, in beide selsisteme geïnduseer word. Die data wat aangebied word dui op 'n sterk ooreenstemming tussen sterkte en doeltreffendheid van transaktivering en die omvang van GR fosforilasie op Ser211 wat deur 'n ligand geïnduseer word by versadigende konsentrasie, ongeag watter selsisteem gebruik word. Die ooreenstemming is egter nie so sterk by onversadigende konsentrasies in COS-1 selle nie, moontlik as gevolg van afwykings deur die gedeeltelike agoniste. Hierdie studie dui ook aan dat daar moontlik ooreenstemming kan wees tussen fosforilering op Ser211 en die sterkte en doeltreffendheid van transonderdrukking. Daar word ook verskille in doeltreffendheid, maar nie in sterkte nie, vir ligand-gestimuleerde transaktivering tussen die natuurlike (wilde-tipe) GR en 'n onfosforileerbare GR mutant (S211A) getoon. Dit dui aan dat fosforilering op Ser211 nie vereis word vir die verskille in doeltreffendheid van verskillende GR ligande nie. Een van die ligande wat ondersoek is, was Verbinding A, 'n nie-steroïedverbinding van plantaardige oorsprong wat onderskei tussen transaktivering en transonderdrukking van geenuitdrukking

('n SEGRA). Aangesien Verbinding A ongewone GR-bindingseienskappe toon, is die binding van Verbinding A aan die GR verder ondersoek. Die resultate dui aan dat Verbinding A nie onderskei tussen die A- en B-isovorms van die GR, of tussen die verskillende subpopulasies van gefosforileerde GR nie. Kennis van die meganismes van ligand-selektiwiteit in GR-bemiddelde transkripsionele regulering kan nuttig wees vir die ontwerp van nuwe middels met beter terapeutiese eienskappe en minder newe-effekte.

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List of abbreviations

3'UTR	3' Untranslated region
5'UTR	5' Untranslated region
AD	Activation domain
AF-1	Activation function-1
AF-2	Activation function-2
AL438	Compound Abbott-Ligand 438
Ald	Aldosterone
AP-1	Activator protein-1
AR	Androgen receptor
ATF	Protein-activating transcription factor
BAF	BRG-associated factor
BRG1	Brahma-related gene 1
CBP	CREB binding-protein
CDK	Cyclin-dependent kinase
Cort	Cortisol
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor II
CpdA	Compound A
CRE	cAMP response element
CREB	CRE-binding protein
CRH	Corticotropin releasing hormone
D06	Abbott-Ligand 082D06
DBD	DNA binding domain
Dex	Dexamethasone
DRIP	Vitamin D receptor-interacting protein
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
G6Pase	Glucose-6-phosphatase
GCs	Glucocorticoids
GILZ	GC-induced leucine zipper
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein-coupled receptor

GR	Glucocorticoid receptor
GRIP1	GR-interacting protein 1
GS	Glutamine synthetase
GSK	Glycogen synthase kinase
GRE	Glucocorticoid response element
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HNF1	Hepatic nuclear factor 1
HPA	Hypothalamic-pituitary-adrenal
HRE	Hormone response element
Hsp	Heat shock protein
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
JNK	c-Jun N-terminal kinase
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MMP-1	Matrix metalloproteinase-1
MMTV	Mouse mammary tumor virus
MPA	Medroxyprogesterone acetate
MR	Mineralcorticoid receptor
NCoR	Nuclear receptor corepressor
NET-A	Norethisterone acetate
NF1	Nuclear factor 1
NF-κB	Nuclear factor-κB
nGRE	Negative GRE
NID	Nuclear receptor interaction domain
NL1	Nuclear localisation signal domain 1
NL2	Nuclear localisation signal domain 2
NOR-1	Neuron-derived orphan receptor
NT/N	Neurotensin/neuromedin N
NurRE	Nur response element

OTFs	Octamer transcription factors
PAH	Phenylalanine hydroxylase
p/CAF	p300/CBP-associated factor
pCMV	Cytomegalovirus promoter
PGC-1	Peroxisome proliferator-activated receptors-γ coactivators 1
PKC	Protein kinase C
PNMT	Phenylethanolamine N-methyltransferase
POMC	Proopiomelanocortin
PR	Progesterone receptor
Pred	Prednisolone
Prog	Progesterone
RU486	Roussel-Uclaf 38486
SEGRAs	Selective glucocorticoid receptor agonists
SRC	Steroid receptor coactivators
TAFs	TBP-associated factors
TAT	Tyrosine aminotransferase
TBP	TATA binding protein
TIF2	Transcriptional intermediary factor 2
TRHR	Thyrotropin releasing hormone receptor
UDCA	Ursodeoxycholic acid
VIPR1	Vasoactive intestinal polypeptide receptor

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CHAPTER 1

Introduction

1.1 Background

This thesis is part of a 5 year NRF funded project investigating the mechanism of ligand-selectivity of glucocorticoid receptor (GR) action. A central question in steroid receptor research is to understand the differential potencies (concentration of compound required for half maximal response) and efficacies (maximal response) observed for different ligands in the transcriptional regulation of the same gene in the same cell. For example, why does dexamethasone behave like a full agonist and RU486 like an antagonist and what determines these differences? The overall purpose of the project is to systematically investigate specific steps in the GR transcriptional regulatory pathway and the behavior of a liganded GR at that specific step. Does the behavior correlate with potency and efficacy for transactivation/transrepression by a specific ligand? The response elicited by the compounds will be measured by dose-response curves for various synthetic promoter-reporter constructs, and the potency and efficacy will be determined from these curves for each compound.

Some specific steps in the GR pathway have been identified in the literature as possible determinants for ligand-selective regulation of gene expression. There are particularly 7 steps that are of great interest to our group, as being likely determinants for the potency and efficacy of a ligand (some of the steps have been hypothesised by others to be involved and some by us). These steps are ligand binding to the receptor (affinity and kinetics), ligand induced stability of the receptor, dimerisation of liganded-GR, nuclear translocation and retention of the liganded-GR, phosphorylation of the liganded-GR, binding of the liganded-GR to DNA and co-factor recruitment by the liganded-GR. Most studies have been conducted with a limited number of ligands, most commonly used is dexamethasone and RU486. In this project a broad panel of GR ligands will be investigated, including several agonists, partial agonists, selective glucocorticoid receptor agonists (SEGRAs) and antagonists, which should provide a good basis for correlating ligand-selective effects at a specific step with transcriptional response.

Understanding what determines ligand-selective potency and efficacy will further our understanding of the physiological responses to endogenous ligands and assist in the design of more effective drugs with fewer side-effects, for many different pharmacological applications.

1.2 The nuclear hormone receptor family

Nuclear receptors were first identified nearly 40 years ago as intracellular receptors for some steroids. More than 20 years passed, however, before it became apparent that these steroid receptors are part of a superfamily of transcription factors. Nuclear receptors are found in vertebrates and invertebrates, however, not in yeast or plants, and 48 members are presently identified in the human genome (reviewed in Berkenstam and Gustafsson, 2005). The 48 human members of this family include both receptors with identified ligands and 'orphan receptors' for which there are, as yet, no known ligands. All nuclear hormone receptors share a common structural organisation consisting of separate DNA- and ligand-binding domains (DBD and LBD) (Evans, 1988). The superfamily includes receptors for hydrophobic molecules such as steroid and thyroid hormones, retinoic acids, and fatty acids. The steroid hormone class of nuclear receptors is divided into two groups, the GR group (including the glucocorticoid, progesterone (PR), mineralcorticoid (MR) and androgen receptor (AR)) and the estrogen receptor (ER) group (including the estrogen-related receptor 1 and 2) (reviewed in Berkenstam and Gustafsson, 2005).

Nuclear hormone receptors are one of the most abundant classes of ligand-dependent transcription factors, capable of exerting transcriptional regulation in the nucleus in response to various extracellular and intracellular signals. The transcriptional activity of many receptors is controlled by the binding of small lipophilic molecules to the LBD. Binding of hormone to its receptor triggers a conformational change in the receptor protein, which facilitates interaction with cofactors and high affinity binding to DNA sequences called hormone response elements (HRE). This leads to either activation or repression of specific genes. The genes regulated by nuclear receptors are involved in a wide variety of cellular processes including metabolism, development, growth and differentiation (reviewed in Aranda and Pascual, 2001; Gronemeyer *et al.*, 2004; Berkenstam and Gustafsson, 2005). The small molecules that bind to the nuclear receptors can easily be modified by drug design. The nuclear receptors control functions associated with major diseases (e.g. cancer, osteoporosis and diabetes) and therefore, they are currently exploited as pharmacological targets.

1.3 Glucocorticoids and their receptor

Cortisol, also known as hydrocortisone, is a small lipophilic steroid hormone and the major endogenous glucocorticoid in humans, and it is synthesised in and secreted from the adrenal cortex. The function of glucocorticoids in the body includes roles in the regulation of the metabolism of carbohydrates, proteins and lipids, suppression of inflammatory and immunological responses and suppression of the HPA axis. Cortisol secretion increases in response to any stress in the body, whether physical (such as illness, trauma, surgery or temperature extremes) or psychological. The effects of natural and synthetic glucocorticoids are mediated through the intracellular GR. GR functions as a hormone-activated transcription factor that regulates the expression of specific target genes. Selective DNA-binding sites for GR, so called glucocorticoid-response elements (GREs), in the promoter of the target genes have been identified. The binding of the hormone-activated GR at these sites results in a positive or negative regulation of gene transcription. Genes regulated by GCs can be as much as 20 % of the human genome (Galon *et al.*, 2002).

The GCs can also mediate rapid nongenomic effects, which occur within minutes of administration via activation of signal transduction pathways and generation of second-messenger systems. These rapid effects may be mediated by the intracellular GR but they may also be mediated by a proposed membrane-bound GR (reviewed in Stellato, 2004). However, this thesis will focus on the classical genomic actions of GR.

Glucocorticoid analogs are widely used in the clinical field as immunosuppressive and anti-inflammatory drugs in the management of inflammatory and autoimmune diseases. Inflammatory diseases, such as asthma, are characterised by an increase in expression of many inflammatory proteins, such as cytokines, chemokines and growth factors. This increased expression is the result of enhanced gene transcription, which is regulated by transcription factors. Hormone activated-GR can either switch on the expression of anti-inflammatory genes or more importantly switch off inflammatory gene expression by targeting and inhibiting pro-inflammatory transcription factors such as activator protein 1 (AP-1) and nuclear factor κB (NF-κB). GR will interact with these transcription factors independently of binding to a GRE and inhibit them from binding to the transcriptional machinery, causing an inhibition of inflammatory gene expression (Göttlicher *et al.*, 1998). However, the anti-inflammatory and immunosuppressive actions of GCs are accompanied by

severe side-effects (Boumpas *et al.*, 1993). The most important side effects are osteoporosis, diabetes and arthrosclerosis. Therefore, the design of new drugs that will dissociate between the important transrepression of inflammatory genes and the unwanted gene expression causing the severe side-effects is crucial for improved therapy.

1.4 The structure of the human glucocorticoid receptor gene and protein

A single human GR gene was identified in 1991 (Encio and Detera-Wadleigh, 1991) and the gene is coded by 9 exons (Figure 1.1A). Exon 1 and the first part of exon 2 contain the 5'untranslated region (5'UTR); protein-coding regions are in exon 2-9, and the 3'untranslated region (3'UTR) in exon 9. Exon 2 codes for most of the receptor N-terminal end including activation domain 1 (AF-1). Exon 3 codes for one zinc-finger motif while exon 4 encodes the second motif that together constitute the DBD. The remaining exons make up the LBD and the activation domain 2 (AF-2) (Encio and Detera-Wadleigh, 1991). Recently, exon 1 has been divided into 3 regions (exon 1A, 1B and 1C) and alternative splicing of exon1A mRNA produces three different 1A transcripts (1A1, 1A2 and 1A3) which all have their own promoter (Breslin *et al.*, 2001). All the isoforms derived from exon 1 and its promoters might be regulated in a cell-specific manner as the different promoters respond to different tissue-specific transcription factors. Promoter 1A has several possible GR binding sites that resemble GREs (Breslin *et al.*, 2001; Geng and Vedeckis, 2004), while promoter 1B and 1C bind various other transcription factors (Webster *et al.*, 2001). GR transcripts containing exon 1A1, 1A2, 1B, and 1C are expressed at various levels in many different cell lines, while the exon 1A3-containing GR transcript is expressed most abundantly in blood cell cancer cell lines (Breslin *et al.*, 2001; Nunez and Vedeckis, 2002).

The GR gene contains two terminal exons 9 (exon 9α and 9β) (Figure 1.1A). Alternative splicing of exon 9 in GR transcripts gives rise to two native mRNA and protein isoforms, hGRα (5.5 kb) and hGRβ (4.3 kb) (Encio and Detera-Wadleigh, 1991). A GR transcript of approximately 7.0 kb has also been identified which derives from exon 1-8 and both the 9α and 9β exons, and it is expected to encode the GRα protein (Oakley *et al.*, 1996). The GR gene has also recently been described as having an alternative translation initiation start site 71 base pairs downstream from the classic translation initiation site, thus producing A and B

translational isoforms of both GRα and GRβ, with the B-isoform containing a 27 amino acids shorter N-terminal region (Yudt and Cidlowski, 2001).

The human GR possesses three functionally independent domains (Figure 1.1B): a N-terminal domain which principal function is transactivation of specific genes (coded by exon 2), a central DBD which recognises specific DNA sequences (coded by exon 3 and 4) and a C-terminal LBD (coded by exon 5-9) (Carlstedt-Duke *et al.*, 1982; Wrange and Gustafsson, 1978).

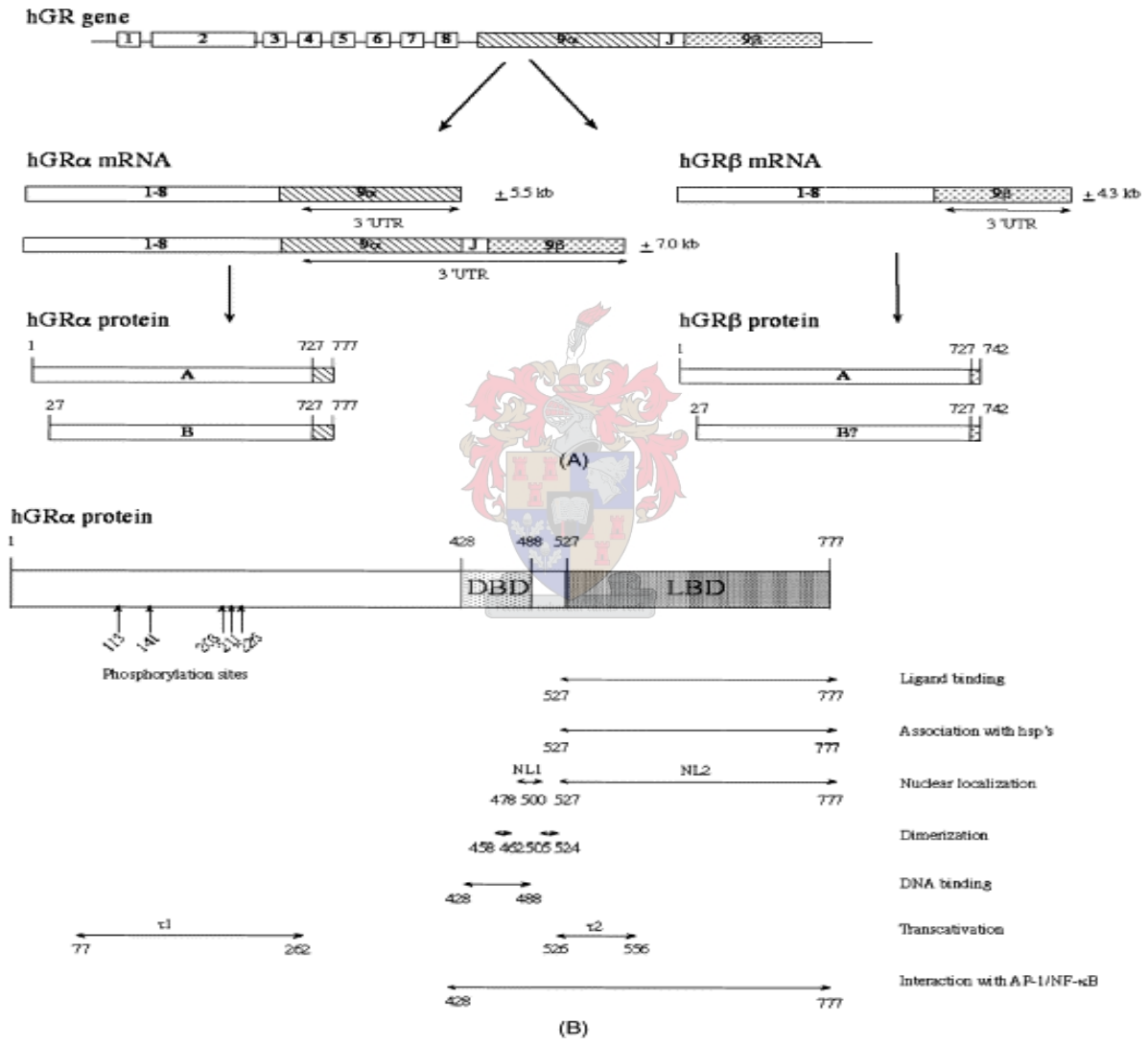


Fig. 1.1. The structure of the human glucocorticoid receptor gene, mRNA and protein. (A) The hGR gene contains nine exons. The two exons 9 are transcribed into two isoforms, the hGRα and hGRβ. Two translational isoforms exists, the A- and the B-isoform, caused by an alternative internal translation site at methionine 27 (B) The hGRα protein is divided into three major domains, the N-terminal domain, the DNA binding domain (DBD) and the ligand binding domain (LBD). Several other functional domains also exist, like the nuclear localisation signal domains and the important transactivation domains. Figure from (De Rijk *et al.*, 2002).

1.4.1 Activation functions 1 and 2

GR has two activation domains (Figure 1.1B), the ligand-independent AF-1 (amino acids 77-262), which resides in the N-terminal region, and the ligand-dependent AF-2 (amino acids 526-556), which is situated in the C-terminal LBD. AF-1 and AF-2 play an important role in the communication between the receptor and molecules necessary for the initiation of transcription, such as coactivators and the basal transcriptional machinery. To achieve transcriptional activation of target genes, coactivators need to bind to the receptor to recruit general transcription factors (Beato and Sanchez-Pacheco, 1996). Most of the known coactivators primarily interact with the AF-2 domain in the presence of activating hormones (Jenkins *et al.*, 2001), however, many have been identified that bind to the AF-1 domain as well (reviewed in McKenna and O'Malley, 2002). In the same way, corepressors are also able to bind both to the AF-1 and AF-2 domain of GR causing gene repression (Schulz *et al.*, 2002; Wang and Simons, Jr., 2005).

1.4.2 DNA-binding domain

The central DNA-binding domain (DBD) corresponds to amino acids 428-488 (Figure 1.1B). It contains two asymmetric zinc-finger motifs, each containing four conserved cysteine residues coordinating binding of a zinc atom that results in the formation of α-helices that interact with specific DNA sequences known as glucocorticoid-response elements (GREs) (Luisi *et al.*, 1991). Each zinc ion may be considered as a separate subdomain, coordinated to four cysteine residues and a α-helix. Several amino acids in the DBD interact with the DNA, keeping GR in the major groove of the DNA α-helix. The N-terminal zinc-finger is responsible for recognising certain nucleotide sequences in the GRE and the C-terminal zinc-finger is responsible for receptor homodimerisation (Dahlman-Wright *et al.*, 1991; Hovring *et al.,* 1999; Luisi *et al.,* 1991).

1.4.3 Ligand-binding domain

The C-terminal ligand-binding domain (LBD) corresponds to amino acids 527-777 (Figure 1.1B). GCs bind to the LBD and the LBD mediates homo-dimerisation and interaction with heat-shock proteins. The LBD plays a critical role in the ligand-induced activation of the receptor (reviewed in Bledsoe *et al.,* 2004). Recently, the GR LBD was crystallised and its structure determined, both in complex with an agonist (dexamethasone) and an antagonist

(RU486) (Bledsoe *et al.*, 2002; Kauppi *et al.*, 2003). The crystal structure results are consistent with the theory that the LBD folds into a structure that creates a hydrophobic ligand-binding pocket through which GR associates with glucocorticoids (Bledsoe *et al.*, 2002). It was proposed that only agonist-bound GR could interact with coactivators (Kauppi *et al.*, 2003), however, other studies have shown otherwise. Both agonist- and antagonist-bound GR can interact with both coactivators and corepressors (He *et al.*, 2002; Schulz *et al.*, 2002) but the conformational change induced by each ligand appears to cause different affinities for coactivators and corepressors (reviewed in Simons, 2003). Therefore, the ratio between coactivators and corepressors present in the cell system is a major determinant for the activity of the ligand-receptor-complex.

1.4.4 α- and β-isoform

The human GRα represents the classical GR composed of a single polypeptide chain of 777 amino acids, which is located primarily in the cytoplasm when not bound to ligand and when bound to ligand it translocates to the nucleus and modulates transcription in a ligand-dependent manner. In contrast, hGRβ, a single polypeptide chain of 742 amino acids, is located primarily in the nucleus and is unable to bind glucocorticoids because it does not contain the full-length LBD (Oakley *et al.*, 1996; Oakley *et al.*, 1997). GRβ can bind to a GRE, however, it cannot activate glucocorticoid-responsive genes and thus is transcriptionally inactive (Oakley *et al.*, 1999).

However, GRβ seems to antagonise the transactivation and transrepression ability of GRα by generating GRα/β heterodimer complexes incapable of binding GREs (Oakley *et al.*, 1996; Bamberger *et al.,* 1997; Oakley *et al,* 1999) and by competition for coactivators (Charmandari *et al.*, 2005a). The dominant negative effect of GRβ on GRα has been located to two residues within the 15 amino acids translated from exon 9β, which helps to form a GRβ/GRα heterodimer (Yudt *et al.*, 2003). The suppressive effect of GRβ on GRα-induced transactivation has been shown to depend on the type and dose of the synthetic glucocorticoid used. Synthetic GCs may each induce different conformational changes in the GRα and results suggest that the binding of GRβ to GRα is dependent on a certain conformational change (Fruchter *et al.*, 2005). In addition, it has been hypothesised that the presence of relatively high levels of GRβ in certain cells could have an influence on the sensitivity to GCs in these cells.

It has been found that there are often large differences in mRNA-expression levels of endogenous GRα relative to GRβ in various tissues in response to either normal physiology or to a disease state (Dahia *et al.*, 1997; Gagliardo *et al.*, 2000; Pujols *et al.*, 2002). Elevated levels of GRβ in certain cells seem to be related to glucocorticoid resistance in asthma, rheumatoid arthritis and colitis ulcerous (Hamid *et al.*, 1999; Honda *et al.*, 2000; Webster *et al.*, 2001; Hauk *et al,* 2000; Orii *et al.,* 2002) as suggested by results showing that overexpression of GRβ in some cells resulted in glucocorticoid insensitivity (Hauk *et al.*, 2002; Leung *et al.*, 1997). The notion that GRβ contributes to GC insensitivity is controversial as the level of endogenous GRβ is low as compared to GRα, which suggests that there would not be sufficient GRβ present to have a dominant negative effect on the GRα (DeRijk *et al.*, 2003). In support of this, there are also studies that show that even a 10-fold excess of GRβ over GRα does not interfere with GRα activated transcription (Hecht *et al.*, 1997) and increased levels of the β-isoform do not influence cytokine-induced glucocorticoid sensitivity (Torrego *et al.*, 2004). Possibly, the effects of GRβ are restricted to certain cells or disease states.

1.4.5 γ-isoform

GR-γ expression is caused by the presence of a constitutive alternative mRNA splicing site, resulting in an additional amino acid insertion between exon 3 and 4 within the DBD (at amino acid 452) of the receptor protein. GRγ makes up about 5 % of all GR transcripts in various tissues tested (Rivers *et al.*, 1999) and it is unlikely to play an important physiological role in glucocorticoid sensitivity. It has previously been shown that an amino acid insertion at the site in GRγ impairs the transcriptional potency of the receptor. It has been suggested that increased expression levels of GRγ occur in acute leukemia during childhood. GRγ expression is not influenced by GCs and therefore, is unlikely to influence the response to glucocorticoid treatment (Stevens *et al.*, 2004).

1.4.6 P-isoform

Another splice variant that is over-expressed in tumor cells was discovered. The hGR-P results from alternative splicing when exon 8 and 9 is replaced by intron G (lacks LBD), giving rise to a shorter protein (676 amino acids) (Krett *et al.*, 1995). Although, the GR P-isoform itself has a low transactivation activity it actually enhances the steroid response in

GRα-mediated transcription. Possibly, GR-P forms dimers with GRα that can have increased transactivation activity as compared to GRα homodimers. Differential expression of the GR-P isoform can explain the increased GC sensitivity observed in certain cells (De Lange *et al.*, 2001).

1.4.7 A- and B-isoform

Besides the splicing isoforms (GRα, GRβ, GRγ and GR-P), two additional translational isoforms have been described (Yudt and Cidlowski, 2001). The longer GR-A is translated from the first AUG codon (Met1) and the shorter GR-B isoform is initiated from an internal, in frame AUG codon (Met27). A weak Kozak translation initiation consensus sequence causes the ribosomal scanning mechanism to not always recognise the first translation initiation codon, producing the GR-B isoform. The isoforms have the same subcellular distribution and nuclear translocation mechanisms (Yudt and Cidlowski, 2001). Both are detected in human cell lines with endogenous GR and cells transfected with wildtype GRα constructs, and the isoforms are also observed with mouse and rat GR constructs (Russcher *et al.*, 2005; Yudt and Cidlowski, 2001). Specifically, the B-isoform is more effective (1.4- to 2-fold more effective) in GR-mediated transactivation than the A-isoform on three different GREs (GRE-CAT, 2 X GRE-Luc and MMTV-CAT) (Russcher *et al.*, 2005; Yudt and Cidlowski, 2001). In gene repression, both isoforms exhibit the same effect (Yudt and Cidlowski, 2001). Recently, the ER22/23EK polymorphism in the GR gene was found to be responsible for the overexpression of the A-isoform (Russcher *et al.*, 2005). Two mutations in exon 2 are present in this polymorphism; at codon 22 the mutation is silent while at codon 23 the mutation results in a change from arginine to lysine, and it is associated with resistance to glucocorticoids (reviewed in Van Rossum *et al.,* 2004). As the A-isoform is less effective in GR-mediated transactivation, the increased expression of the A-isoform could explain the glucocorticoid resistance caused by this polymorphism.

1.5 Nuclear translocation, localisation, dimerisation and phosphorylation of the receptor

1.5.1 Nuclear localisation signal domains 1 and 2

GR also contains two nuclear localisation (NL) signal domains, NL1 (amino acids 478-500) and NL2 (amino acids 527-777) (Figure 1.1B). NL1 contains a classic basic-type nuclear localisation signal structure that overlaps with and extends the C-terminal of the DBD (Picard and Yamamoto, 1987). The basic sequence adjacent to the DBD is required for NL1 function, while two smaller clusters of basic amino acids at the C terminus of the DBD appear to contribute to increasing the strength of the NL1 and thus the efficiency with which the receptor is imported into the nucleus (Tang *et al.*, 1997). NL1 is dependent on importin α and importin 7 nuclear import receptors, protein components of the nuclear translocation system, which is energy-dependent and facilitates the translocation of the activated receptor to the nucleus through the nuclear pore (Freedman and Yamamoto, 2004; Savory *et al.*, 1999). The exact localisation of the second nuclear localisation signal, NL2, is unknown, however it spans over almost the entire LBD (Picard and Yamamoto, 1987). NL2-mediated nuclear translocation is slower than translocation mediated by NL1 which is hormone-dependent but importin α-independent (Savory *et al.*, 1999).

1.5.2 Receptor localisation

In the absence of hormone, GR resides in the cytoplasm as part of a large chaperone complex composed of a receptor monomer, a dimer of heat shock proteins, Hsp90 and Hsp70, immunophilins, p23 and several different protein factors (Howard *et al.*, 1990; Murphy *et al.,* 2003; Owens-Grillo *et al.,* 1995). The interaction between the hsp90 and the LBD of the receptor contributes to the maintenance of the ligand binding pocket in an optimal, high-affinity configuration, keeping it transcriptionally inactive until activated by hormone (Pratt *et al.*, 1988; Cadepond *et al.,* 1991). In addition, p23 and Hsp70 are required for optimal stabilisation of the receptor-Hsp90 complex (Dittmar *et al.*, 1997; Murphy *et al.*, 2003). Ligand binding to the receptor induces conformational alterations, which result in dissociation of the Hsp90 complex (Giannoukos *et al.*, 1999) exposure of the receptor's nuclear localisation signal that promotes translocation of the GR–ligand complex from the cytoplasm into the nucleus (Savory *et al.*, 1999). Importin 7 and importin α/β are responsible for the

import of GR to the nucleus by binding to the nuclear localisation signal domains (Freedman and Yamamoto, 2004).

After treatment with potent agonistic (like dexamethasone) and antagonistic (like RU486) ligands, GR is completely translocated to the nucleus within minutes (Galigniana *et al.*, 1998; Jewell *et al.*, 1995) while less potent agonists (like progesterone and aldosterone) induce translocation at a slower rate (Htun *et al.*, 1996; Yoshikawa *et al.*, 2002). The receptor localises to specific foci within the nucleus when bound to agonists but not when bound to partial agonists or antagonists (Htun *et al.*, 1996). The mobility of the receptor inside the nucleus is dependent on the ligand with which it is associated. High affinity ligand-bound GR has a lower mobility in the nucleus as compared to low affinity-bound GR, possibly because of different conformational changes leading to increased binding of high affinity-bound GR to certain nuclear structures (Schaaf *et al.*, 2005). Liganded-GR remains in the nucleus for hours before returning to the cytoplasm and relocalisation also appears to be ligand-specific (Vicent *et al.*, 2002).

1.5.3 Receptor dimerisation

As a DNA-binding transcription factor, GR functions and activates transcription as a homodimer on GREs. A GR homodimer has a 10-times higher affinity for a GRE than a monomer, and a homodimer:GRE complex is much more stable than a monomer:GRE complex (Drouin *et al.*, 1992). It is unclear whether the formation of a homodimer at the DNA is caused by cooperative binding of GR monomers or the coordinate binding of preformed GR dimers. Early on it was thought that GR had a higher affinity for one of the half-sites on a GRE and that the binding to the other site was dependent on occupancy of the first site (Dahlman-Wright *et al.*, 1990). However recent studies have shown that GR dimerisation followed by GRE binding is more likely (Savory *et al.*, 2001; Segard-Maurel *et al.*, 1996). Receptor dimerisation is mediated in part through the LBD and in part through the homodimer interface on the C-terminal end of the DBD. Interestingly the GR LBD alone has been shown to be capable of forming a homodimer prior to DNA binding, possibly causing the formation of dimers in the cytoplasm independent of ligand binding (Savory *et al.*, 2001). A mutation in the homodimer interface in the DBD causes a loss in the transactivational ability of the receptor but does not affect transrepression (Reichardt *et al.*, 1998). In GR^{dim} mice (mice that have a GR mutant that cannot dimerise), it has been shown that no GRE-

dependent transcription occurs but that all the inflammatory responses are still repressed by GCs via interaction with NF-κB and AP-1 (Reichardt *et al.*, 2001).

1.5.4 Phosphorylation of GR

Initial work focused on potential phosphorylation sites in the GR and it was proposed that phosphorylation of the receptor was responsible for the level of active receptor present in the cell. Several groups reported that the rat and mouse GR were hyperphosphorylated at multiple residues upon hormone treatment by using [^{32}P]-ATP incubation and Western blot analysis (Dalman *et al.*, 1988; Housley and Pratt, 1983; Singh and Moudgil, 1985). It was discovered that the phosphorylation of GR was most likely agonist-induced *in vivo* since the antagonist RU486 was not able to result in phosphorylation of the mouse GR (Orti *et al.*, 1989) however it was unknown at the time where the phosphorylation sites are located.

Identification and cloning of the mouse GR allowed for a more detailed analysis of GR phosphorylation. Seven phosphorylation sites (Ser122, Ser150, Ser212, Ser220, Ser234, Ser315 and Thr159) were discovered on the mouse receptor. The sequences around the mouse GR phosphorylation sites at Ser122, Ser150, Ser212, Ser220 and Ser234 and Thr159 are conserved in the human GR (homologous at Ser113, Ser141, Ser203, Ser211 and Ser226, not conserved Ala at 150) and rat GR (homologous at Ser134, Ser162, Ser224, Ser232 and Ser246, and Thr171) and all phosphorylation sites reside within the AF-1 domain (Bodwell *et al.*, 1991). The hormone-induced phosphorylation at each site was quantified and all sites were hyperphosphorylated significantly on the mouse receptor, except Ser150 and Thr159. Ser212, Ser220 and Ser234 are in a highly acidic region that is necessary for full transcriptional activity, suggesting a role for phosphorylation in transactivation (Bodwell *et al.*, 1995).

To understand whether GR phosphorylation status contributes to its transcriptional activity, Mason *et al.* investigated the role of phosphorylation sites in transactivation by the mouse GR. Mutant receptors were tested in promoter-reporter transactivation assays (MMTV-LTR-CAT promoter-reporter construct) in COS-1 cells and it was found that individual residues were not critical for activity (Mason and Housley, 1993). Unexpectedly, receptors mutated at all seven sites exhibited only a 22 % decrease in transcriptional activity (Mason and Housley, 1993). Similar results were found by Almlof *et al.* with mutant human GR on a 1 X GRE-lacZ reporter gene in yeast cells (Almlöf *et al.*, 1995). Consistent with this, a later study by

Webster *et al.* showed that mutant mouse receptors were equally potent in transactivation as the wildtype receptor on a similar MMTV promoter-reporter construct. However, phosphorylation mutants showed a significant decrease in transcriptional activity of a minimal reporter promoter construct, the GRE-CAT, indicating that the effect is promoter-specific (Webster *et al.*, 1997).

The functional significance of phosphorylation at specific sites in GR was studied in more depth when phospho-specific antibodies against human GR Ser203 and Ser211 were developed (Wang *et al.*, 2002). Wang *et al.* showed a stronger basal phosphorylation of Ser203 than of Ser211 and both sites showed increased phosphorylation upon treatment with 100 nM Dex. Phosphorylation at Ser203 was not agonist-dependent. However, Ser211 phosphorylation was more extensively induced by GR agonists (dexamethasone, prednisolone and fluocinolone) and minimal phosphorylation was induced by GR antagonists (RU486 and ZK299), all tested with 100 nM for one hour (Wang *et al.*, 2002). Similarly, the GR ligands were tested in GR-dependent transcription on a MMTV-luc promoter-reporter construct at 100 nM for one hour. Wang *et al.* therefore suggested that agonists in transactivation induced more phosphorylation at Ser211 than antagonists. It was also shown that phosphorylation at Ser211 was more robust. Upon hormone treatment the site specific phosphorylation at Ser211 was sustained for up to 6 hours while with Ser203 it was sustained only for 2 hours. Wang *et al.* also found that GR phosphorylated at Ser203 resided in the cytoplasm while GR phosphorylated at Ser211 was predominantly observed in the nucleus, supporting the idea that phosphorylation at Ser211 is important for transactivation (Wang *et al.*, 2002).

The phosphorylation and adjacent sites are conserved between the mouse GR, rat GR and human GR; the serine residue is followed by a proline (reviewed in Ismaili and Garabedian, 2004). This consensus motif is recognised by either mitogen-activated protein kinases (MAPKs) or cyclin-dependent kinases (CDKs) (Figure 1.2). As predicted, GR is a substrate for both the MAPKs and the CDKs (reviewed in Ismaili and Garabedian, 2004). Firstly, it was determined that the rat GR was phosphorylated by both MAPKs and CDKs by *in vitro* kinase assays (Krstic *et al.*, 1997). MAPKs induced phosphorylation at Thr171 and Ser246, and CDKs at Ser224 and Ser232 (Krstic *et al.*, 1997). It was later found that c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), but not p38 kinase from the MAPK family, were responsible for the phosphorylation at Ser246 in the rat GR *in vitro*, but JNK had primarily an effect *in vivo* in SAOS2 cells by stimulating the JNK and ERK

pathways by serum stimulation (Rogatsky *et al.*, 1998b). In addition, phosphorylation at T171 was found to be mediated by glycogen synthase kinase (GSK-3) *in vitro* (Rogatsky *et al.*, 1998a). On the human Ser226, JNK is responsible for phosphorylation, as determined *in vivo* in COS-7 cells (Itoh *et al.*, 2002). In contrast, the MAPK p38 phosphorylates the human GR Ser211 *in vivo* in CEM-C7-14 cells, not a CDK as expected (Miller *et al.*, 2005).

	Consensus sequence	Sequence in the human GR
CDK family	**S/T (P)-P-X-R/K**	**S/T (P)-P-X-R/K at Ser203 and Ser211**
MAPK family	**nonpolar-X-S/T (P)-P**	**non-polar-X-S/T (P)-P at Ser226**

Figure 1.2. The consensus sequences for MAPK and CDK phosphorylation sites and corresponding residues phosphorylated on the human GR (adopted from Ismaili and Garabedian, 2004).

1.6 Mechanisms of transcriptional regulation by GR

GR can positively or negatively regulate gene expression via several different mechanisms. As mentioned above GR usually activates transcription by binding as a homodimer to specific DNA response elements in the regulatory region of its target gene. Consensus response elements have been identified for the receptor, called glucocorticoid response elements, GREs. Small variations in the sequence of the GREs do affect the binding affinity of the receptor and the extent of transcriptional activity, allowing differential control of gene transcription (reviewed in Schoneveld *et al.,* 2004). The GREs are divided into different categories which will be discussed in the section below. However, the GR can also transactivate genes independently of DNA binding by interacting with other transcription factors, e.g. Oct-1. Most importantly, transcriptional repression by the GR is generally regulated by protein-protein interactions between the receptor and particularly the transcription factors NF-κB and AP-1, via a so-called tethering mechanism.

1.6.1 Simple glucocorticoid response element

Analysis of a number of GREs defined a consensus GRE (Figure 1.3A and 1.4a) for GR as two inverted repeats of a half-site, 5'-AGAACAnnnTGTTCT-3', separated by a three base-pair spacer in which the 3' half is most conserved (Nordeen *et al.*, 1990). The 5' half, however, can tolerate substitutions in its sequence. This flexibility does not necessarily imply a reduced GC response, since GR only contacts the GRE at certain positions (Cairns *et al.*,

1991; Truss *et al.*, 1990), however, it has been shown that the positions -3, -2, +2, +3 and +5 in the GRE are most critical for GR activation of gene transcription (Nordeen *et al.*, 1990). GR has a higher affinity for the 3' half-site and binding will occur at this site first followed by binding at the 5' half-site to form a DNA-bound dimer (La Baer and Yamamoto, 1994; Dahlman-Wright *et al.,* 1990). This cooperative binding of the two GR monomers to the palindromic GRE is lost if the 3-basepair spacer between both half-sites is changed (Dahlman-Wright *et al.*, 1991). As mentioned above, it is unclear when dimerisation of the receptor occurs, as recent studies have shown that possibly dimer formation takes place before DNA binding (Savory *et al.*, 2001; Segard-Maurel *et al.*, 1996).

It was discovered early on that the mouse mammary tumor virus (MMTV) (Hutchison *et al.*, 1986) and the tyrosine aminotransferase (TAT) (Schmid *et al.*, 1987) genes were regulated by multiple GREs in their promoters and these genes have proven to be useful models for GC-induced gene expression. Various other genes that are regulated by simple GREs have been identified as well (reviewed in De Bosscher *et al.,* 2003; Schoneveld *et al.,* 2004).

Figure 1.3. Glucocorticoid response element consensus sequences. (A) The DNA sequence of a simple GRE is an inverted repeat of a half-site where N represents any nucleotide. (B) The consensus sequence for a GRE half-site.

1.6.2 Composite glucocorticoid response element

In a number of genes, composite response elements are present where the GC response is not only dependent on GR binding to a simple GRE but also the binding of another transcription factor to an adjacent binding site (Figure 1.4b). In this manner different transcription factors collaborate to confer transcriptional regulation, such as shown with AP-1 and GR for several genes, including the neurotensin/neuromedin N (NT/N) (Harrison *et al.*, 1995), proliferin (Miner and Yamamoto, 1992), corticotrophin releasing hormone (CRH) (Malkoski and Dorin, 1999) and thyrotropin-releasing hormone (TRH) (Cote-Velez *et al.*, 2005) genes.

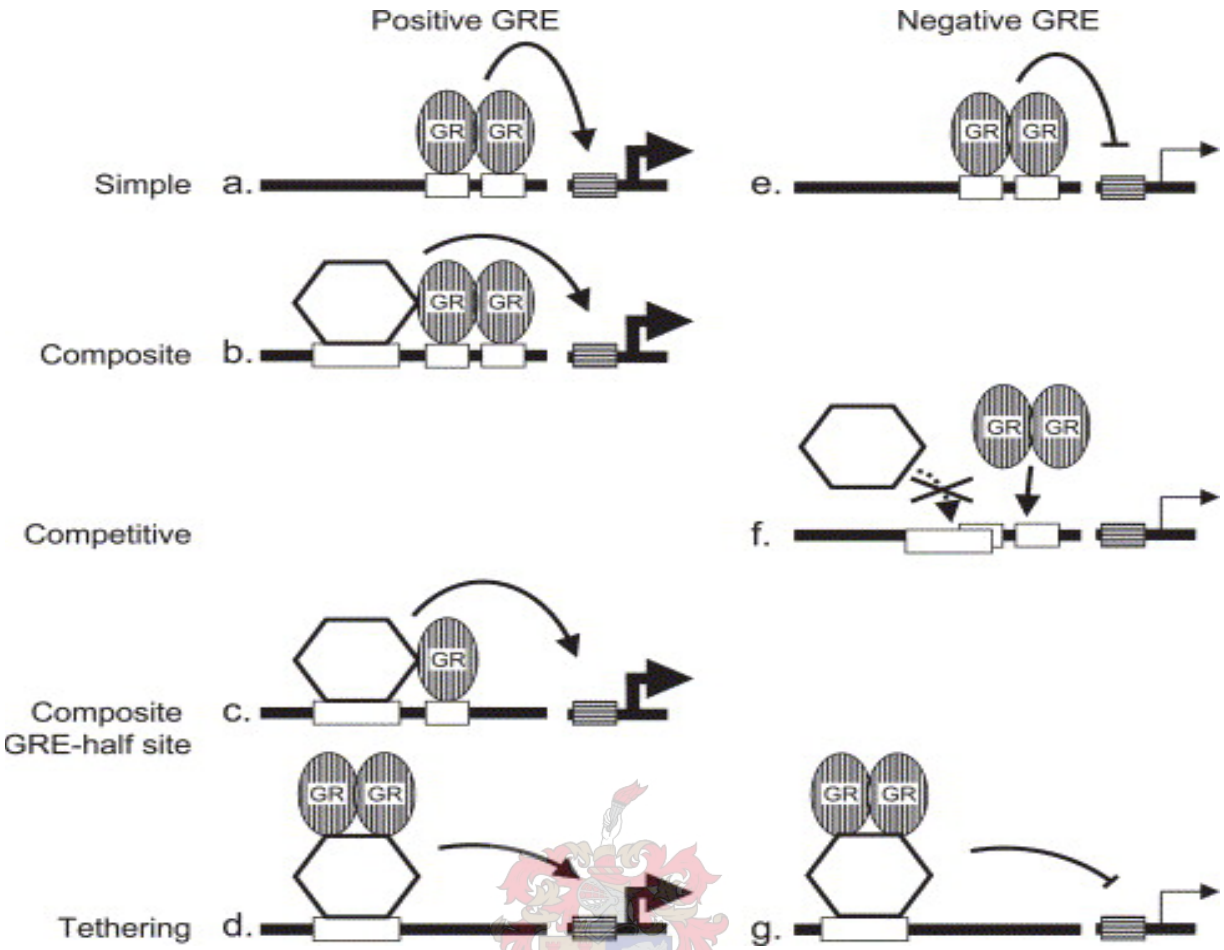


Figure 1.4 Proposed models of GR-mediated transcriptional regulation. (a) Simple GRE (b) A composite response element (c) A composite half-site response element (d + g) Positive and negative tethering response element (e) A negative GRE (f) A competitive response element. Figure from (Schoneveld *et al.*, 2004).

On the neurotensin/neuromedin N (NT/N) gene, AP-1, CRE and GRE elements are all required for a complete transcriptional response. The maximal induction of the promoter, however, depends on the AP-1 complex present. C-Jun together with GR potently activates the promoter, while c-Fos has a more limited but still positive effect on gene expression (Harrison *et al.*, 1995). Similarly, on the proliferin gene promoter GR can bring about either transactivation or transrepression, through interaction with AP-1 complexes depending on the subunit composition of AP-1 (Miner and Yamamoto, 1992). GR can regulate activated AP-1 and enhance transcription of the proliferin gene if AP-1 consists of c-Jun homodimers, but represses when AP-1 consists of c-Jun/c-Fos heterodimers. Transactivation by GR of the proliferin gene will occur if GRE and the AP-1 site are more than 26 base pairs apart, regardless of the AP-1 composition, while transrepression (c-Jun/c-Fos heterodimers) and transactivation (c-Jun homodimers) occurs only if the elements are 14-18 base pairs apart (Pearce *et al.*, 1998). A composite mechanism also occurs within the CRH promoter, where

binding sites for both GR and AP-1 are found on adjacent elements within an nGRE, and mutations at either site lead to loss of GR-dependent repression. The mechanism by which GR and AP-1 interact at the nGRE to repress gene transcription remains uncertain (Malkoski and Dorin, 1999). A similar mechanism is seen on a GRE half site in the TRH promoter (Figure 1.3c). The promoter consists of two cAMP response elements (CRE), a GRE half-site and two AP-1 sites, however, the interaction between the different transcription factors is poorly understood (Cote-Velez *et al.*, 2005).

GR can also bind to DNA as a monomer at GRE half-sites (Figure 1.3B). To mediate a GC response, additional elements or multiple GRE half-sites need to be present (Figure 1.4c). For example, on the phenylalanine hydroxylase (PAH) gene, the binding of the GR to three GRE half-sites is dependent on the binding of hepatocyte nuclear factor (HNF1), a liver transcription factor, to sites in the enhancer and cAMP to mediate a maximal response (Bristeau *et al.*, 2001; Faust *et al.,* 1996). A GRE half- site together with a simple GRE can also confer transactivation as in the thyrotropin releasing hormone receptor (TRHR) gene (Hovring *et al.*, 1999). In the phenylethanolamine *N*-methyltransferase (PNMT) promoter, multiple GRE half-sites are responsible for the GC-dependent gene regulation (Adams *et al.*, 2003; Aumais *et al.*, 1996).

1.6.3 Tethering glucocorticoid response element

The most common and most studied mechanism of repression is caused by a tethering effect by GR, which does not involve binding to a GRE, but instead binding to another transcription factor bound to DNA and blocking of the binding of that transcription factor or blocking of the recruitment of the basal transcription machinery to the regulatory region of the gene. It has been shown that GR can tether transcription factors like NF-κB, AP-1, Nur-77 and Oct1, independently of DNA-binding, on different gene promoters (Figure 1.4g).

NF-κB-dependent gene expression is one signaling pathway that is repressed by GR in a GRE-independent manner. Direct protein-protein interaction between GR and NF-κB have been demonstrated and the repression is mutual (Nissen and Yamamoto, 2000; Ray and Prefontaine, 1994; Wissink *et al.*, 1997). One possible mechanism is the physical interaction between GR and NF-κB and therefore inhibition of the binding of NF-κB to its response element in a target gene. This mechanism is responsible for the repression in gene expression by GR of several pro-inflammatory cytokines relevant to inflammatory diseases. Both the

interleukin 6 (IL-6) and interleukin 8 (IL-8) promoters contain a NF-κB response elements where NF-κB binds to initiate transcription, however, GR can interfere with the binding of NF-κB to its site effectively suppressing the transcription (De Bosscher *et al.*, 1997; De Bosscher *et al.*, 2000; Mukaida *et al.,* 1994). Another proposed mechanism is that the receptor represses NF-κB-driven genes by disturbing the interaction of the p65 subunit with the basal transcription machinery. GR represses the E-selectin promoter by binding to the DNA-bound NF-κB complexes, possibly interfering with the binding of important cofactors to the promoter, as overexpression of CBP and SRC-1 abolishes the GR-mediated repression (Sheppard *et al.*, 1998). NF-κB-dependent activation can also be inhibited by GR while NF-κB remains bound to its response element in the promoter region. On the intercellular adhesion molecule-1 (ICAM-1) promoter, NF-κB is still bound to its DNA binding site however the complex is changed into a transcriptionally inactive form by GR (Liden *et al.*, 2000). Both the DBD and the LBD of GR are important for repression via NF-κB (Wissink *et al.*, 1997). Thus, although direct DNA binding of GR is not required for a tethering mechanism, the DBD of GR seems to be essential (Heck *et al.*, 1994; Liden *et al.,* 1997).

The mechanisms for GR-mediated transrepression through AP-1 are similar to the repression of NF-κB dependent transactivation. The mutual cross-talk between GR and AP-1 was first described on the collagenase promoter and a direct protein-protein association between GR and AP-1 was shown (Yang-Yen *et al.*, 1990). It was later found that AP-1 remains DNA-bound to the promoter however the interaction between GR and AP-1 prevents the binding of the transcriptional initiation machinery or essential cofactors (Karin and Chang, 2001). However, it was found by De Bosscher *et al*. that GR-mediated repression of AP-1 upregulated genes was not due to competition for the same coactivators (De Bosscher *et al.*, 2001), in contrast to NF-κB transcriptional regulation. AP-1, like NF-κB is also responsible for the transcriptional regulation of proinflammatory cytokines. Transcription of the human interleukin 2 (IL-2) gene is inhibited by GR through interference with AP-1 preventing its binding to the IL-2 promoter (Paliogianni *et al.*, 1993). On the interferon-γ (IFN-γ) promoter, a complex of AP-1 and protein-activating transcription factor (CREB-ATF) is essential for promoter activity and GR inhibits the activity of this complex to negatively regulate IFN-γ gene expression (Cippitelli *et al.*, 1995).

Other transcription factors are also targets for GR-mediated repression via a tethering mechanism. Nur77 is a mediator in proopiomelanocortin (POMC) gene transcription and glucocorticoids antagonise this positive effect at two levels. Firstly, glucocorticoids repress Nur77 mRNA synthesis and secondly, GR prevents Nur77 from binding to the Nur response element (NurRE) element in the POMC gene (Philips *et al.*, 1997). Two other related orphan nuclear receptors, Nurr1 and neuron-derived orphan receptor (NOR-1), are also targets of GR antagonism (Martens *et al.*, 2005). Furthermore, by interaction with Oct1 proteins, GR transrepress the gonadotropin-releasing hormone (GnRH) gene independent of a GRE (Chandran *et al.*, 1996; Chandran *et al.*, 1999).

1.6.4 Negative glucocorticoid response element

Beside the classical GREs responsible for transactivation, a number of negative GREs (nGRE) have been identified which mediate transrepression with direct binding of GR to the nGRE required (Figure 1.4e). The nGREs are related to the well-defined GREs described above, however, the DNA sequence of the nGRE differs significantly from the GRE consensus sequence. A strong consensus sequence for receptor binding within the nGRE has not yet been defined and nGRE can either be a full GRE or GRE half-sites (reviewed in Dosert and Heinzel, 2004). In five keratin genes, four negative GRE half-sites, with homology with the 5' half-site in a simple GRE, were identified where each half-site binds a GR monomer to suppress gene expression (Radoja *et al.*, 2000). Similarly, in the human corticotrophin-releasing hormone (CRH) gene, three negative GRE half-sites, with GRE half-site homology, were identified and the core binding site was determined to be important for GR-mediated transrepression (Malkoski and Dorin, 1999). In the POMC promoter, an nGRE half-site (homology to GRE half-site) is also responsible for GR-mediated transrepression (Drouin *et al.*, 1989). A sequence similar to the POMC nGRE is also found in the vasoactive intestinal polypeptide receptor (VIPR1) gene (Pei, 1996). Recently, a new response element was discovered in the mouse glucose-6-phosphatase (G6Pase) gene (Vander Kooi *et al.*, 2005). The promoter contains both functionally positive (three simple GREs) and one negative GRE elements. This is believed to ensure a stricter control of the response to GCs in the same cellular environment (Vander Kooi *et al.*, 2005).

1.6.5 Competitive glucocorticoid response element

On a competitive GRE, the GR binding site overlaps with the binding site for a required transcription factor in the gene promoter. When GR binds to the GRE, it prevents the binding of the other transcription factor that would normally induce transcription of that specific gene, causing repression of gene expression (Figure 1.4f). This mechanism is present in the human osteocalcin gene, which is transcriptionally repressed by GCs, as a GRE overlaps a weak TATA box preventing TATA binding protein (TBP) from binding to this site (Meyer *et al.*, 1997).

1.6.6 Mechanisms of transcriptional activation by GR

GR utilises several mechanisms to activate transcription of hormone responsive target genes. Firstly, binding to the GREs allows GR to directly interact with components of the basal transcriptional machinery that are part of the preinitiation complex. The AF-1 domain of GR has been reported to interact with basal transcription factors, such as transcription factor IID (Ford *et al.*, 1997) and TATA box binding protein (TBP) (Kumar *et al.*, 2001). Secondly, the transcriptional activity of GR can be regulated by coactivators that activate transcription by remodeling chromatin and by facilitating the recruitment and stabilisation of the basal transcriptional machinery. Thirdly, GR may interact with cellular factors that act as bridging factors to the preinitiation complex, or with proteins that modify chromatin structure.

1.6.6.1 Chromatin remodeling and histone modifications

DNA, in the nucleus, is organised into chromatin with histone and non-histone proteins. The basic unit of chromatin is the nucleosome, which consists of DNA wrapped around histone molecules thereby compacting the DNA (Luger *et al.*, 1997). Chromatin has an inhibitory effect on transcription in preventing the access of the general transcriptional machinery to DNA. Thus, chromatin rearrangements are required to activate genes. Hyperacteylated histones are linked to active chromatin, since acetylation of histones results in an unpacking of the local DNA structure, thereby enabling interaction with proteins important for transcriptional activation of the promoter. Histone acetylation levels are determined by the equilibrium between the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Disruption of chromatin structure to lift the repressive effect can be mediated by two general classes of chromatin remodeling factors: ATP-dependent chromatin remodeling complexes and factors that contain histone acetyltransferase activity (Fischle *et*

al., 2003; Narlikar *et al.*, 2002). However, GR has the ability to access its GRE within the chromatin structure and recruit necessary coactivators that have HAT activity and chromatin remodeling complexes to rearrange the chromatin structure (Hebbar and Archer, 2003).

GR is known to cause changes in the chromatin structure of GC-regulated promoters such as the TAT gene promoter and the MMTV gene promoter, in an ATP-dependent manner (Fletcher *et al.*, 2002). The MMTV promoter has four GREs and binding sites for nuclear factor NF1, octamer transcription factors (OTFs) and the TATA binding protein (TBP). The transcription factor NF-1 is necessary for recruitment of both GR and the Brahma-related gene 1 (BRG1) chromatin remodeling complex to the promoter (Hebbar and Archer, 2003). On the MMTV promoter, the GR AF-1 domain recruits the BRG1 complex (Wallberg *et al.*, 2000) via protein-protein interactions with BRG-associated factor (BAF) 250 (Nie *et al.*, 2000) and BAF60a (Hsiao *et al.*, 2003), and it is essential for MMTV transcriptional activation (Fryer and Archer, 1998; Trotter and Archer, 2004). GR then recruits TBP to the promoter for transcription to occur (Hebbar and Archer, 2003). Another chromatin remodeling complex, p/CAF, is also recruited via the AF-1 domain and it is important for GR-mediated transcriptional activation. p/CAF has HAT activity, that regulates chromatin structure and the complex contains TBP-associated factors (TAFs) that facilitate the recruitment of the basal transcriptional machinery (Henriksson *et al.*, 1997). In addition, the P/CAF complex can be recruited to the receptor via interaction with CBP/p300 or p160 coactivators.

1.6.6.2 Interaction with coactivators

Nuclear receptor coactivator complexes are generally defined as proteins that "glue" the DNA-bound nuclear receptors and the basal transcriptional apparatus together and thereby enhance their transcriptional activation function. These cofactors interact with nuclear receptors in a ligand-dependent manner and enhance transcriptional activation by recruitment of additional cofactors such as CBP/p300 or P/CAF, promoting chromatin remodeling via histone acetylation/demethylation. This ensures that the access of the basal transcriptional machinery to the DNA and direct protein-protein interactions between the cofactors and the general transcription factors stabilise the basal transcriptional machinery (reviewed in Edwards, 2000).

Several nuclear receptor coactivator proteins that interact with GR have been identified and to mention a few: the p160 family of proteins (Xu and Li, 2003), chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) (Jiang *et al.*, 2004), peroxisome proliferator-activated receptors gamma coactivators 1 (PGC-1) (Borgius *et al.*, 2002), GT198 (Ko *et al.*, 2002b) and Cdc25B (Chua *et al.*, 2004). Prominent among these coactivators is the p160 coactivator family, which consists of three closely related members, SRC-1 (SRC-1a, SRC-1e), SRC-2 (GR-interacting protein (GRIP1, mouse), transcriptional intermediary factor (TIF-2, human)) and SRC-3 (activator for thyroid hormone and retinoid receptors (ACTR), receptor-associated coactivator-3 (RAC3), thyroid hormone receptor activator molecule 1 (TRAM-1) and amplified in breast cancer 1 (AIB1) (Xu and Li, 2003). A brief summary of what is known about the p160 coactivator family and especially the SRC-1 follows.

The steroid receptor coactivator (SRC) proteins contain multiple transcription activation domains and nuclear receptor domains, and the p160 family members show significant amino acid sequence homology and have similar domain organisation (Xu and Li, 2003). The activation domains (AD) have been located C-terminal to the receptor-interacting domain (RID). The ADs binds CBP or p300, that serve as secondary coactivators which acetylate histones and general transcription factors (Korzus *et al.*, 1998). The RID contains three LXXLL motifs (NR boxes) (Heery *et al.*, 1997), which are differentially recognised by receptors (Ding *et al.*, 1998). The SRC proteins do not themselves appear to have DNA-binding activity however they are recruited to promoters of steroid responsive target genes via protein-protein interactions with nuclear receptors. Although SRC proteins were originally identified as AF-2 interacting proteins, there is increasing evidence they can also interact with and enhance the AF-1 activity of steroid receptors (Onate *et al.*, 1998). Coactivators may preferentially utilise specific ADs depending on the receptor or activation function (AF-1 or AF-2) that is mediating the response to hormone (Ma *et al.*, 1999).

One of the first steroid receptor coactivators that was described for GR is SRC-1. Subsequent studies have identified two functionally distinct SRC-1 isoforms, SRC1-a and SRC-1e (Kalkhoven *et al.*, 1998) which cause specific effects in GR-mediated transcription. SRC-1e is more potent on multiple response elements containing promoters while SRC1-a coactivates the partial agonist activity of RU486-bound GR better than SRC-1e (Meijer *et al.*, 2005). SRC-1 interacts in a ligand-dependent manner with and enhances AF-2 transcriptional activation of GR (Kucera *et al.*, 2002). SRC-1 has been demonstrated to interact with general

transcription factors, such as TBP, TFIIB, CBP and p300 (Yao *et al.*, 1996) thus effectively bridging the gap between the GR and the basal transcriptional machinery.

1.7 Basic principles for evaluating ligand-receptor complexes

The parameters that characterise ligand-receptor complexes are affinity, potency and efficacy that can be determined quantitatively for a particular ligand for a particular receptor in a particular cell. A major aim of this thesis is to determine the potency and efficacy for a panel of proposed GR ligands in transcriptional regulation, which will be used as a basis for the remainder of the GR project. All data compiled from the transactivation and transrepression studies will be correlated to each step, which is believed to be important for ligand-selectivity, in the GR transcriptional regulatory pathway. Therefore, some basic principles and definitions of some pharmacological terms will be discussed.

1.7.1 Affinity

The strength of binding interaction between a ligand and a receptor is affinity. Affinity measures the relative occupation of a receptor at a specific ligand concentration. The interaction of a ligand with its receptor should not be viewed simply as a static process of binding and occupation, but rather, as a kinetic process in which molecules move towards and away from the receptor at various rates (Figure 1.5). The fraction of receptors occupied by a drug at a given instant is dependent on the relative rates of onset (k_{on}) and offset (k_{off}) of ligand attachment to the receptor. Equilibrium is reached when the rate of formation of new ligand-receptor complexes equals the rate at which existing ligand-receptor complexes dissociate. The equilibrium dissociation constant, K_D, is the concentration of ligand that, at equilibrium, will cause binding to half the receptors (reviewed in Neubig *et al.,* 2003).

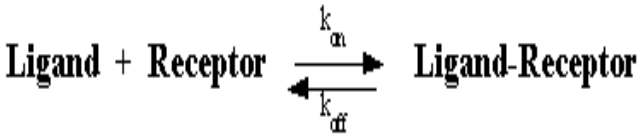


Figure 1.5. The affinity of a ligand for its receptor is determined by the association and dissociation rates as the K_D equals the ratio of K_{on} and K_{off}

Fractional occupancy describes relative receptor occupancy at equilibrium as a function of ligand concentration and K_D (Figure 1.6).

$$\text{Fractional occupancy} = \frac{[\text{Ligand}]}{[\text{Ligand}] + K_D}$$

Figure 1.6. The equation used to calculate fractional occupancy. If no ligand is present the occupancy will be zero; at saturating ligand concentrations (>> K_D), the fractional occupancy is close to 100 %; when ligand concentration equals K_D, the fractional occupancy will be 50 %. This equation assumes equilibrium.

1.7.2 Potency

Potency is a measure used to describe and quantify the concentration of ligand needed to produce a defined level of response; the lower the concentration required the greater the potency. The potency is often referred to as an EC_{50} value, the molar concentration of a ligand which produces 50 % of the maximal possible response for that ligand. A semi-log plot of a sigmoidal dose-response curve (a $\log_{10}$ scale of ligand concentration against percentage response) is almost linear between 20 and 80 % of maximum obtained, and an EC_{50} is determined from the dose-response curve by reading of the ligand concentration at 50% of maximal response (Figure 1.7). It is important to realise that the potency of a ligand does not give any information about its affinity for the receptor, because the pharmacological response is rarely directly proportional to receptor occupancy. The relative potency is the ratio of the potency of a specific ligand to that of a standard ligand (reviewed in Neubig *et al.,* 2003).

1.7.3 Efficacy

Efficacy is used to characterise and quantify the ability of different ligands to produce a maximal response in transcriptional regulation (Figure 1.7). Relative efficacy compares the relative activity of one ligand against a standard ligand at maximal response. The activity at maximal response for the standard ligand is set as 100 %. A pure antagonist will have zero efficacy. A partial agonist will have an efficacy between a full agonist (100 %) and antagonist (0 %) (reviewed in Neubig *et al.,* 2003).

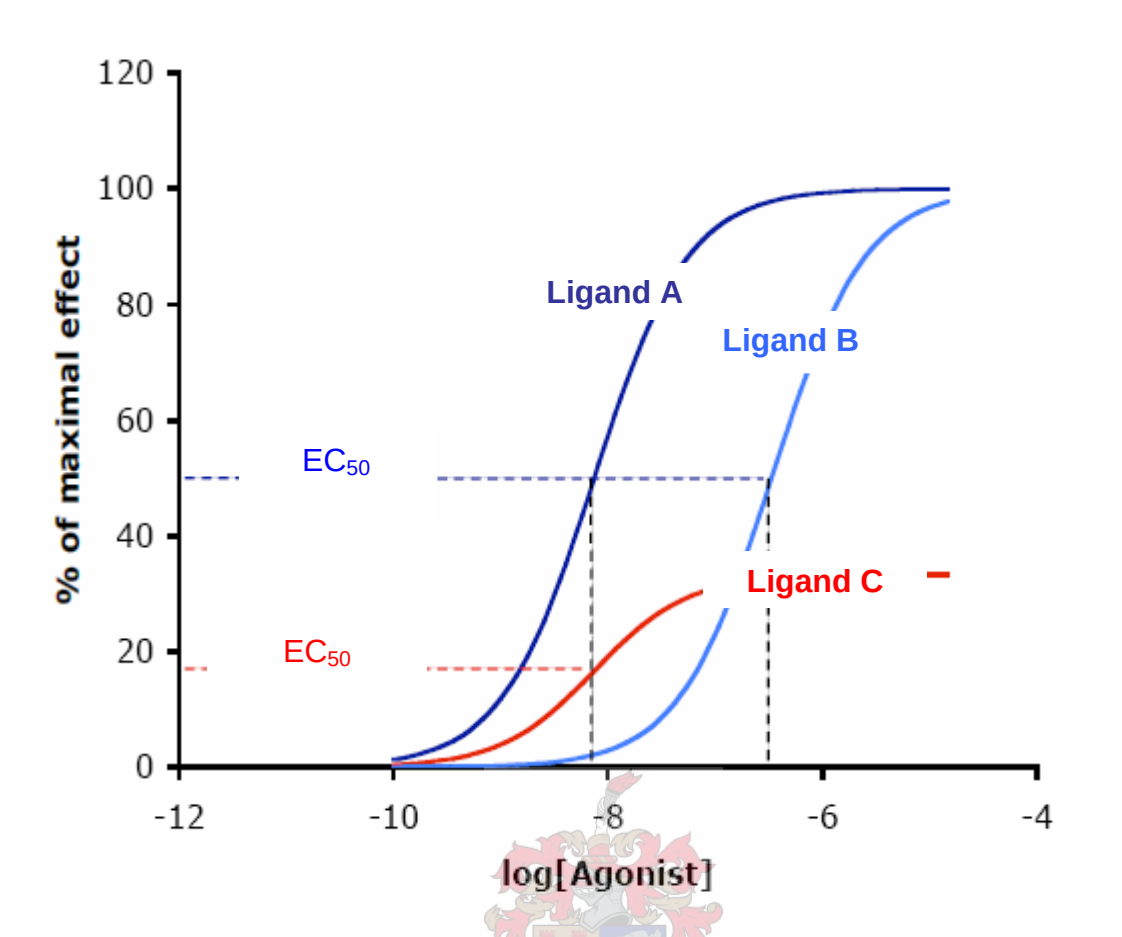


Figure 1.7. Potency versus efficacy. Potency refers to the different concentrations of two ligands needed to produce the same effect which is half of the maximal response. Efficacy is the maximum effect of a specific ligand, which specifies the agonist activity. Ligand A and B have the same efficacy, behaving as full agonists. Ligand A has a greater potency than B because the concentration of B must be larger to produce the same effect as A. Ligand C has a lower efficacy, behaving as a partial agonist however it is more potent than B. Figure from http://glutxi.umassmed.edu/lectures/dynamics.pdf

1.8 Factors affecting the potency, efficacy and agonist activity in transcriptional regulation

Initially, the EC_{50} value for a receptor-agonist complex and the partial agonist activity of an antagonist for a specific gene were thought to be constant and determined by the steroid itself. However, neither the EC_{50} value nor the partial agonist activity is constant among genes induced by a given receptor-ligand complex, even within the same cell. The effect seems to be independent of the cell line and species origin of the receptor (Szapary *et al.*, 1996) and reporter gene, promoter and enhancer (Szapary *et al.*, 1999).

Increasing the density of the receptor in cells, shifts the EC_{50} value of agonists to a lower steroid concentration and it increases the partial agonist activity of antagonists, without changing the relative maximal induction caused by saturating concentrations of ligand. Szapary *et al.* was the first group to examine this phenomenon in HeLa cells with endogenous GR and CV-1 cells lacking endogenous receptor. Co-transfecting more receptors into the cells, caused a significant left shift in the dose-response curve and the agonist activity of an antagonist increased 10-fold at saturating concentrations on a GREtkCAT reporter construct (Szapary *et al.*, 1996). This effect was later shown by the same group to be independent of the reporter, promoter or enhancer indicating that possibly other transcription factors or cofactors are being titrated by the additional receptor (Szapary *et al.*, 1999).

Therefore, it was tested whether increased expression of certain coactivators could mimic the same effect as increased receptor concentration. It was shown that TIF2, SRC-1 and AIB1 could increase the agonist activity of dexamethasone mesylate (an antagonist) and clearly left-shift the dose-response curve (Szapary *et al.*, 1999). Also increased concentrations of CBP and P/CAF can reduce the EC_{50} value and increase the partial agonist activity of an antagonist, dexamethasone mesylate (He *et al.*, 2002; Szapary *et al.*, 1999). Interestingly, the effects of increased GR levels are saturable, consistent with the titration of an unknown factor or saturation of a step in the transcriptional activation process (Chen *et al.*, 2000).

The same concept can also be applied to GR-mediated transrepression. Work done by Zhao *et al.*, in COS-7 cells, showed that both the potency and efficacy in GR-mediated transrepression was dependent on the receptor concentration (Zhao *et al.*, 2003). RU486 and MPA switched from antagonists to full agonists in GR-mediated transrepression with increasing amounts of receptor, while this did not happen to cortisol or budesonide (Zhao *et al.*, 2003).

1.9 GR ligands

In humans, the most important physiological ligand for GR is cortisol. Along with the endogenous glucocorticoids, several compounds have been synthesised that exhibit high affinity to GR. These synthetic glucocorticoids either mimic (agonist) or suppress (antagonist) the activity of the endogenous ligand. Nonsteroidal ligands have been synthesised for ER, PR and AR but few selective nonsteroidal ligands for GR have been developed. There are more than 10 GR ligands currently in use for treatment of various diseases, such as asthma, allergy and autoimmune diseases (reviewed in Boers, 2004; Heasman *et al.,* 2003; Walsh *et al.,* 2003).

A large panel of ligands was chosen for this study, including natural and synthetic agonists, partial agonists, SEGRAs and antagonists. Some were chosen because they are extensively used in treatment for diseases (dexamethasone and prednisolone), some as the natural ligands for steroid receptors (cortisol, progesterone and aldosterone) and some because they have been of particular interest in the lab. MPA and NET-A have been extensively studied in the lab over the last couple of years. They are both synthetic progestins used in contraceptives and hormone replacement therapy, however, they display very different glucocorticoid properties (Koubovec *et al.*, 2005). CpdA was synthesised at the University of Stellenbosch in the late 90s (Schalk de Kock, PhD thesis, University of Stellenbosch) and it has been proposed to have GC like properties but it has not yet been shown to be a ligand for the receptor.

Table 1.1, 1.2 and 1.3 (in section 1.13.5) summarises the binding properties and the potency and efficacy values for transcriptional regulation for the panel of GR ligands.

1.9.1 Full agonists

An agonist binds to its target receptor, changes the receptor into a conformationally active form and this activates transduction pathways, which result in a biological response. Full agonists, irrespective of their very different receptor-binding affinities, are all capable of eliciting a full maximum response (100-70 %, as defined in this thesis) at saturating concentrations (reviewed in Neubig *et al.,* 2003).

1.9.1.1 Dexamethasone (Dex)

Dexamethasone (Dex) is a potent, steroidal, synthetic GR agonist used primarily in the treatment of chronic inflammatory diseases, rheumatoid arthritis (Stein *et al.*, 1999) and cancers, such as multiple myeloma (Cook *et al.*, 2004; Richardson *et al.*, 2005) and prostate cancer (Koutsilieris *et al.*, 2004). The scientific data describing the glucocorticoid agonist properties of Dex are extensive. They are based on several *in vitro* and *in vivo* tests under various conditions and different parameters, therefore it is impossible to compare them in a reliable way. A few examples of recent binding affinity data, and potency and efficacy for transactivation and transrepression will be given in table 1.1-1.3.

The binding affinity for Dex for the glucocorticoid receptor ranges from 5 nM to 10 nM, depending on cell systems used in *in vivo* and conditions used during the *in vitro* assays. The potency of Dex for transactivation depends on the type of promoter or receptor concentration and cell system used, causing the EC_{50} values to vary from about 1 nM to 10 nM. Dex is also a potent agonist for gene transrepression with a potency of between about 0.05 nM and 15 nM, depending on cell and promoter type and receptor concentration.

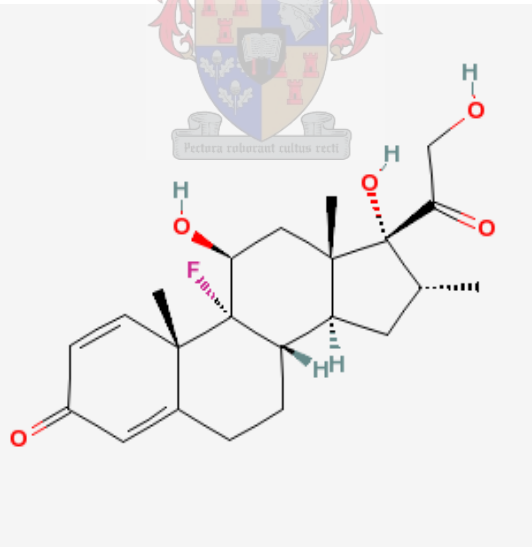


Figure 1.8. Structure of dexamethasone [(9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one)]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.1.2 Cortisol (Cort)

Cortisol is the primary endogenous steroid ligand for the glucocorticoid receptor in humans. The production of cortisol is regulated by the hypothalamic-pituitary-adrenal (HPA) axis and cortisol controls several processes involved in glucose metabolism and the immune function in the body. Excessive endogenous production of cortisol causes Cushing's disease, leading to hypertension, obesity and diabetes (reviewed in Whitworth *et al.,* 2000). Insufficient production of cortisol is called Addison's disease, leading to cardiovascular disorders, diarrhea and weakness (reviewed in Lovas and Husebye, 2003).

Cort has a high binding affinity for GR and it is a potent GC agonist for both transactivation and transrepression with EC_{50} values varying from about 10 nM to 40 nM, depending on the system investigated.

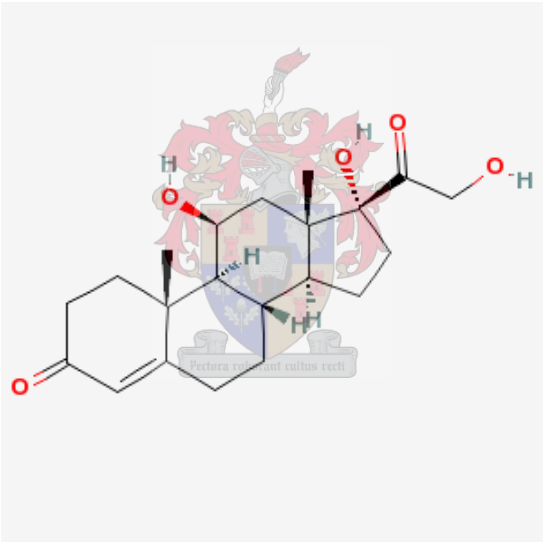


Figure 1.9. Structure of cortisol [11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydrocyclopenta[a]phenanthren-3-one]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.1.3 Prednisolone (Pred)

Prednisolone (Pred) is a synthetic steroidal drug with predominantly glucocorticoid properties. Pred is administered as a drug for asthma (Qureshi *et al.*, 2001) and rheumatic disorders (Haugeberg *et al.*, 2004).

The binding affinity of Pred to GR in several cell lines with endogenous receptor has been determined, with IC_{50} values ranging from about 20 nM to 85 nM. Pred is a potent agonist in GR-mediated transcriptional regulation. In transactivation, the potency of Pred varies from about 1 nM to 200 nM depending on the cell system and promoter investigated. In transrepression, the EC_{50} values for Pred ranges from about 5 nM to 20 nM.

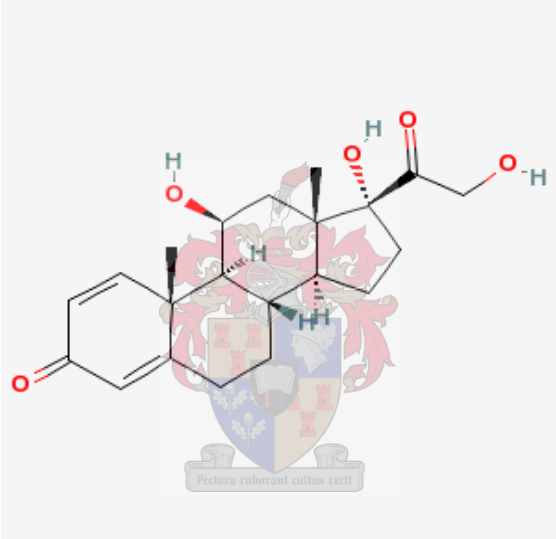


Figure 1.10. Structure of prednisolone [11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.2 Partial agonists

Maximal receptor occupation does not necessarily produce a maximal response. An agonist that in a given cell system, at maximal activity, cannot elicit as large an effect as a full agonist is called a partial agonist (reviewed in Zhu, 2005). A partial agonist can switch to a full agonist dependent on the cell system, e.g. if more receptors or cofactors are expressed. When more receptors are present, the absolute numbers of receptors occupied by the partial agonist will be higher and hence, the maximum response increases. The molecular basis of partial agonism is unknown, however, two theories have been proposed (Cho *et al.*, 2005a; Simons, 2003; Zhao *et al.*, 2003). Firstly, the partial agonist may fit the receptor-binding site well but

it is less able to promote the receptor conformational change leading to transactivation (Zhao *et al.*, 2003). Secondly, the receptor may exist in two states, the inactive form (R_i) and the active form (R_a). If an agonist has a higher affinity for the active form, the equilibrium will shift in favor of the liganded-R_a and full transactivation may occur. If an agonist has similar affinity for the two forms, both liganded-R_a and liganded-R_i will be formed, however, only the liganded-R_a will produce partial effect. Weak partial agonists can also be competitive antagonists. If a full agonist is administered together with a partial agonist, and they both have the same affinity for the receptor, as the concentration of the partial agonist increases, it will displace the full agonist occupying the receptor and reduce the overall response of the system. Partial agonists are administered when the production of the endogenous ligand is low, to boost the response, or when the production of the endogenous ligand is too high, to repress the stimulation of the receptor (reviewed in Zhu, 2005).

1.9.2.1 Progesterone (Prog)

Progesterone is the natural ligand for the progesterone receptor. In women, Prog is produced primarily by the ovaries and adrenal glands. Prog is also the precursor of most other hormones, like estradiol, testosterone and cortisol. Prog is responsible for a normal monthly menstrual cycle and is also important in the maintenance of the function of the nervous system, the cardiovascular system and skeletal system. The hormone is often used in the treatment of menopausal and infertility disorders (reviewed in Panay and Studd, 1997).

Prog binds only weakly to GR with K_i values ranging between about 95 nM and 215 nM. Prog can switch between partial agonist activity and no agonist activity in GR-mediated transcriptional regulation depending on cell and promoter type and receptor concentration. Therefore, Prog is described as a partial, steroidal, natural GR agonist or antagonist, depending on the cell system. In HEK293 cells, Prog behaves like a full agonist for both transactivation and transrepression (Koubovec *et al.,* 2005). In L929 cells, Prog behaves more like a SEGRA with weak agonistic activity for transactivation but with significant agonistic activity for gene repression (Koubovec *et al.,* 2004). In human lymphocytes, Prog behaved more like an antagonist (Bamberger *et al.,* 1999).

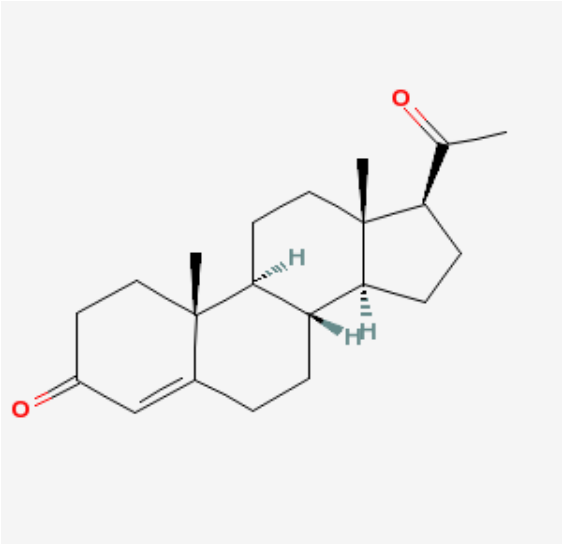


Figure 1.11. Structure of progesterone [17-acetyl-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetra-decahydrocyclopenta[a]phenanthren-3-one]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.2.2 Medroxyprogesterone acetate (MPA)

Besides the natural progestin, progesterone, there are different classes of synthetic progestins, like MPA and NET-A. The synthetic progestin, MPA (named Depo provera when used as such) is widely used in 3 months injectable contraception (Affandi, 2002) and hormone replacement therapy (Brunelli *et al.*, 1996; Irahara *et al.*, 2001; Muneyyirci-Delale and Karacan, 1998; Taitel and Kafrissen, 1995). Recent evidence, however, has shown that these synthetic progestins confer severe side-effects, such as increased risk of cardiovascular complications, stroke and breast cancer (Beral, 2003; McKenzie *et al.*, 2004; Rossouw *et al.*, 2002; Valdivia *et al.*, 2004). Both exhibit progestogenic effects and they have longer bioavailability and half-life than progesterone. However, the synthetic progestins have also been shown to have non-progestogenic effects mediated through other nuclear receptors (Schindler *et al.*, 2003). The glucocorticoid activity of these progestins is not well-known and this has been a major research area for our lab in recent years.

MPA has been shown to bind not only to the progesterone but also to the glucocorticoid receptor with relatively high affinity. MPA appears to act as a partial or full agonist for transactivation depending on the cell system, with a potency ranging from about 10 nM to 90 nM. MPA also switches agonistic activity in transrepression, with EC_{50} values varying from about 2.5 nM to 90 nM.

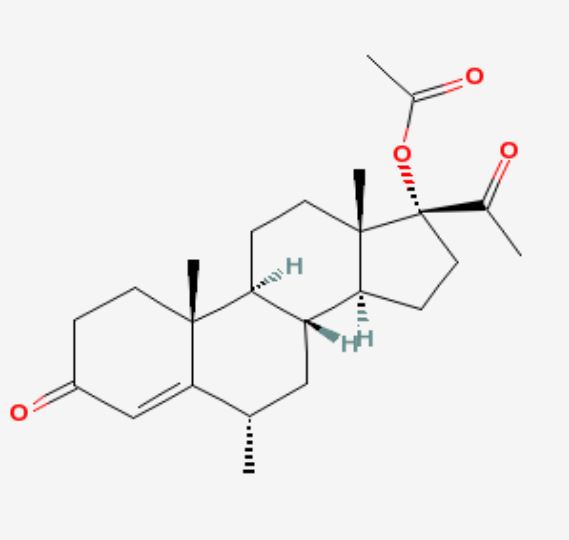


Figure 1.12. Structure of MPA [acetic acid (17-acetyl-6,10,13-trimethyl-3-oxo-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetra-decahydrocyclopenta[a]phenanthren-17-yl) ester]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.2.3 Norethisterone acetate (NET-A)

NET-A, a progesterone derivative, is extensively used in 2-month injectable contraceptives (named Noristerat when used as such) and hormone replacement therapy, as described above. NET-A is a steroidal, synthetic progestin that binds very weakly to the human GR. NET-A appears to be a weak partial agonist for GR-mediated transrepression, however, NET-A has no agonistic activity in transactivation, therefore it might possibly behave more like a SEGRA (Koubovec *et al.*, 2005).

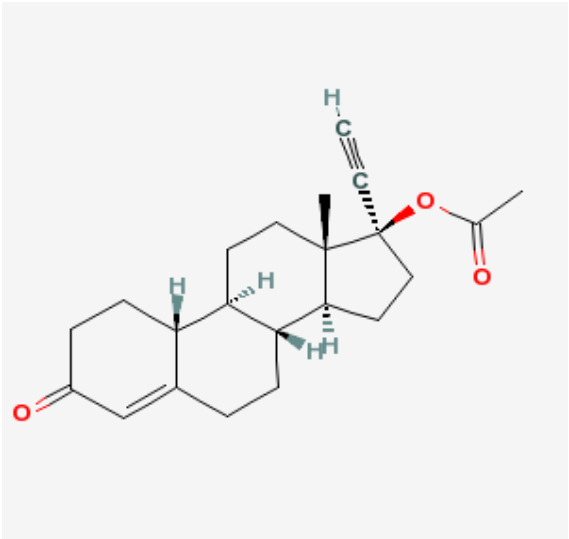


Figure 1.13. Structure of NET-A [acetic acid (17-ethynyl-13-methyl-3-oxo-1,2,6,7,8,9,10,11,12,13,14,15,16,17 tetra-decahydrocyclopenta[a]phenanthren-17-yl) ester]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.2.4 Aldosterone (Ald)

Aldosterone is a mineralocorticoid produced by the adrenal cortex. The amount of Ald in the body is monitored by the kidneys, which secrete hormones to increase or decrease Ald production. Ald regulates electrolyte levels (such as sodium and potassium) in the blood. This helps to maintain blood pressure and heart function. GR and MR exhibit ligand cross-reactivity and the two receptors share 56 % identity in their LBD therefore Ald binds to both the MR and GR (reviewed in Heymes *et al.,* 2004).

Ald binds weakly to GR and it is a partial agonist with low potency in GR-mediated transactivation, with EC_{50} values varying from about 150 nM to 500 nM, depending on cell system studied. It appears that no studies have been done on aldosterone agonist activity in transcriptional repression via GR.

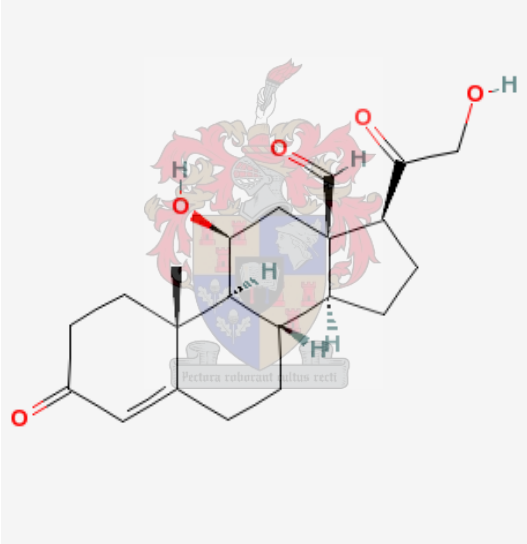


Figure 1.14. Structure of aldosterone [11-hydroxy-17-(2-hydroxyacetyl)-10-methyl-3-oxo1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydrocyclopenta[a]phenanthrene-13-carbaldehyde]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.3 Antagonists

An antagonist binds to the receptor, but fails to initiate transcriptional regulation. Furthermore, an antagonist can compete with agonists for occupancy of the receptor. Reversible competitive antagonism involves competition between agonist and antagonist for the same receptor, resulting in reduced binding of the agonist to the receptor, although addition of increased amounts of agonist can reverse the blockade fully. Irreversible competitive antagonism also involves competition between agonist and antagonist for the

same receptor, but stronger binding forces, usually involving covalent binding of the antagonist to the receptor, ensure that even at high agonist concentrations, the effect of the antagonist cannot be fully reversed. Antagonists have been classified according to the conformational change that they induce in the receptor after ligand binding. Type I antagonists only slightly change the conformation of the receptor, resembling the inactive conformation. Type II antagonists cause a conformational change similar to agonists (reviewed in Neubig *et al.,* 2003).

1.9.3.1 RU486 (Roussel-Uclaf 38486)

RU486, also known as mifepristone, is a derivative of norethindrone and it binds strongly to the progesterone receptor (stronger than progesterone itself), behaving like a full antagonist. It is used as emergency contraception and in abortions as RU486 blocks the action of the hormone, progesterone, essential for maintaining pregnancy (reviewed in Sarkar, 2005). RU486 also has antiglucocorticoid effects by binding to glucocorticoid receptors with approximately 4-fold higher relative binding affinity than dexamethasone (reviewed in Cadepond *et al.,* 1997). RU486 is a potent, steroidal, synthetic Type II antagonist or partial agonist, depending on context, like cell system, promoter and receptor concentration, in both transactivation and transrepression.

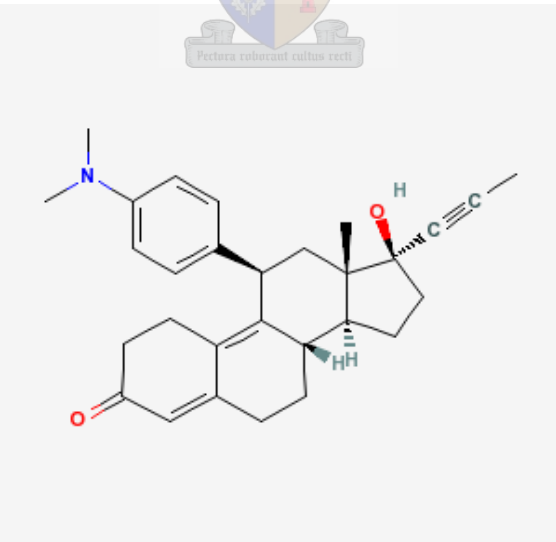


Figure 1.15. Structure of RU486 [11-(4-dimethylaminophenyl)-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,13,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.3.2 D06 (Abbott-Ligand 082D06)

D06 is one of the first nonsteroidal type I antagonists described for GR (Miner *et al.*, 2003). It might be useful in treating Cushing's disease, depression and diabetes. *In vitro*, D06 binds to GR with low affinity, with no cross-reactivity with the AR, PR, MR or ER, unlike RU486. D06 has antagonistic activity in GR-mediated transactivation and transrepression, but no agonistic activity itself (Miner *et al.*, 2003). In addition, D06 has no agonist or antagonist activity through AR, PR, MR or ER (Miner *et al.*, 2003).

Unlike RU486, that causes receptor-DNA binding and that has some agonist activity in certain cell systems, D06 does not induce DNA binding by GR *in vitro* or *in vivo.* This might be due to lack of nuclear translocation of the D06-bound GR as compared to RU486-bound GR (Miner *et al.*, 2003).

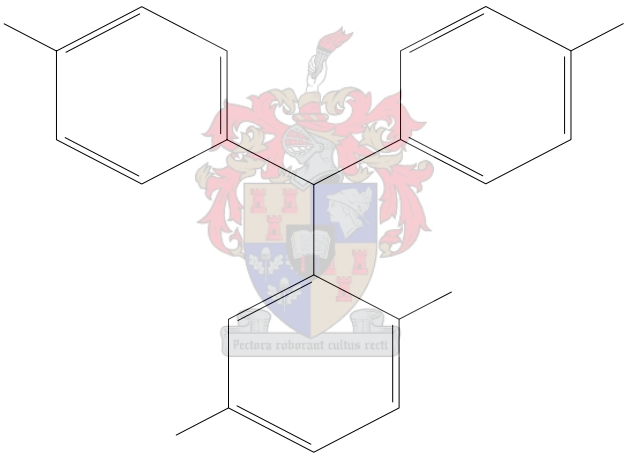


Figure 1.16. Structure of D06 [bis (4-N, N-dimethylaminophenyl) (2-chloro-5-nitrophenyl) methane]

1.9.4 Selective glucocorticoid receptor agonists (SEGRAs)

In recent years, investigations into GR ligands with improved therapeutic effects and less side-effects have been conducted. The anti-inflammatory effects of glucocorticoids result from repression of inflammatory pathways and some of the unwanted side-effects from up-regulation of certain metabolic genes. New glucocorticoids that are able to dissociate between transactivation and transrepression, thus lowering the potential of unwanted side-effects, would be promising drug candidates. Most of these ligands are non-steroidal and they are called selective glucocorticoid receptor agonists, SEGRAs.

1.9.4.1 Compound A (CpdA)

Compound A is a non-steroidal plant derivative that was developed at the University of Stellenbosch in the Department of Biochemistry in 1997 (Schalk de Kock, PhD thesis, University of Stellenbosch). It was initially found to interact with steroidogenic enzymes (Louw *et al.*, 2000a) and steroid-binding globulins (Louw *et al.*, 2000b). Therefore, because of its steroid-like activity, CpdA has been of great interest in the lab as it has been shown that CpdA exhibits anti-androgenic and anti-progestogenic properties (Tanner *et al.*, 2003) and recently, further investigation into CpdA and its glucocorticoid activity has been performed (De Bosscher *et al.*, 2005; Fatima Allie-Reid, PhD thesis, University of Stellenbosch; personal communication with Prof. Hapgood and Dr. Louw, Department of Biochemistry, University of Stellenbosch).

It is unclear whether CpdA is a ligand for GR as it has shown atypical binding properties for the receptor (for more detail see Chapter 4). CpdA is unable to activate synthetic GREs, both minimal and physiological promoter-reporter constructs, or endogenous gluconeogenic genes via GR in several different cell lines, including mouse fibroblast cells (L929), human lung carcinoma cells (A549) and liver cells (BWTG3) (De Bosscher *et al.*, 2005). However, CpdA does repress NF-κB mediated gene transcription of the IL-6, IL-8 and ICAM promoters, although it exerts no effect on AP-1 mediated gene expression in the presence of GR (De Bosscher *et al.*, 2005). CpdA also transrepresses other genes: via a tethering mechanism, for example the POMC gene promoter via Nur77 and the GnRH promoter via Oct1 (personal communication Prof. Hapgood) and through at yet unknown mechanisms, for example CBG (personal communication Dr. Louw).

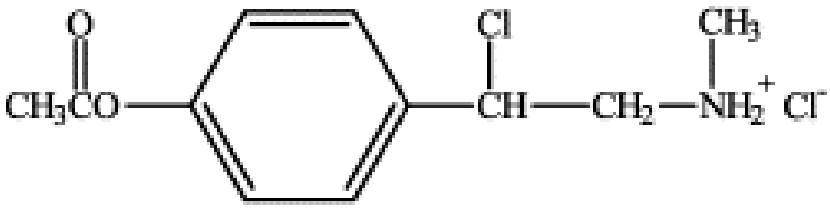


Figure 1.17. Structure of CpdA [2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride] Structure from (Tanner *et al.*, 2003)

1.9.4.2 AL438 (Abbott-Ligand 438)

AL438 has a high affinity for GR with some affinity for the MR. However, AL438 has only slight antagonist activity in MR-mediated activation, and none for the PR, AR and ER (Coghlan *et al.*, 2003). AL438 is a potent agonist for selective promoters, however, a partial agonist for other promoters, in both transactivation and transrepression (Coghlan *et al.*, 2003). AL438 also has antagonistic activity in GR-mediated transactivation (Coghlan *et al.*, 2003).

AL438 causes a conformational change in the receptor such that it binds less, than the potent agonist prednisolone, of the coactivator PGC-1, which is important for the increase of the glucose production by GCs, while AL438 bound-GR binds equally well, compared to prednisolone, to the coactivator GRIP1, which is important for GR-mediated repression of proinflammatory genes. This differential binding of coactivators could be responsible for the dissociative behavior of AL438 in GR transcriptional regulation (Coghlan *et al.*, 2003).

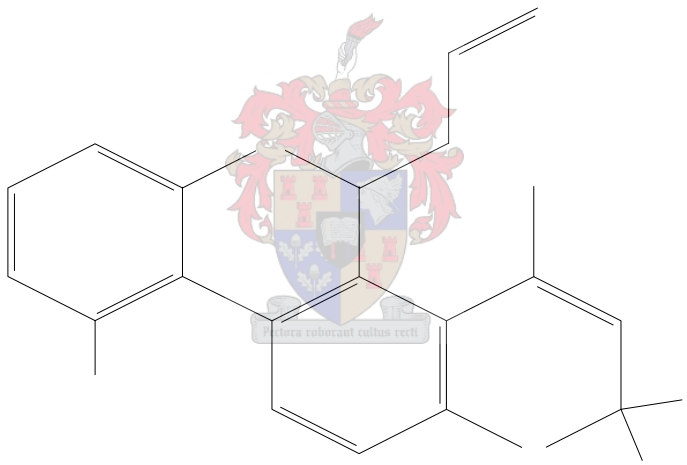


Figure 1.18. Structure of AL438 [2, 5-dihydro-10-methoxy-5-(2-propenyl)-2, 2, 4-trimethyl-1H-[1] benzo-pyrano [3, 4-f] quinoline]

1.9.4.3 Ursodeoxycholic acid (UDCA)

The hydrophilic bile acid ursodeoxycholic acid (UDCA) has been widely used for dissolution of gallstones and chronic liver diseases. UDCA does not bind to GR, as no association of radiolabelled UDCA with GR was found in 293-T cells (Weitzel *et al.*, 2005). Despite no direct binding of UDCA to GR, UDCA behaves like a weak partial agonist in GR-mediated transactivation and a full agonist in transrepression.

Even though UDCA appears not to bind directly to the GR, it is also able to promote nuclear translocation and DNA-binding of the receptor in CHO cells (Tanaka *et al.*, 1996), hepatocyte cells (Weitzel *et al.*, 2005) and COS-7 cells. However, the translocation occurs more slowly than with Dex (Miura *et al.*, 2001). UDCA also causes a conformational change in the receptor that disrupts the binding of the coactivator TIF-2, which can explain the partial agonistic effect that UDCA has on gene transcription (Miura *et al.*, 2001).

The effect of UDCA on GR-mediated transcriptional regulation may involve other mechanisms than classical ligand-receptor interactions. The hydrophilic bile acid, UDCA, may not readily penetrate the cell membrane and one can speculate that UDCA interacts with a cell membrane receptor thereby activating a secondary intracellular pathway that can activate GR downstream, like the protein kinase C (PKC) pathway (Rao *et al.*, 1997) or other intracellular receptors, like orphan nuclear receptors (Parks *et al.*, 1999).

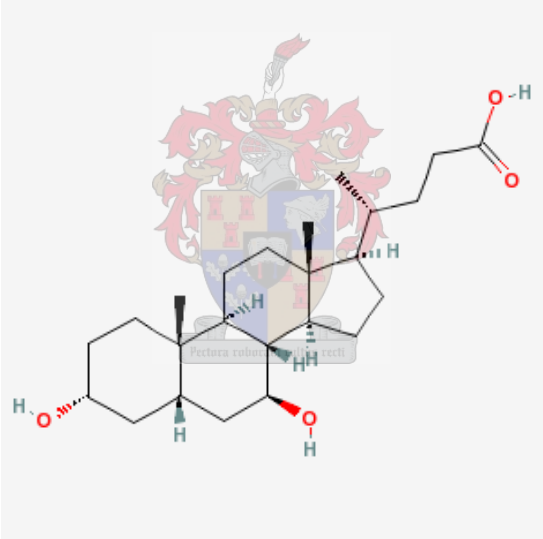


Figure 1.19 Structure of UDCA [4-[(3,7-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17-hexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)]pentanoic acid]
Structure from http://pubchem.ncbi.nlm.nih.gov

1.9.5 Summary of the functional properties of the panel of test compounds

Table 1.1. Summary of the binding affinities for GR for the panel of test compounds.

Test compound	Binding affinity (nM)	Cell system	Species of receptor	Reference
Dex	6.6 (K_D)	Placental cytotrophoblast	Human	(Driver *et al.*, 2001)
	9.4 (K_D)	Blood lymphocytes	Human	(Then Bergh *et al.*, 1999)
	9.4 (K_D)	COS-7	Human	(Charmandari *et al.*, 2005b)
	5.6 (K_D)	COS-7	Human	(Hammer *et al.*, 2003)
	8.2 (K_D)	(*in vitro*)	Rabbit	(Attardi *et al.*, 2004)
Cort	51.0 (IC_{50})	COS-7	Human	(Lind *et al.*, 2000)
	17.5 (K_D)	Blood leukocytes	Human	(Mulatero *et al.*, 1997)
Pred	17.6 (IC_{50})	COS-7	Human	(Lind *et al.*, 2000)
	33.4 (IC_{50})	HTC	Rat	(Ko *et al.*, 2000)
	85.3 (IC_{50})	264.7	Mouse	(Ko *et al.*, 2002a)
	68 (IC_{50})	(*in vitro*)	Human	(Schacke *et al.*, 2004)
Prog	215 (K_i)	A549	Human	(Koubovec *et al.*, 2005)
	95.2 (K_i)	(*in vitro*)	Canine	(Selman *et al.*, 1996)
MPA	31 (K_i)	Blood leukocytes	Human	(Kontula *et al.*, 1983)
	10.8 (K_i)	A549	Human	(Koubovec *et al.*, 2005)
	43.8 (IC_{50})	IM9	Human	(Kurata *et al.*, 2005)
	3.7 (K_i)	(*in vitro*)	Canine	(Selman *et al.*, 1996)
NET-A	270 (K_i)	A549	Human	(Koubovec *et al.*, 2005)
	0.8 % (RBA)	IM-9	Human	(Schoonen *et al.*, 2000)
Ald	290 (K_D)	COS-7	Human	(Martinez *et al.*, 2005)
	100 (K_i)	COS-7	Human	(Yoshikawa *et al.*, 2002)
	14.4 (K_D)	(*in vitro*)	Human	(Hellal-Levy *et al.*, 1999)
	140 (K_i)	(*in vitro*)	Human	(Rebuffat *et al.*, 2004)
RU486	0.68 (K_D)	(*in vitro*)	Human	(Wagner *et al.*, 1999)
	9.1 (IC_{50})	(*in vitro*)	Rabbit	(Attardi *et al.*, 2004)
D06	210 (K_i)	(*in vitro*)	Human	(Miner *et al.*, 2003)
CpdA	6.4 (IC_{50})	L929	Mouse	(De Bosscher *et al.*, 2005)
AL438	2.5 (K_i)	(*in vitro*)	Human	(Coghlan *et al.*, 2003)
UDCA	No binding	293-T	Rat	(Weitzel *et al.*, 2005)

Table 1.2. Summary of the potencies and relative efficacies in GR-mediated transactivation for the panel of test compounds.

Test compound	Potency EC_{50} (nM)	Relative efficacy (% of Dex)	Cell system	Gene promoter	Species of receptor	Reference
Dex	1	n/d	CV-1	MMTV	Human	(Charmandari *et al.*, 2005b)
	1	n/d	COS-7	MMTV	Human	(Muller *et al.*, 2004)
	12	n/d	A549	MMTV	Human	(Einstein *et al.*, 2004)
	4.1	n/d	PBMC	GILZ	Human	(Smit *et al.*, 2005)
	5.2	n/d	H4IIE	TAT	Rat	(Einstein *et al.*, 2004)
	2.4	n/d	H4IIE	GS	Rat	(Einstein *et al.*, 2004)
Cort	12.0	113	CV-1	MMTV	Human	(Grossmann *et al.*, 2004)
	33	n/d	CV-1	MMTV	Human	(Lim-Tio *et al.*, 1997)
	10	n/d	COS-7	MMTV	Human	(Muller *et al.*, 2004)
	38	n/d	E8.2	MMTV	Mouse	(Rebuffat *et al.*, 2004)
Pred	88.5	125	PBMC	GILZ	Human	(Smit *et al.*, 2005)
	211	81	Hep2G	TAT	Human	(Ali A *et al.*, 2004)
	32	97	Hep2G	GS	Human	(Ali A *et al.*, 2004)
	6.9	78	CV-1	MMTV	Human	(Grossmann *et al.*, 2004)
	11.6	n/d	HTC	TAT	Rat	(Ko *et al.*, 2000)
	1.5	100	H4IIE	TAT	Rat	(Schacke *et al.*, 2004)
Prog	n/a	n/d	Lymphocytes	TAT	Human	(Bamberger *et al.*, 1999)
	n/a	n/d	CV-1	MMTV	Human	(Grossmann *et al.*, 2004)
	280	64.3	HEK293	TAT	Rat	(Koubovec *et al.*, 2005)
	930	16	L929	TAT	Mouse	(Koubovec *et al.*, 2004)
MPA	10	55	A549	HRE	Human	(Zhang *et al.*, 2000)
	7.2	72.2	HEK293	TAT	Rat	(Koubovec *et al.*, 2005)
	92	50	L929	TAT	Mouse	(Koubovec *et al.*, 2004)
NET-A	n/a	n/d	CHO	MMTV	Human	(Schoonen *et al.*, 2000)
	n/a	n/d	HEK293	TAT	Rat	(Koubovec *et al.*, 2005)
Ald	500	50	COS-7	MMTV	Human	(Hellal-Levy *et al.*, 1999)
	166	111	CV-1	MMTV	Human	(Grossmann *et al.*, 2004)
	400	50	E8.2	MMTV	Mouse	(Rebuffat *et al.*, 2004)

RU486	n/a	n/d	COS-7	MMTV	Human	(Muller *et al.*, 2004)
	n/d	20	U20S	MMTV	Human	(Fryer *et al.*, 2000)
	n/a	n/d	T47D	MMTV	Human	(Fryer *et al.*, 2000)
	39	10	H4IIE	TAT	Rat	(Einstein *et al.*, 2004)
	n/a	n/d	H4IIE	GS	Rat	(Einstein *et al.*, 2004)
	n/d	20	COS-7	GRE	Rat	(Prima *et al.*, 2000)
D06	n/a	n/d	CV-1	MMTV	Human	(Miner *et al.*, 2003)
CpdA	n/a	n/d	L929	MMTV	Mouse	(De Bosscher *et al.*, 2005)
AL438	800	100*	HepG2	TAT	Human	(Coghlan *et al.*, 2003)
	500	50*	Skin fibroblast	Aromatase	Human	(Coghlan *et al.*, 2003)
UDCA	n/a	n/d	Hepatocytes	TAT	Human	(Mitsuyoshi *et al.*, 1997)
	n/a	n/d	COS-7	Gal4-luc	Human	(Miura *et al.*, 2001)
	n/d	10	Hepatocytes	MMTV	Rat	(Weitzel *et al.*, 2005)
	n/d	10	CHO	MMTV	Rat	(Tanaka *et al.*, 1996)

Table 1.3. Summary of the potency and relative efficacy GR-mediated transrepression for the panel of test compounds.

Test compound	**Potency EC_{50} (nM)**	**Relative efficacy (% of Dex)**	**Cell system**	**Gene promoter**	**Species of receptor**	**Reference**
Dex	0.05	n/d	COS-7	5 X NF-κB	Human	(Zhao *et al.*, 2003)
	14.3	n/d	PBMC	IL-2	Human	(Smit *et al.*, 2005)
	2.2	n/d	A549	GM-CSF	Human	(Adcock *et al.*, 1999)
	3	n/d	A549	MMP-1	Human	(Einstein *et al.*, 2004)
Cort	10	n/d	COS-7	5 X NF-κB	Human	(Zhao *et al.*, 2003)
Pred	6.1	50	PBMC	IL-2	Human	(Smit *et al.*, 2005)
	4.5	102	A549	IL-6	Human	(Ali A *et al.*, 2004)
	18	100	H4IIE	IL-8	Rat	(Schacke *et al.*, 2004)
Prog	n/a	n/d	Lymphocytes	IL-2	Human	(Bamberger *et al.*, 1999)
	n/a	n/d	KTC-2	IL-6	Human	(Kurebayashi *et al.*, 2003)
	26	88.7	HEK293	IL-8	Rat	(Koubovec *et al.*, 2005)
	470	50	L929	IL-8	Mouse	(Koubovec *et al.*, 2004)

MPA	5	n/d	COS-7	5 X NF-κB	Human	(Zhao *et al.*, 2003)
	n/d	73.3	Lymphocytes	IL-2	Human	(Bamberger *et al.*, 1999)
	2.7	95.4	HEK293	IL-8	Rat	(Koubovec *et al.*, 2005)
	90	50	L929	IL-8	Mouse	(Koubovec *et al.*, 2004)
NET-A	0.2**	23.0	HEK293	IL-8	Rat	(Koubovec *et al.*, 2005)
Ald	n/d					
RU486	0.3	28	A549	MMP-1	Human	(Einstein *et al.*, 2004)
	1.0	n/d	COS-7	5 X NF-κB	Human	(Zhao *et al.*, 2003)
D06	n/a	n/d	Skin fibroblast	E-selectin	Human	(Miner *et al.*, 2003)
CpdA	n/d	100	L929	E-selectin	Mouse	(De Bosscher *et al*., 2005)
	n/d	100	L929	IL-6	Mouse	(De Bosscher *et al*., 2005)
AL438	10	100*	HepG2	E-selectin	Human	(Coghlan *et al.*, 2003)
	60	100*	Skin fibroblast	IL-6	Human	(Coghlan *et al.*, 2003)
	n/a	n/d	Osteoblast	osteocalcin	Human	(Coghlan *et al.*, 2003)
UDCA	n/d	100	HeLa	NF-κB	Human	(Miura *et al.*, 2001)

n/a: no activity

n/d: not determined

*: relative efficacy to Pred

**: relative potency to Dex

COS-7 = Transformed African green monkey kidney fibroblast cell line

HTC = Rat hepatoma cell line

264.7 = Mouse macrophage cell line

A549 = Human lung carcinoma cell line

PBMC = Peripheral blood mononuclear cell line

IM-9 = Human lymphocyte cell line

L929 = Mouse fibrosarcoma cell line

293-T = Human embryonic kidney cell line

HEK293 = Human embryonic kidney cell line

CV-1 = Normal African green monkey kidney fibroblast cell line

H4IIE = Rat hepatoma cell line

E8.2 = Mouse fibroblast cell line

Hep2G = Human hepatoma cell line

CHO = Hamster ovary cell line

U20S = Human osteosarcoma cell line

T47D = Breast cancer cell line

KTC-2 = thyroid cancer cell line

HeLa = Human cervical adenocarcinoma cell line

1.10 Aim of thesis

The question of why different ligands for GR have different potencies and efficacies for transcriptional response on the same gene in the same cells remains unanswered. The 5 year GR project will look at seven steps in the GR transcriptional regulation pathway that can possibly determine the biological characteristics of glucocorticoids.

The main aim of this thesis is to correlate the behaviour of a liganded-GR at a specific step in the GR transcriptional regulation pathway with the potency and efficacy for transcriptional regulation measured by the ability to induce transactivation and transrepression on synthetic promoter-reporter constructs. This thesis contains results from a two-year project that focuses on the phosphorylation of the liganded-GR at Ser211.

The first aim is to determine the relative potency and efficacy for the panel of chosen GR ligands in transcriptional activation and repression in the same model cell system chosen on the same promoter-reporter construct, thereby excluding promoter and cell-type specific effects. These data will be used in future by others throughout the five year project to further investigate several possible steps involved in ligand-selectivity of transcriptional regulation by the GR.

Secondly, the extent of GR phosphorylation at Ser211 of the different ligand-receptor complexes will be investigated in this thesis by using an antibody specifically recognising GR phosphorylated at Ser211, as it has been hypothesised that the phosphorylation at Ser211 correlates with agonist activity in transactivation.

Finally, correlation analyses between the potency and efficacy of the ligands in transactivation and transrepression and the extent of phosphorylation at Ser211 will be performed to test the hypothesis.

CHAPTER 2

Materials and methods

2.1 Plasmids

The plasmid, pTAT-GRE-E1b-luc, driven by the E1b promoter that contains two copies of the rat TAT-GRE, was a kind gift from Dr. G. Jenster at Erasmus University of Rotterdam, Rotterdam, The Netherlands (Sui *et al.,* 1999). The expression vectors for β-galactosidase (i.e. pSVβ-gal) and for the empty vector (i.e. pGL2-basic) were obtained from Promega, Madison, WI, USA. The IL-8 promoter variant, p546hIL-8luc+, was kindly provided by Dr. N. Mukaida at the Cancer Research Institute, Japan (Mukaida *et al.,* 1990). The expression vectors for the wildtype HA-tagged human GR (i.e. pCMV-HA-hGR) and the S203A, S211A and S226A HA-tagged human GR mutants (i.e. pCMV-HA-hGR$_{S203A}$, pCMV-HA-hGR$_{S211A}$ and pCMV-HA-hGR$_{S226A}$, respectively), all cloned into the pCMV-HA mammalian expression vector which contains a human cytomegalovirus promoter (pCMV), were obtained from Prof. M. J. Garabedian at New York University, School of Medicine, USA (Wang *et al.,* 2002). The expression vectors for the A- and B-isoform of human GR (i.e. pCMV-hGRα$_{M27T}$ and pCMV-hGRα$_{M1T}$, respectively) were gifts from Prof. Cidlowski at Laboratory of Signal Transduction, NIH, USA (Yudt and Cidlowski, 2001).

2.2 Transformation of plasmid DNA

Plasmid DNA was transformed into competent DH5α cells. 5 ng of plasmid DNA was aliquoted into an eppendorf tube and kept on ice. All the following steps were performed on ice. The electrocompetent cells were diluted 1:1 in ice-cold 10 % (v/v) glycerol and 40 µl of the cell suspension were dispensed into the tube containing plasmid DNA. The DNA-cell mixture was transferred into a 1 mm gap width electroporation cuvette (BTX, San Diego, CA, USA) and subjected to an electric pulse (1800 V) in a Savant GTF100 gene transformer. Immediately afterwards, 1 ml of SOC medium (Sambrook *et al*., 1989) was added to the transformed cells and transferred to an eppendorf tube before shaken at 200 rpm for 1 hour at 37°C. Different dilutions of the transformation culture were aliquoted and plated out on antibiotic LB agar plates (Sambrook *et al*., 1989) and incubated overnight at 37°C.

2.3 Plasmid preparation

Positive clones were picked from the LB agar plates (Sambrook *et al*., 1989) streaked with transformed cells. A culture was grown in 5 mL 2 X YT200 (Sambrook *et al*., 1989) plus 50 µg/mL ampicillin (Sigma-Aldrich, St. Louis, MO, USA) medium for 8 hour at 37°C before 100 µl of the culture was transferred to 500 mL of LB (Sambrook *et al*., 1989) plus 50 µg/mL ampicillin medium and grown overnight at 37°C while shaking at 200 rpm. Plasmid DNA was isolated with the Promega QIAGEN kit or Pure yield plasmid midi prep kit (Promega, Madison, WI, USA), according to the protocol provided. The purity and integrity of the plasmids were analysed by agarose gel electrophoresis.

2.4 Test compounds and antibodies

Dexamethasone, cortisol, progesterone, MPA, aldosterone, prednisolone, RU486, UDCA and NET-A were purchased from Sigma-Aldrich, St. Louis, MO, USA and all compounds were made up in 96 % EtOH . AL438 and D06 were a kind gift from Dr J. Miner from Ligand Pharmaceuticals, San Diego, CA, USA. AL438 was dissolved in 50 % DMSO and 50 % EtOH and D06 in 100 % DMSO. CpdA was synthesised at the University of Stellenbosch, Department of Biochemistry (Louw *et al*., 1997) and dissolved in 96 % EtOH. All compounds were made up in 10 mM stock solutions and serial dilutions were made from the stock solutions. The test compounds were added to the cells such that the final concentration of EtOH was less than 0.1 %.

The anti-phospho-211 GR antibody was a kind gift from Prof. M. J. Garabedian (New York University, School of Medicine, USA). The wildtype GR antibody (H-300) was obtained from Santa Cruz biotechnology (Santa Cruz, CA, USA). The secondary antibody was an anti-rabbit HRP conjugate purchased from Amersham Biosciences (Buckinghamshire, England).

2.5 Maintenance of cell cultures

2.5.1 A549 cells

The human lung carcinoma A549 cell line with endogenous GR was a kind gift from Prof. S. Okret (Karolinska Institute, Sweden). The cells were maintained at 37°C with 90 % humidity

and 5 % CO_2 in 50 % high glucose (1 g/mL) Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) and 50 % F12/HAMs supplemented with 1 mM glutamine (Invitrogen, Paisley, UK), 50 IU/mL penicillin and 50 µg/mL streptomycin (termed P/S, Invitrogen, Paisley, UK) and 10 % (v/v) fetal bovine serum (Delta Bioproducts, Johannesburg, South Africa), termed complete medium.

2.5.2 COS-1 cells

COS-1 cells (monkey kidney fibroblasts) were purchased from American Type Culture Collection (ATCC), Manassas, USA. COS-1 cells were cultured at 37° C in high glucose (1 g/mL) DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1 mM glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin (Invitrogen, Paisley, UK) and 10 % (v/v) fetal bovine serum (Delta Bioproducts, Johannesburg, South Africa), termed complete medium.

2.5.3 U2OS-hGR cells

U2OS cells stably transfected with a plasmid expressing the HA-tagged human GR (i.e. U20S-hGR) were a kind gift from Prof. Garabedian (New York University, School of Medicine, USA) (Wang *et al.,* 2002). The cells were maintained at 37°C in high glucose (1 g/mL) DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1 nM glutamine and G418 (Sigma-Aldrich, St. Louis, MO, USA) used to select for cells stably transfected with DNA, 50 IU/mL penicillin and 50 µg/mL streptomycin (Invitrogen, Paisley, UK) and 10 % (v/v) fetal bovine serum (Delta Bioproducts, Johannesburg, South Africa).

2.6 Transactivation assays

Transactivation studies were performed in transiently transfected A549, COS-1 and U2OS-hGR cells. The cells were seeded in 10-cm tissue culture dishes (Nunc, Roskilde, Denmark) at a density of 3 X 10^6 for the A549 and 2 X 10^6 for the COS-1 and U2OS-hGR cells/dish in respective complete medium. On day two, the A549 cells were transiently transfected with 7.5 µg pTAT-GRE-E1b-luc, the COS-1 cells with 10.0 µg pCMV-HA-hGR or pCMV-HA-hGR_{S211A} and 3.75 µg pTAT-GRE-E1b-luc and the U2OS-hGR cells with 5.0 µg pTAT-GRE-E1b-luc using FuGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. In COS-1 cells, if fewer cells were plated, for example in 24-well plates, proportionally less DNA was added accordingly, keeping the ratio of 10 µg receptor/ 2 X 10^6

cells. If so, it is stated in the figure legend of the appropriate experiment. β-galactosidase co-transfections to normalise for transfection efficacy were not done as the transfected cells were replated into 24-well plates 24 hours after transfections, ensuring equivalent transfection efficiency per well.

24 hours later, the medium was removed from the dish and the cells were washed with PBS (Sambrook *et al*., 1989). The cells were trypsinised by incubating them for 2 to 3 min in trypsin (Highveld Biologicals, South Africa) at 37°C before washing with complete medium and counted. The cells were replated into 24-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 1 X 10^5 cells/well. After 6 hours, the complete medium was removed and the cells were washed with PBS (Sambrook *et al*., 1989). Serum-free medium (DMEM+P/S) was added to the cells. The cells were then incubated with increasing concentrations of test compounds for 16 hours. The cells were washed with PBS (Sambrook et al., 1989) and lysed with 50 µl reporter lysis buffer (Promega, Madison, WI, USA). The plates were shaken for 15 min at room temperature before being frozen. 10 µl of the lysates were assayed for luciferase activity (Promega luciferase assay system, Promega, Madison, WI, USA) in a Veritas microplate luminometer. In addition 5 µl of the lysates were used to determine protein concentration by Bradford assay. The luciferase values were normalised to the protein concentration for each sample.

The normalised transactivation value for 10 µM Dex was set as 100 % and values for all the other samples were calculated relative to the maximal induction by Dex at 10 µM. Relative efficacy for transactivation data were plotted against log M concentration of inducing compound and analysed using GraphPad Prism software. The following parameters were chosen for dose-response curve analysis: non-linear regression (curve fit) and sigmoidal dose-response curve with fixed slope with each replicate y value considered as an individual point. The relative efficacy is given as the maximal induction point and the EC_{50} was obtained from the dose-response curve in GraphPad Prism. The relative potency was calculated by setting the EC_{50} for Dex as 100 % and values for all the others samples were calculated relative to the EC_{50} value of Dex using the nanomolar concentrations for each individual experiment. For example: if the EC_{50} (Dex) = 1 nM and the EC_{50} (Cort) = 10 nM, the relative potency of Cort is 1/10 x 100 = 10 % compared to Dex.

2.7 Transrepression assays

COS-1 cells (2 X 10^6 cells/10-cm dish) were transiently transfected with pCMV-HA-hGR (2.5 µg DNA/dish) and p546hIL-8luc+ (2.5 µg DNA/dish) with FuGENE 6 (Roche, Basel, Switzerland) according to manufacturer's instructions. The cells were replated as described above. 24 hours later, cells were untreated or pre-treated with increasing concentrations of test compound in the absence or presence of 2000 IU/mL TNF (the origin and activity of TNF has been described previously (Vanden Berghe *et al.*, 1998)). Test compounds were added 2 hours before TNF, for a total period of 8 hours, after which cells were lysed with 50 µl reporter lysis buffer (Promega, Madison, WI, USA) and assayed for luciferase expression (Promega luciferase assay system, Promega, Madison, WI, USA) in a Veritas microplate luminometer. Luciferase activity was normalised to total protein concentration measured by Bradford assay to account for differences in cell number per well after replating.

The value for induction with TNF only was set as 100 % and induction of all other samples was calculated accordingly. The data were analysed as described above, except that the relative efficacy was determined from the bottom plateau on each dose-response curve generated by GraphPad Prism software.

2.8 Western blot analysis

COS-1 cells and U2OS-hGR cells (2 X 10^5 cells/well) were plated in 12-well tissue culture plates (Nunc, Roskilde, Denmark). COS-1 cells were transiently transfected with 1 µg per well of pCMV-HA-hGR using FuGENE 6 according to manufacturer's instructions (Roche, Basel, Switzerland) whereas U2OS-hGR cells remained untransfected as they contain stably transfected hGR. The cells were washed with prewarmed PBS (Sambrook *et al.*, 1989) (37°C) 48 hours post transfections and then cultured in DMEM plus P/S without fetal bovine serum. The cells were incubated for 1 hour, with or without 100 nM or 10 µM of test compounds. Subsequently, the cells were placed on ice and twice washed with ice-cold PBS (Sambrook et al., 1989) before being lysed in 200 µl lysis buffer (20 mM Tris (pH = 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate) containing phosphatase (1 mM Na_3VO_4, 10 mM NaF and 1 mM PMSF) and protease inhibitors (1 complete mini protease inhibitor cocktail tablet/10 ml, Roche, Basel, Switzerland) and shaken for 15 minutes on ice. Protein content of each sample was

determined by Bradford assay and the samples were boiled in 1 X SDS sample buffer (5 mL 10 % (v/v) SDS, 2 mL glycerol, 1 mL 1 M Tris (pH = 6.8), 0.5 mL mercaptoethanol, 1.5 mL autoclaved water, 0.1 % (w/v) bromophenolblue). Proteins (20 µg protein per lane) were separated on an 8 % resolving gel (4.6 mL autoclaved water, 2.7 mL 30 % (v/v) acrylamide, 2.5 mL 1.5 M Tris (pH = 8.8), 100 µl 10 % (v/v) SDS, 100 µl 10 % (w/v) ammonium persulfate, 6 µl TEMED), using Bio-Rad Protean III gel apparatus in 1 X SDS gel running buffer (200 mL 10 X running buffer and 20 mL 10 % (v/v) SDS/2L). Rainbow marker (1.5 µl) (Amersham Biosciences, Buckinghamshire, England) was also loaded on the gel. The separated proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, England) by electroblotting for 1 hour at 100 mV in 1X transfer buffer (200 mL 10 X running buffer and 200 mL methanol/2L) using the MINI protean III blotting systems (BioRad, Hercules, CA, USA). The membranes were blocked with blocking buffer (2 % (w/v) ECL Advance blocking agent (Amersham Biosciences, Buckinghamshire, England), 0.1 % (v/v) Tween and 10 mL 1 X TBS (Sambrook *et al*., 1989)) overnight at 4°C and then incubated for 1 hour with antibodies (1:10 000 dilution of anti-phospho-211 or 1:3000 dilution of anti-total-GR) at room temperature. Incubation with secondary antibody (anti-rabbit) was performed for 1 hour at room temperature at 1:10 000 dilution. All antibody dilutions were made in 2 % (w/v) ECL Advanced blocking agent (Amersham Biosciences, Buckinghamshire, England) and 10 mL 1 X TBS (Sambrook *et al*., 1989). The antibody incubations were followed by 3 X 5 min wash in TBS (Sambrook *et al.*, 1989) containing 0.1 % (v/v) Tween and 2 X 5 min washes in TBS (Sambrook *et al*., 1989) at room temperature. Specific protein-antibody complexes were visualised by the ECL advance Western blotting detection kit (Amersham Biosciences, Buckinghamshire, England), according to the protocol provided with the product. Briefly, equal volumes of ECL detection solution A and B were mixed, drizzled onto the membrane, after which the membrane was wrapped and exposed to Hyperfilm (Amersham Biosciences, Buckinghamshire, England) at room temperature. Western blots with the anti-phospho-211 GR antibody were performed first after which total GR levels were detected on the same blot after stripping as described previously (Ismaili *et al.*, 2005).

The intensity of the bands was quantified with photoimaging using a Kodak DC290 camera. The intensity of the phospho-211 band was normalised to the intensity of the corresponding total GR band. Fractional occupancy of GR for each test compound was calculated as [ligand]/ ([ligand] $+K_i$). The correlation analysis was conducted using linear regression, using

GraphPad prism software, where each independent experiment represents a replicate y value that was treated as an individual point. The correlation was calculated with two-tailed p-value and 95 % confidence interval.

2.9 Whole cell binding assays

Competitive whole cell binding assays were performed in transfected COS-1 cells using [1,2,4,6,7-^{3}H]-Dex (89 Ci/mmol, Amersham Biosciences, Buckinghamshire, England) as described in (Koubovec *et al.*, 2005). The cells were seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 5 X 10^4 cells/well in complete medium. On day two, the cells were transiently transfected with pCMV-HA-hGR, pCMV-HA-hGR$_{S203A}$, pCMV-HA-hGR$_{S211A}$, pCMV-HA-hGR$_{S226A}$, pCMV-hGR$_{M27T}$ (A-isoform) or pCMV-hGR$_{M1T}$ (B-isoform) (all with 0.375 µg DNA/well) and pSVβ-gal (0.15 µg DNA/well) using FuGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The cells were then incubated overnight in complete medium. On day three, the cells were washed three times with prewarmed (37° C) PBS then incubated for 90 minutes at 37°C with 5 nM [^{3}H]-Dex, in the absence or presence of 10 µM of unlabelled test compounds diluted in DMEM, in a final volume of 500 µl per well. Working on ice at 4°C, cells were washed three times with ice-cold PBS containing 0.2 % (w/v) BSA for 15 minutes. Cells were then lysed with 100 µl reporter lysis buffer (Promega, Madison, WI, USA) and frozen overnight. The next day the thawed suspension was added to scintillant and total binding was determined in a Beckman LS 3801 liquid scintillation counter. Specific bound [^{3}H]-Dex was calculated as the difference between total and non-specific binding, which was determined by incubating cells in the presence of [^{3}H]-Dex plus 500-fold excess unlabelled Dex. The β-galactosidase assay (Galacto-Star^{TM} assay system) was performed in a Veritas microplate luminometer and used to normalise for transfection efficacy. Specific binding was normalised to β-galactosidase activity. Complete displacement of [^{3}H]-Dex was achieved by unlabelled Dex, which was set as 100 %.

Ki for each ligand was determined by whole cell competition binding assays from previously obtained data performed in COS-1 cells transfected with pCMV-HA-hGR done in our lab. The K_i was calculated according to the Cheng-Prussoff equation: $K_i = EC_{50}/1 + ([ligand]/K_D)$, where the EC_{50} value is the value determined for unlabelled competing ligand, [ligand] is the

concentration of radioligand used (Dex) and K_D was determined from homologous competitive binding experiments.

2.10 Statistical analysis of experimental data

Statistical analyses were carried out with GraphPad Prism software, using one-way ANOVA with Dunnett (compares all columns versus control column) posttests. In most figures, statistical significance of differences is denoted by * $p<0.05$, ** $p<0.01$ or *** $p<0.001$, respectively. The correlation analyses were also carried out with GraphPad Prism software. A linear regression was calculated and a correlation analysis was performed to calculate the r^2 value.

CHAPTER 3

Results and discussion

Transcriptional activity and phosphorylation of the glucocorticoid receptor

3.1 Transcriptional activity of the panel of test compounds

The specific aim of this section is to determine the relative potency and efficacy for the panel of GR ligands in transactivation and transrepression on the same promoter-reporter construct in the cell system chosen. The relative potency and efficacy will be determined from classical sigmoidal dose-response curves. These data will be used for the correlations between the behaviour of liganded-GR at specific steps in the GR transcriptional regulation pathway and the potency and efficacy for the ligands in transactivation and transrepression throughout the project.

As far as possible, all the studies of the steps investigated in the project should be performed in the same cell system as the transactivation and transrepression studies, to exclude cell-specific effects. Therefore, the first aim was to identify an appropriate cell model where it would be possible to do most of the studies planned, including deciding on type and species of cell line, levels of endogenous GR and other steroid receptor expression, and sensitivity of the cells to steroids.

Preferably, a human cell line containing endogenous GR should be chosen to ensure physiological relevance. In addition, low levels of expression of other steroid receptors are crucial to avoid cross-reactivity. A cell system where it would be possible to transfect and express various receptor concentrations is also of interest, as it has become more evident in the literature that some ligands switch from partial to full agonist activity depending on the receptor level in the cell system (Simons, 2003; Zhao *et al.*, 2003).

As the main part of this thesis is to investigate phosphorylation of GR at Ser211, it was important to compare the results with the previous study done on phosphorylation at Ser211

by Wang et al. (Wang *et al.*, 2002). In that study a U2OS cell line stably transfected with the human GR was used and it was therefore decided to include studies with that cell line in this thesis as well.

The tyrosine aminotransferase (TAT) gene promoter was chosen as a model promoter for the transactivation studies. The TAT gene is a well characterised glucocorticoid-responsive liver-specific gene, which exhibits the most studied mechanism for transactivation where GR binds to simple GREs (Hashimoto *et al.*, 1984). The same promoter-reporter construct will be used in all the transactivation studies in different cell systems to exclude promoter-specific effects.

The interleukin-8 (IL-8) gene promoter was chosen as a model promoter for the transrepression studies. The IL-8 gene is a well-known inflammatory gene and the mechanism for transrepression of this gene is well studied. This involves a tethering mechanism (as described in section 1.6.3) where GR does not bind to DNA itself but interferes with other transcription factors such as NF-κB and AP-1 (Nissen and Yamamoto, 2000).

3.1.1 Transactivation assays in A549 cells

The A549 cell line was chosen as a model system because it is a human cell line (lung carcinoma) with significant levels of endogenous GR (Waters *et al.*, 2004), but no endogenous PR (Zhang *et al.*, 2000), MR or ER (Austin *et al.*, 2002) and low levels of the AR (Provost *et al.*, 2000). Also, the glucocorticoid responsive genes in this cell line have been identified and are well characterised (Wang *et al.*, 2004a). A preliminary panel of test compounds was decided on, including some agonists (Dex and Cort), partial agonists (Prog, MPA and NET-A), a SEGRA (CpdA) and an antagonist (RU486). The effect of this panel of test compounds on GR-dependent transcription using a synthetic promoter-reporter construct (pTAT-GRE-E1b-luc), containing two copies of a GRE from the TAT gene promoter, linked to a luciferase reporter gene, was analysed.

To compare the relative potencies and efficacies of the preliminary panel of test compounds for transactivation, A549 cells were transiently transfected with the synthetic promoter-reporter construct (pTAT-GRE-E1b-luc). Cells were then treated with increasing concentrations of the test compounds. Dose-response curves were analysed and the EC_{50} (potency) and maximal values (efficacy) for each test compound were determined (Table 3.1). Figure 3.1 shows a representative experiment.

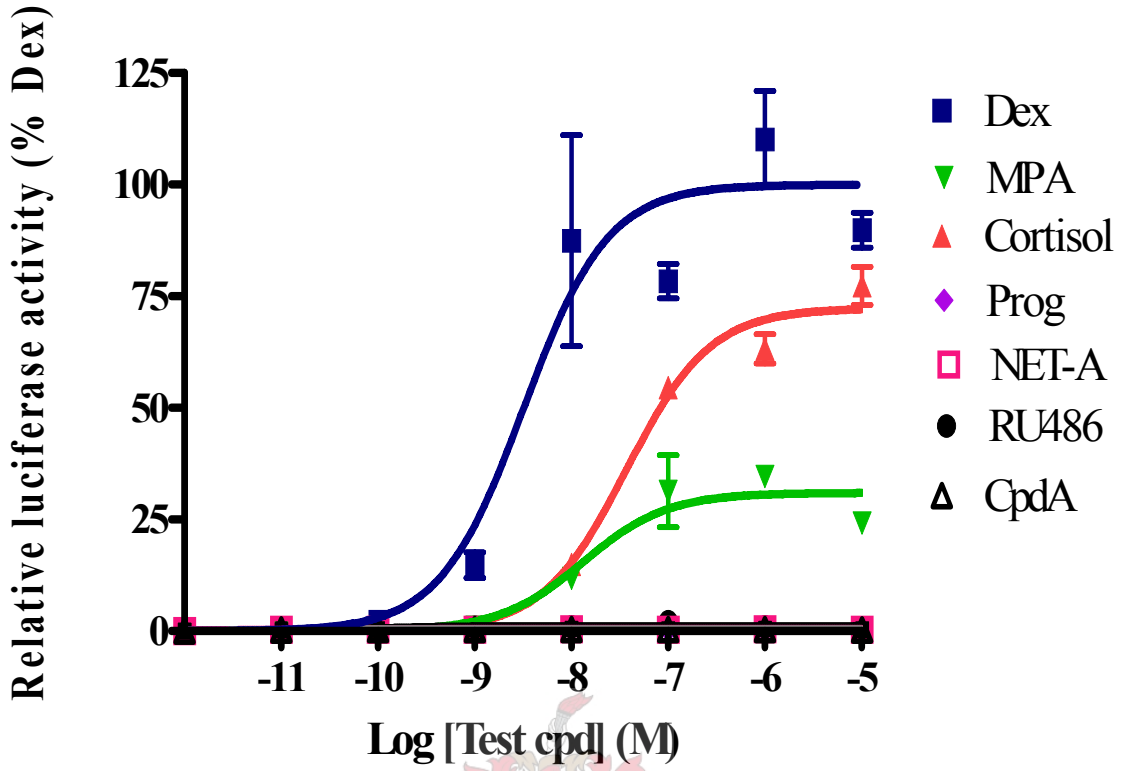


Figure 3.1. The effect of a preliminary panel of test compounds on GR-dependent transactivation in A549 cells. A549 cells were transfected with a pTAT-GRE-E1b-luc promoter-reporter construct and treated with various concentrations of test compounds. After 16 h, cells were harvested for luciferase assay. Experiments were performed three times. Graph shows results of one representative experiment, expressed as relative luciferase activity (Dex set as 100 %), and triplicates are plotted as mean ± SEM.

Table 3.1. Functional properties of test compounds on GR-dependent transactivation in A549 cells. Data shown are the mean EC_{50}, relative potency and relative efficacy values from two independent experiments presented as mean ± SD. The EC_{50} and relative efficacy were determined by GraphPad Prism software. The relative potency was calculated as described in Chapter 2. n/a: no ability to activate transcription.

Test compound	EC_{50} (nM)	Relative potency to Dex (%)	Relative efficacy to Dex (%)
Dexamethasone	2.77 ± 0.63	100	100
MPA	15.6 ± 3.40	18.6 ± 8.06	35.0 ± 5.65
Cortisol	74.2 ± 52.7	5.38 ± 4.66	83.5 ± 15.8
Progesterone	n/a	n/a	n/a
NET-A	n/a	n/a	n/a
RU486	n/a	n/a	n/a
Compound A	n/a	n/a	n/a

Results showed greatest potency for transactivation with Dex with an EC_{50} of 2.77 ± 0.63 nM, followed by MPA (EC_{50} = 15.6 ± 3.40 nM) with a relative potency of 18.6 ± 8.06 % as compared to Dex, followed by Cort (EC_{50} = 74.2 ± 52.7 nM) with a relative potency of 5.38 ± 4.66 % as compared to Dex. Cort behaves like a full agonist with a relative efficacy of 83.5 ± 15.8 % as compared to Dex while MPA behaves like a partial agonist with a relative efficacy of 35.0 ± 5.65 % as compared to Dex in this cell system. Prog, RU486, NET-A and CpdA showed no ability to activate in GR-dependent transcription, even at a concentration of 10 µM. These transactivation results are consistent with those obtained previously in A549 cells for Dex and Cort (Austin *et al.*, 2002; Jaffuel *et al.*, 2001; Webster *et al.*, 2002). It appears that no previous studies have investigated the agonist activity in GR-dependent transcription of the other test compounds in A549 cells.

As the EC_{50} for Dex in the A549 cells is quite high as compared to in other cell systems where the EC_{50} of Dex can be as low as 0.5 nM (Grossmann *et al.*, 2004), it would be difficult to obtain full dose response curves with less potent agonists when a larger panel of GR ligands is included. This is because there is a limit to the maximum concentration of ligand that can be added in aqueous solution, i.e. about 10 µM. Therefore, it was decided to switch from A549 cells to another cell line where it would be possible to transiently transfect different receptor concentrations to obtain an optimal EC_{50} for Dex. It is well established, that the level of GR expression, in addition to several other factors, determines the potency of Dex in GR-mediated gene activation, as described in the introduction (reviewed in Simons, 2003). A possible explanation for the high EC_{50} observed in A549 cells could thus be low levels of endogenous GR or coactivators.

3.1.2 Transactivation assays in COS-1 cells

COS-1 cells were also tested as a possible model system because the cells normally express low levels of endogenous GR (de Lange *et al.*, 1997) as well as other steroid receptors, and thus it is possible by transient transfections to vary the GR levels in the cells. The cells, in the absence of transfected GR, can also serve as a negative control to establish if the effects seen are mediated by GR. To ensure that the COS-1 cell system does indeed not contain interfering amounts of endogenous GR, the transactivation activity of Dex was measured in COS-1 cells that had not been transfected with the GR expression vector (pCMV-HA-hGR). About 7 % relative activity was seen for Dex in cells not transfected with receptor as compared to

transfected cells (Figure 3.2). Background due to endogenous receptors was also checked for all the other test compounds, except AL438 and D06, at saturating concentrations (10 µM) to determine if any other endogenous steroid receptors present in the cells would result in interfering amounts of luciferase activity (Figure 3.3). The results showed that the low backgrounds were insufficient to significantly change the values measured for the test compounds in response to expressed GR.

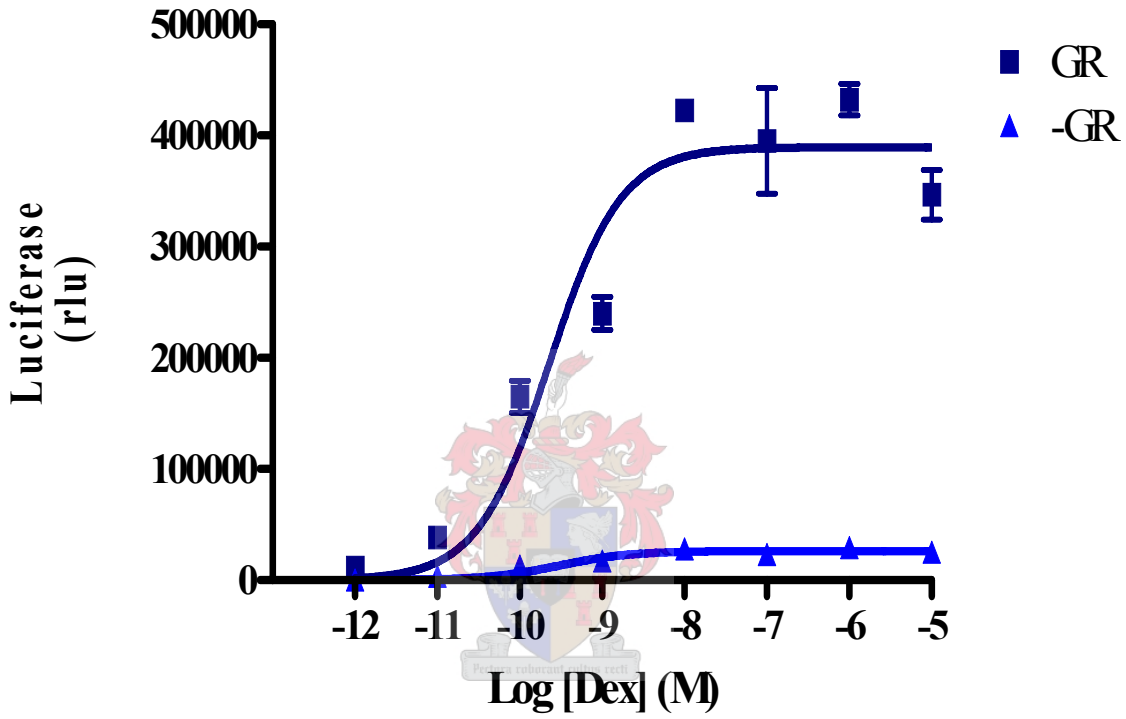


Figure 3.2. GR-dependent transactivation of transcription in COS-1 cells. COS-1 cells (2.5 X 10^4) were plated in a 24-well plate and transfected with 0.125 µg pCMV-HA-hGR or 0.125 µg empty vector, (pGL2-basic), and 0.047 µg pTAT-GRE-E1b-luc promoter-reporter construct. Thirty hours later the cells were treated with the indicated concentrations of Dex. After 16 h, the cells were collected for assay of luciferase activity. Results are plotted as relative light units. One experiment was performed and each point is in triplicate, represented by the mean ± SEM.

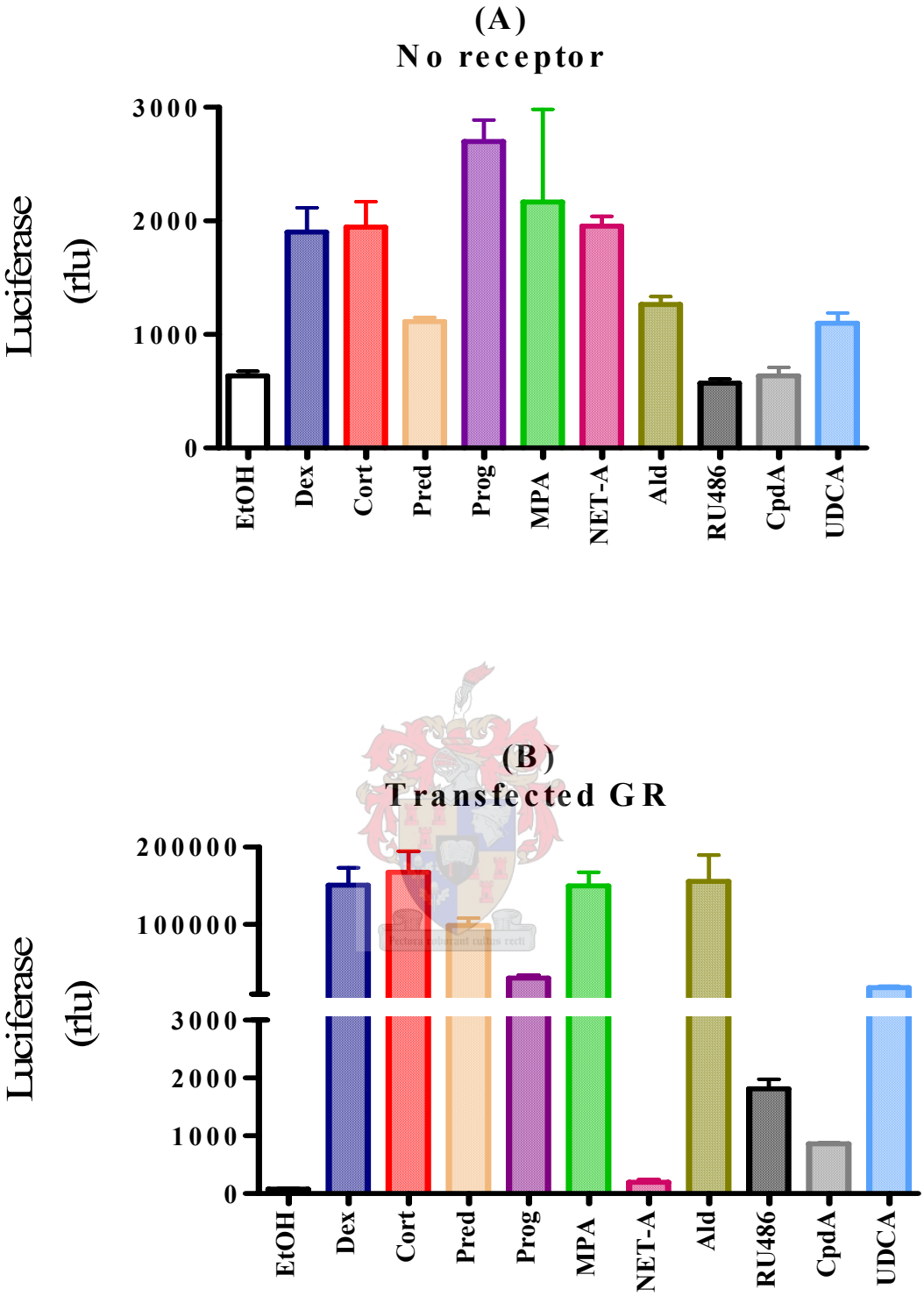
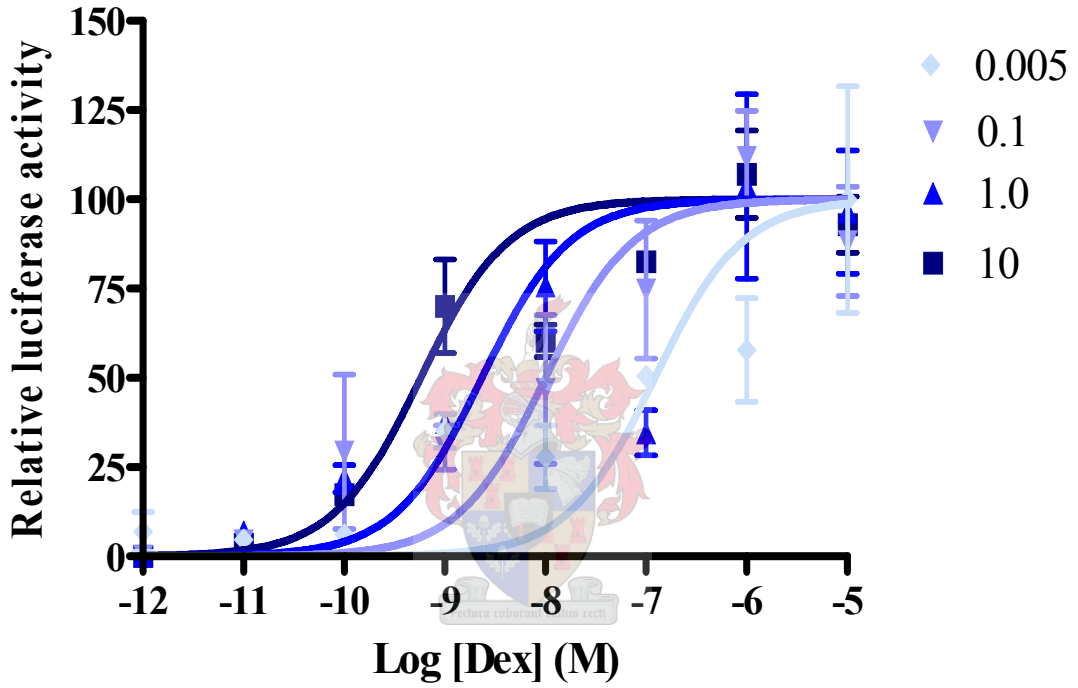


Figure 3.3. GR-dependent transactivation of transcription in COS-1 cells. COS-1 cells (2.5 X 10^4) were plated in 24-well plates and transfected with 0.047 µg pTAT-GRE-E1b-luc promoter-reporter construct plus (A) 0.125 µg empty vector, (pGL2-basic) or plus (B) 0.125 µg pCMV-HA-hGR. Thirty hours later the cells were treated with 10 µM of test compounds and after 16 h, the cells were collected for assay of luciferase activity. Results are plotted as relative light units. One experiment was performed and each point is in triplicate. Each point represents the mean ± SEM.

As mentioned in section 1.8, by increasing the concentration of the receptor, it is possible to shift the dose-response curve further to the left, which would lead to a bigger range to work in. To determine if changing the concentration of GR would affect the potency of Dex in activating transcription, COS-1 cells were co-transfected with the same amount of the synthetic promoter-reporter construct and varying amounts of GR expression vector (pCMV-HA-hGR). After transfections (30 h), cells were treated with different concentrations of Dex (Figure 3.4).



µg of GR transfected	0.005	0.1	1.0	10
Potency (EC_{50} in nM)	122	9.99	2.33	0.57
Relative potency to 10 µg GR (%)	0.47	5.71	24.5	100

Figure 3.4. Receptor concentrations determine potency of Dex in GR-mediated transactivation. COS-1 cells (2 X 10^6) were plated in a 10-cm dish and co-transfected with 3.75 µg of pTAT-GRE-E1b-luc promoter-reporter construct and different amounts of pCMV-HA-hGR with FuGENE 6. The total amount of DNA for each transfection is 13.75 µg following supplementation with different amounts of empty vector (pGL2-basic). Thirty hours later, the cells were treated with increasing amounts of Dex. After an additional 16 h, the cells were harvested for luciferase activity. The results were plotted as relative luciferase activity where the maximal response by Dex with 10 µg receptor transfected is set as 100 %. The results shown are from one experiment, where each point represents a triplicate. The EC_{50} was determined in GraphPad Prism software and the relative potency was calculated as described in Chapter 2.

As shown in Fig. 3.4, increasing the amount of transfected GR DNA enhances the potency of Dex in activating transcription in the model system. Increasing the amount of transfected GR DNA by 10-fold, decreases the EC_{50} for Dex by almost 4-fold. Similarly, the efficacy of Dex increases with increasing amounts of GR transfected into the cells (data not shown). As the dose-response curve was maximally shifted to the left when 10 μg of receptor was transfected per 2 X 10^6 cells, it was decided to use that ratio of receptor/cells for the remainder of the project.

After the initial pilot experiments, a larger panel of test compounds was included in the following experiment, extending the preliminary panel with an agonist (Pred), a partial agonist (Ald), two SEGRAs (AL438 and UDCA) and another antagonist (D06). The glucocorticoid potencies and efficacies in activation of transcription were evaluated for the bigger panel of test compounds using the same promoter-reporter construct, pTAT-GRE-E1b-luc, in COS-1 cells. Fig. 3.5 shows dose–response curves for all test compounds with agonist activity, and Table 3.2 shows the EC_{50} (potency) and maximal (efficacy) response determined from each curve in the transactivation assay.

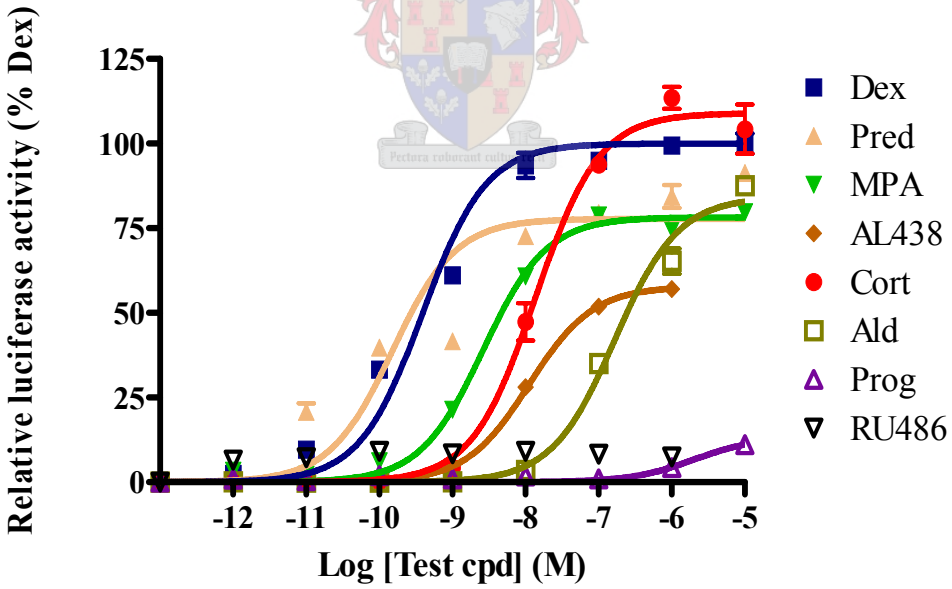


Figure 3.5. The effect of the panel of test compounds on GR-dependent transactivation in COS-1 cells. COS-1 cells were transfected with pCMV-HA-hGR and TAT-luciferase promoter-reporter construct (pTAT-GRE-E1b-luc). The following day, the cells were replated into 24-well plates and 6 hours later treated with the indicated concentrations of test compounds. After 16 h, the cells were collected to assay for luciferase activity. Experiments were performed three times. Graph shows results of one representative experiment where each point represents the mean ± SEM of triplicate. Data were plotted as relative luciferase activity, relative to the values obtained for Dex, where the response of Dex was set to 100 %.

Table 3.2. Functional properties of the test compounds on GR-dependent transactivation in COS-1 cells. Data are from three independent experiments and the mean EC_{50}, relative potency and relative efficacy are presented as mean ± SD. The EC_{50} and efficacy values were determined from the dose-response curves in GraphPad Prism software. The relative potencies were calculated as described in Chapter 2. Statistical analysis on the relative efficacy was done with Dunnett's multiple comparison test, comparing test compounds to Dex. n/a: no ability to activate transcription.

Test compound	EC_{50} (nM)	Relative potency to Dex (%)	Relative efficacy to Dex (%)	Statistical analysis of relative efficacy
Dexamethasone	0.228 ± 0.12	100.0	100.0	
Prednisolone	0.104 ± 0.06	225.5 ± 114	90.32 ± 11.0	
MPA	2.501 ± 0.42	12.06 ± 2.45	73.25 ± 7.08	*
AL438	8.559 ± 3.35	2.307 ± 1.10	67.35 ± 12.6	**
Cortisol	16.77 ± 12.2	2.415 ± 1.25	101.0 ± 16.8	
Aldosterone	138.5 ± 31.6	0.140 ± 0.08	90.65 ± 10.6	
Progesterone	1688 ± 498	0.019 ± 0.01	17.56 ± 15.0	**
RU486	n/a	n/a	8.385 ± 1.56[δ]	**
NET-A	n/a	n/a	n/a	
Compound A	n/a	n/a	n/a	
UDCA	n/a	n/a	n/a	
D06	n/a	n/a	n/a	

[δ] The relevance of this 8 % efficacy is unclear since it is observed even at 1 pM and RU486 exhibits no dose response, suggesting that the constant low level of activity is not due to GR-mediated effects. It thus appears that RU486 has zero GR-mediated agonist activity.

The order of relative transactivation potency of the different test compounds was Pred > Dex > MPA > AL438 = Cort > Ald > Prog > RU486. There was no evidence for agonist activity with NET-A, CpdA, UDCA and D06 (EC_{50} > 10 μM).

As expected, treatment with Dex induced GR transactivational activity in a dose-dependent manner in COS-1 cells with an EC_{50} of 0.228 ± 0.12 nM (n = 3) (Table 3.2), similar to what is seen in Figure 3.4, also with 10 μg transfected receptor.

Cort, Ald and Pred all behave like full agonists in this system, showing efficacies not significantly different from Dex ($p > 0.05$). Pred is the agonist with the highest potency (EC_{50}

= 0.104 ± 0.06 nM). The relative potency of cortisol and aldosterone, however, differed by nearly two (2.415 ± 1.25 %) and three (0.140 ± 0.08 %) orders of magnitude, respectively, from that of Dex.

The efficacies of MPA and AL438 are significantly different from that of Dex ($p < 0.05$ and $p < 0.01$, respectively) and thus they behave like partial agonists with 73.25 ± 7.08 % and 67.35 ± 12.6 % efficacy, respectively, relative to Dex. Their potencies are, however, much lower than that of Dex with MPA (EC_{50} of 2.501 ± 0.42 nM) displaying at 4 times higher potency than AL438 (EC_{50} of 8.559 ± 3.35 nM). An efficacy around 70 % illustrates that both MPA and AL438 have a high degree of agonist activity, albeit slightly lower than that of Dex in COS-1 cells transfected with a high amount of receptor.

Prog displays weak partial agonist activity in the system with a maximum efficacy of 17.56 ± 15.0 % as compared to Dex (significantly different, $p < 0.01$). Prog has the lowest potency (EC_{50} = 1688 ± 498 nM), only 0.019 ± 0.01 % as compared to Dex, of all the compounds displaying transactivational potency. RU486 is described as an antiglucocorticoid however it does exhibit significant agonist activity in certain cell systems. In this model system, RU486 has a very weak partial agonist activity with a relative efficacy of 8.385 ± 1.56 % as compared to Dex (significantly different, $p < 0.01$). No EC_{50} value could be determined for RU486. Already at 1 pM, RU486 is able to activate the reporter gene by 6-8 % even though this is not saturating concentrations of RU486 as the IC_{50} is 1.2 nM for RU486 in COS-1 cells transfected with human GR (data not shown), suggesting that the effect is not GR-specific.

It was decided to continue with COS-1 cells as the model cell system in the project as relative potency and efficacy data were obtained for transactivation for all the chosen test compounds in this system. This data will be used throughout the project for correlation analysis with the specific steps in the GR transcriptional regulation pathway that will be investigated.

3.1.3 Transactivation assays in U2OS-hGR cells

It was decided to also do transactivation assays in U2OS-hGR cells, since this would allow investigation of cell-specific effects, as well as direct comparison of transactivation results with phosphorylation results (section 3.2.2). Previous phosphorylation studies at Ser211 by Wang et al. were performed in these cells, stably transfected with the human GR wildtype receptor (pCMV-HA-hGR) (Wang *et al.*, 2002). The U2OS-hGR cells are a human

osteosarcoma cell line and the glucocorticoid responsive genes in the cell line have been studied (Rogatsky *et al.*, 2003). Again, the potency and efficacy in GR-mediated transactivation were determined for the panel of test compounds using the synthetic promoter-reporter construct, pTAT-GRE-E1b-luc. Figure 3.6 shows a representative experiment and Table 3.3 shows the calculated potency and efficacy data for the test compounds in GR-mediated transactivation in the U2OS-hGR cells.

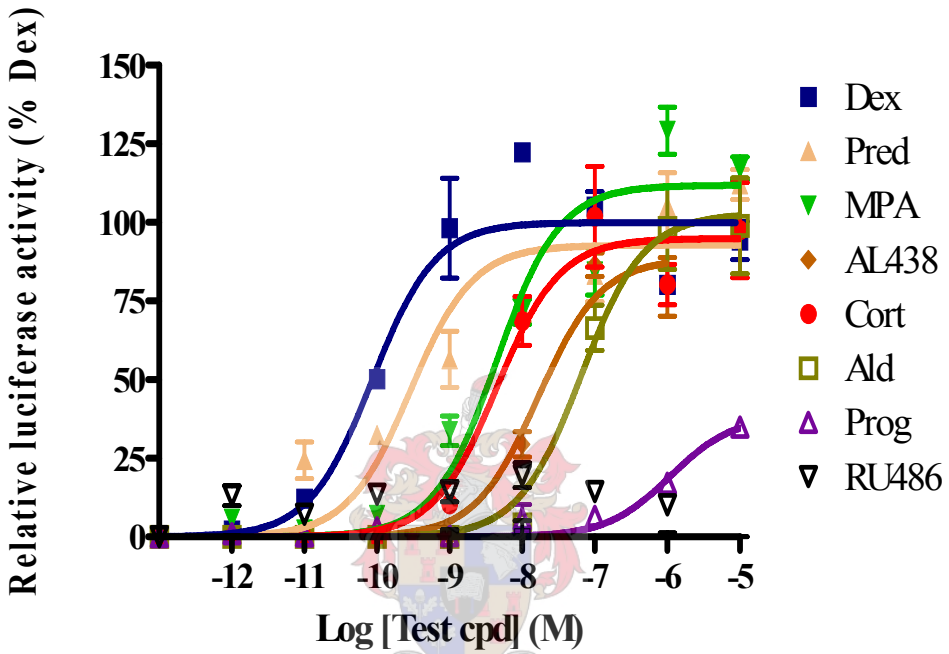


Figure 3.6. The effect of the panel of test compounds on GR-dependent transactivation in U2OS-hGR cells. U2OS-hGR cells were transfected with pTAT-GRE-E1b-luc promoter-reporter construct and treated with various concentrations of test compounds. After 16 h, cells were harvested for luciferase assay. Experiments were performed three times. Graph shows results of one representative experiment, expressed as relative luciferase activity (Dex set as 100 %), and triplicates are plotted as means ± SEM.

The order of relative transactivation potencies of the different test compounds was Dex > Pred > MPA > Cort = AL438 > Ald > Prog > RU486. There was no evidence for agonist activity with NET-A, CpdA, UDCA and D06 (EC_{50} > 10 µM). This is very similar to what was seen in COS-1 cells, except for the switch in potency between Dex and Pred. This may be attributed to cell-specific effects of Pred in GR-mediated transactivation.

Table 3.3. Functional properties of the test compounds on GR-dependent transactivation in U2OS-hGR cells. Data shown are the mean EC_{50}, relative potency and relative efficacy from three independent experiments presented as mean ± SD. The EC_{50} and relative efficacy were determined from the dose-response curves generated by GraphPad Prism software. The relative potency was calculated as described in Chapter 2. Statistical analysis on the relative efficacy was done with Dunnett's multiple comparison test, comparing test compounds to Dex. n/a: no ability to activate transcription.

Test compound	EC_{50} (nM)	Relative potency to Dex (%)	Relative efficacy to Dex (%)	Statistical analysis of relative efficacy
Dexamethasone	0.18 ± 0.09	100	100	
Prednisolone	0.28 ± 0.13	77.8 ± 54.9	98.2 ± 16.8	
MPA	3.63 ± 1.10	5.64 ± 3.37	127 ± 17.5	
Cortisol	10.9 ± 5.95	1.74 ± 0.18	112 ± 18.4	
AL438	14.6 ± 5.44	1.56 ± 1.34	96.9 ± 11.5	
Aldosterone	139 ± 126	0.18 ± 0.11	116 ± 14.1	
Progesterone	700 ± 350	0.04 ± 0.03	32.6 ± 8.19	**
RU486	n/a	n/a	12.3 ± 1.88[δ]	**
NET-A	n/a	n/a	n/a	
Compound A	n/a	n/a	n/a	
UDCA	n/a	n/a	n/a	
D06	n/a	n/a	n/a	

[δ] The relevance of this 12 % efficacy is unclear since it is observed even at 1 pM and RU486 exhibits no dose response, suggesting that the constant low level of activity is not due to GR-mediated effects. It thus appears that RU486 has zero GR-mediated agonist activity.

Dex was the most potent agonist with an EC_{50} of 0.18 ± 0.09 nM closely followed by Pred with an EC_{50} of 0.28 ± 0.13 nM and a relative potency of 77.8 ± 54.9 %. The EC_{50} of MPA was 3.63 ± 1.10 nM with a relative potency of 5.64 ± 3.37 %. Cort and AL438 had similar potencies. Cort has a relative potency of 1.74 ± 0.18 % (EC_{50} = 10.9 ± 5.95 nM) and AL438 a relative potency of 1.56 ± 1.34 % (EC_{50} = 14.6 ± 5.44 nM). Ald and Prog have low potencies relative to Dex, 0.18 ± 0.11 % (EC_{50} = 139 ± 126 nM) and 0.04 ± 0.03 % (EC_{50} = 700 ± 350 nM), respectively.

In contrast to what was found in the COS-1 cells, Dex, Pred (98.2 ± 16.8 %), MPA (127 ± 17.5 %), Cort (112 ± 18.4 %), AL438 (96.9 ± 11.5 %) and Ald (116 ± 14.1 %) all behaved

like full agonists relative to the efficacy of Dex in the U2OS-hGR cells ($p > 0.05$). Prog (32.6 ± 8.19 %) and RU486 (12.3 ± 1.88 %) were both weak partial agonists, however they exhibited significantly ($p < 0.01$) more partial agonist activity, relative to Dex, than in COS cells.

The consistent higher efficacy in GR-mediated transactivation in the U2OS-hGR cells compared to the COS-1 cells for all the test compounds with agonist activity suggested that the GR receptor level may be higher in the U2OS-hGR cells compared to transiently transfected COS-1 cells. This hypothesis was tested in the following experiment.

3.1.4 Expression of the glucocorticoid receptor in the cell lines

A Western blot analysis was performed to examine the levels of GR in each cell line used in the transactivation studies (Figure 3.7). The A549 cell line has a lower level of endogenous receptor than COS-1 cells transfected with 10 µg receptor expression vector. No endogenous GR was detected in untransfected COS cells. The expression of the 98 kDa receptor isoform is similar between the transfected COS-1 cells and the U2OS-hGR cells. However, the COS-1 cells express a more heterologous GR population, such that the total amount of GR expressed in the transfected COS cells is more than that found in the U20S-hGR cells. The two bands detected in transfected COS-1 cells are most likely the A- (98 kDa) and the B-isoforms (94 kDa) (see section 1.4.7), both of which bind ligand and are transcriptionally active. Thus the greater potency observed for ligands in the U20S-hGR cells is not due to higher GR expression levels, but most likely due to other cell-specific differences (see section 1.8).

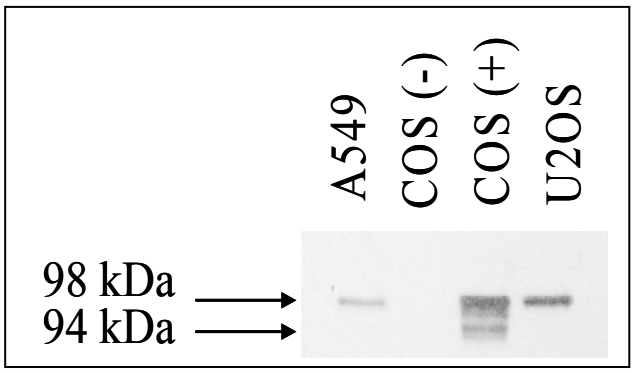


Figure 3.7. Western blot analysis of receptor levels in cell lines used for transactivation assays. Cell lysates were prepared from A549 cells, untransfected COS-1 cells (denoted COS (-)), COS-1 cells transfected with 10 µg pCMV-HA-hGR (denoted COS (+)) and U2OS-hGR cells, and 20 µg protein was resolved on an 8 % SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and examined by Western blot analysis with an antibody specific to GR wildtype (H-300). The sizes of the GR bands are denoted by arrows in the margin.

3.1.5 Transrepression assays in COS-1 cells

The effect of the preliminary panel of test compounds on GR-mediated transcriptional repression was studied in COS-1 cells to directly compare with the effects established in the transactivation assays for the same test compounds. COS-1 cells were co-transfected with a GR expression vector (pCMV-HA-hGR) and IL-8 promoter-reporter construct. The IL-8 gene promoter is activated by tumor necrosis factor (TNF) and repressed by glucocorticoids via GR interaction with AP-1 and NF-κB, two transcription factors binding to the IL-8 gene promoter (De Bosscher *et al.*, 2003). In the present study, TNF stimulation gave rise to about 1.7-fold induction of IL-8 mediated luciferase activity (Figure 3.8). This is an extremely low induction by TNF on this promoter compared to other data of a 50-fold induction (Harant *et al.*, 1996).

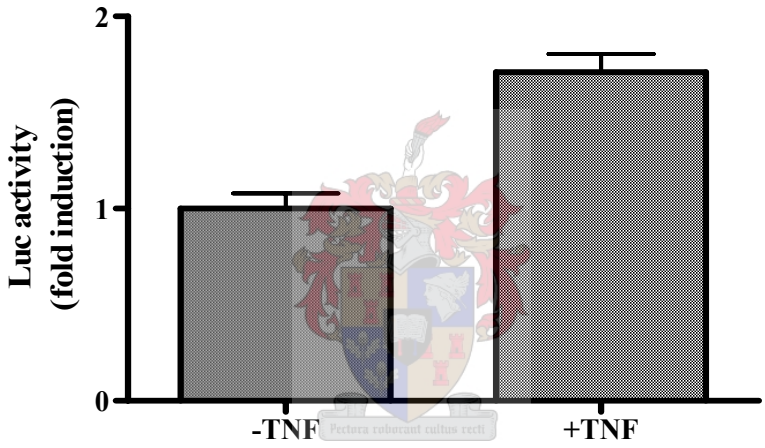


Figure 3.8. TNF induces the IL-8 promoter-reporter construct by about 1.7-fold induction. COS-1 cells were co-transfected with p546hIL-8luc+ and pCMV-HA-hGR. The following day, the cells were treated with 2000 IU/mL TNF or EtOH. After additional 6 h, the cells were harvested for luciferase assay. The results shown are fold induction of luciferase activity in transfected COS-1 cells following stimulation with TNF. The results shown are from one experiment performed in triplicate.

To compare the relative potencies and efficacies of the preliminary panel of test compounds for transrepression, COS-1 cells were transiently transfected with a human GR expression vector (pCMV-HA-hGR) and IL-8 promoter-reporter construct (p546hIL-8luc+). Cells were then treated with increasing concentrations of test compounds in the presence of TNF. To determine the relative potencies and efficacies between the test compounds, dose-response curves were analysed (Figure 3.9) and EC_{50} and maximal repression values for each test compound were determined (Table 3.4).

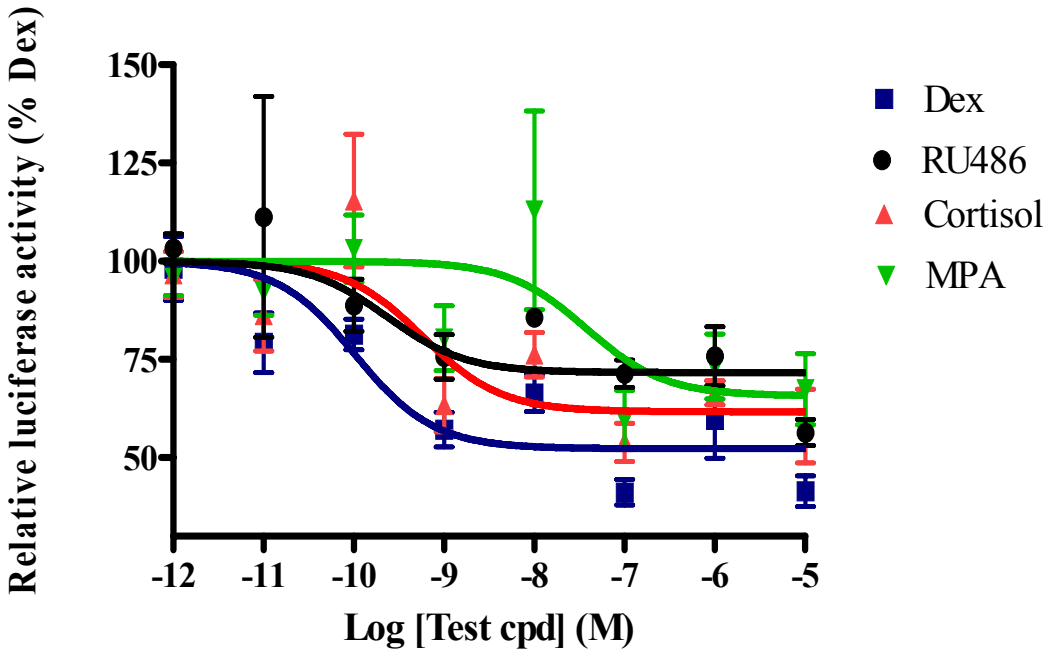


Figure 3.9. The effect of a preliminary panel of test compounds on GR-dependent transrepression in COS-1 cells. COS-1 cells were co-transfected with pCMV-HA-hGR and p546hIL-8luc+ promoter-reporter construct. Cells were incubated in the absence and presence of increasing concentrations of test compounds with or without 2000 IU/ml TNF. Test compounds were added two hours before TNF, for a total period of eight hours, after which cells were lysed and assayed for luciferase expression. Results shown are from one experiment, expressed as relative luciferase activity (Dex set as 100 %, calculations described in Chapter 2) and each condition is performed in triplicate. Prog, NET-A and CpdA data are not shown as no agonist activity was seen for these test compounds.

Table 3.4 Functional properties of test compounds in GR-dependent transrepression in COS-1 cells. Data shown are EC_{50} and relative efficacy determined by GraphPad Prism software from one experiment. The relative potency was calculated as described in chapter 2. n/a: no activity for repression of transcription.

Test compound	EC_{50} (nM)	Relative potency to Dex (%)	Relative efficacy to Dex (%)
Dexamethasone	0.10	100	100
RU486	0.23	43.5	72.9
Cortisol	0.58	17.2	84.8
MPA	37.7	0.27	79.6
Progesterone	n/a	n/a	n/a
NET-A	n/a	n/a	n/a
Compound A	n/a	n/a	n/a

As expected, Dex is a potent agonist in GR-mediated repression with an EC_{50} of 0.1 nM along with Cort with an EC_{50} of 0.58 nM with an efficacy of 84.8 % compared to Dex. RU486 had a relative potency of 43.5 % compared to Dex with an EC_{50} of 0.23 nM, behaving like an agonist with a relative efficacy of 72.9 % compared to Dex. MPA was a less potent agonist with an EC_{50} of 37.7 nM and a relative efficacy of 79.6 % compared to Dex. No agonist activity was detected for Prog, NET-A or CpdA in GR-mediated transrepression in COS-1 cells.

Zhao et al. have previously shown that receptor concentrations affect the potency and efficacy in Dex-mediated transrepression from different promoter-reporter constructs (containing 5 X NF-κB and 7 X AP-1 binding sites) in COS- 7 cells. At high receptor concentrations, the EC_{50} value for Dex was 0.1 nM (Zhao *et al.*, 2003). Since the same potency was achieved for Dex in the COS-1 studies presented here, it suggests that the density of the receptor is high in this study. This is consistent with the extent of agonist activity obtained for MPA and RU486. As for transactivation, it has been shown for RU486 and MPA that a switch occurs between partial agonist and antagonist activity for these ligands, depending on the receptor concentration in transrepression. Again, Zhao et al. showed that RU486 behaves like a full agonist with an efficacy of 80 % compared to Dex in GR-mediated transrepression in COS-7 cells at high receptor concentrations (Zhao *et al.*, 2003) and likewise for MPA, Zhao et al. showed that MPA behaves like a full agonist at high receptor concentrations with similar efficacy compared to Dex in COS-7 cells (Zhao *et al.*, 2003).

Some of the results in Fig 3.9 appear to differ with that of the published literature. As Prog has shown varying ability to induce transrepression in different cell systems (described in section 1.9.2.1), the lack of agonist activity in this study by Prog on gene repression at first appears not to be surprising. However, given the high agonist activity of MPA and RU486 in this system, indicative of high GR density, it would be expected that Prog, a partial GR agonist, would display more partial agonist activity in this system. It appears that only one study has investigated the glucocorticoid properties of NET-A in transrepression via GR, where it was found that NET-A acts as a weak partial agonist (Koubovec *et al.*, 2005). In the present study, NET-A shows no agonist activity in GR-mediated transrepression on the IL-8 promoter. CpdA has previously been shown to have agonist activity in repressing the NF-κB mediated gene transcription of the IL-8 promoter (De Bosscher *et al*. 2005) however in this study on the same IL-8 promoter no agonist activity was detected for CpdA. The reasons for

these apparent differences are most likely due to the low sensitivity and large errors of the repression assay in the present study.

It was found to be difficult to establish reproducible and accurate data in the COS-1 cells transfected with GR and the IL-8 promoter-reporter constructs, due to low levels of TNF-induced expression in the system. The reasons for these low levels are most likely due to low levels of components of the appropriate signaling pathway in these cells. As Dex only inhibited the TNF-induced expression of IL-8 by 50 %, the range of the actual luciferase values that were obtained in the experiments was very small. For example, if the basal expression of the promoter-reporter construct is 1000 relative light units (rlu) and the TNF induction is 1.7-fold (as in this study), the maximal rlu will be 1700. Then Dex inhibits 50 % which will give rlu of 1350, so the working range is very narrow. If the TNF induction would be 50-fold (as in some studies), the maximal rlu would be 50 000 and Dex inhibition would give readings of 25 000, increasing the working range significantly. As the error within the triplicates for each point on the graph was sometimes more than 350 rlu, thereby ranging from full inhibition to zero inhibition within a triplicate, it was impossible to get accurate data.

3.2 Correlation between phosphorylation and transactivation

It was initially believed that phosphorylation of GR was responsible for the active state of the receptor since it was discovered that multiple GR residues, all within the AF-1 domain, were phosphorylated upon hormone treatment (see section 1.5.4). Only one published study has previously examined the effect of GR ligands on phosphorylation at Ser211 in U2OS-hGR cells, where the effects of different GR agonists (Dex, Pred and fluocinolone) and antagonists (RU486 and ZK299) was examined (Wang *et al.*, 2002). These authors investigated the extent of phosphorylation at Ser211 induced by 100 nM ligand for one hour, as compared to the extent of luciferase activity in GR-mediated transactivation from a MMTV promoter-reporter construct at 100 nM for one hour. They showed that phosphorylation at Ser211 induced by the agonists was greater than phosphorylation induced by antagonists. Therefore, they proposed that the extent of phosphorylation at Ser211 induced by a ligand might correlate with the activity of the ligand for transactivation (Wang *et al.*, 2002).

The main aim of this thesis is to test the hypothesis that a correlation exists between the extent of phosphorylation induced by ligands at Ser211 and the potency and efficacy of GR ligands

for transactivation by using the same antibodies as Wang et al. that are specific for GR phosphorylated at Ser211 (Wang *et al.*, 2002). The panel of test compounds has been extended, to include a much broader range of types of ligands, including three full agonists (Dex, Cort, and Pred), four partial agonists (MPA, NET-A, Ald and Prog), two antagonists (RU486 and DO6) and three possible SEGRAs (CpdA, UDCA and AL438) than that used by Wang *et al.* and the test compounds will be investigated using both 100 nM (subsaturating concentration for most ligands), and 10 µM concentrations (saturating for all ligands). The rationale for using the two different concentrations of ligand was to be able to detect possible differences in phosphorylation that may only be apparent at either saturating or subsaturating concentrations. The data from the phosphorylation studies will be correlated with the potency and efficacy of the test compounds in transactivation of the synthetic TAT promoter-reporter construct, determined from full dose-response curves in section 3.1. Another aspect that will be investigated is whether the correlation holds for both COS-1 cells transiently transfected with hGR, as well as in U2OS-hGR cells containing stably transfected hGR.

3.2.1 Effects in COS-1 cells

The site-specific antibody used in this study was raised against the 202-215 residues, containing a phosphorylated Ser211, of the hGR in order to study the phosphorylation of Ser211. A mutant receptor (S211A hGR) unable to phosphorylate at the specific site was used to control the specificity of the antibody (Figure 3.10) (Wang *et al.*, 2002). The anti-phospho-Ser211 antibody recognises the wildtype GR, but not the mutant GR, in extracts from transfected cells treated with Dex. Thus, the specificity of the antibody is high. An antibody that recognises the total amount of GR independent of its phosphorylation status is used to control for levels of total receptor. A basal level of phosphorylation is recognised with the anti-phospho-Ser211 GR antibody when cells are treated with EtOH (see Figure 3.11).

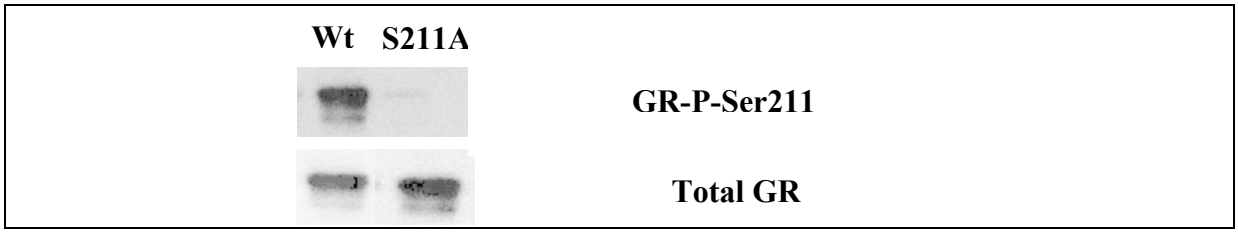


Figure 3.10. The specificity of the GR-P-Ser211 Ab. The Western blot shows human GR detected with GR-P-Ser211 antibody and total GR antibody. Whole cell extracts were prepared from COS-1 cells transfected with either pCMV-HA-hGR or pCMV-HA-hGR$_{S211A}$ treated with 10 µM Dex for 1 hour. Equal amounts of protein (20 µg) of each sample was separated by an 8 % SDS-PAGE gel, transferred to a nitrocellulose membrane and was analysed by immunoblotting with anti-phospho-Ser-211 or antibody recognising total GR.

Next, the phosphorylation of GR at Ser211 in response to the panel of test compounds was investigated in COS-1 cells transiently transfected with pCMV-HA-hGR and stimulated with 100 nM or 10 μM of each test compound for 1 hour (Figure 3.11). When analysing the data with 100 nM test compound, it is necessary to normalise for the different fractional occupancy of the receptor by the different ligands. The fractional occupancy (equation in Figure 1.6) was calculated by using theoretical K_i values determined in whole cell binding assays in COS-1 cells (Table 3.5). For example at 100 nM, Ald only occupies 42 % of the receptor molecules whereas Dex occupies 97 %. Hence, the blots were quantified and the amount of phosphorylation induced at Ser211 was normalised to the total GR level, as a loading control. Then the normalised data were expressed either as GR phosphorylated at Ser211 as a percentage of total GR (ligand bound plus unbound) or GR phosphorylated at Ser211 as a percentage of ligand-bound GR only (Figure 3.12).

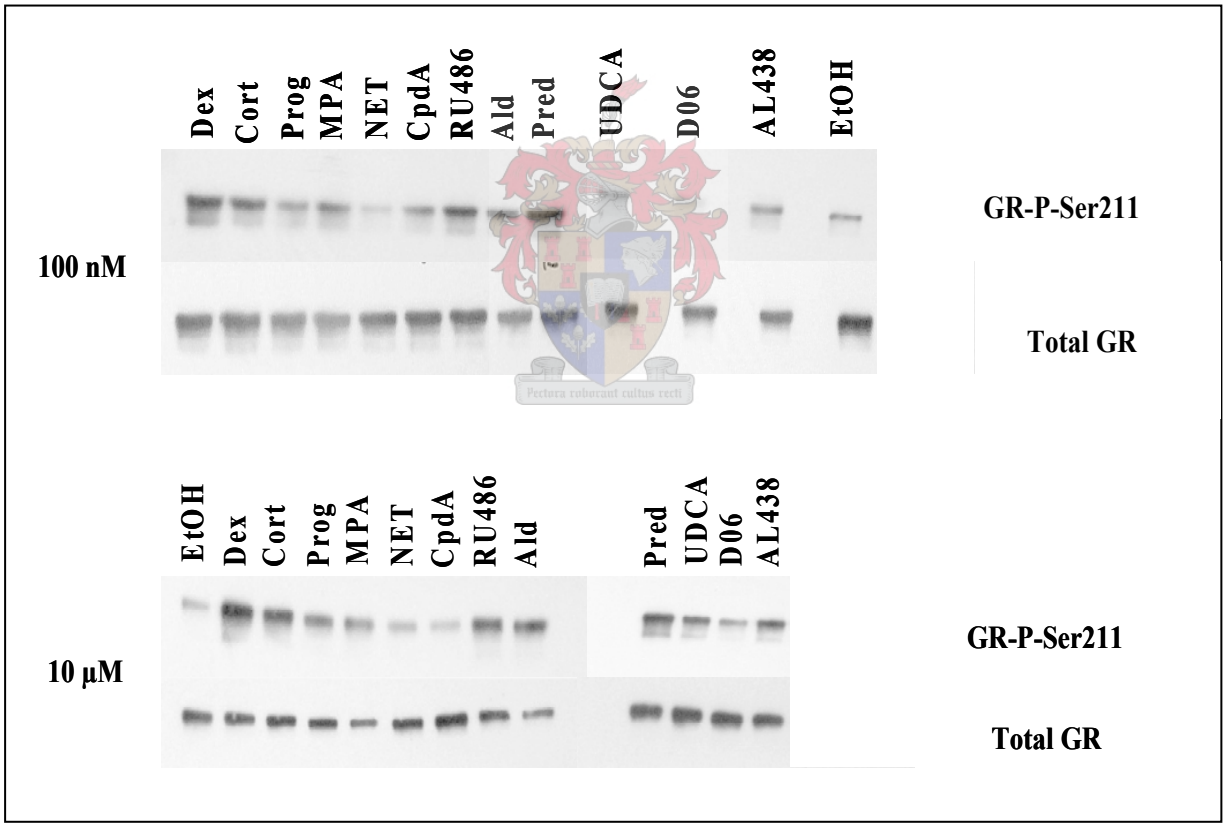


Figure 3.11. The effect of the panel of test compounds on GR phosphorylation at Ser211 in COS-1 cells. COS-1 cells were transfected with wildtype GR (pCMV-HA-hGR) and treated with ethanol or the test compounds indicated at 100 nM or 10 μM for one hour. Whole cell extracts were prepared. Equal amounts of protein (20 μg) from each sample were analysed by Western blot with anti-phospho-Ser211 antibody or an antibody to detect total GR. The data shown are from a single experiment representative of at least three independent experiments for 10 μM test compound.

Table 3.5. The K_i and fractional occupancy of the receptor for all test compounds. The theoretical K_i values were calculated from the IC_{50} values determined in whole cell binding assays in COS-1 cells with transfected human GR (performed by Dr. K. Ronacher, data not shown). The K_D value of 3 nM for Dex and the concentration of radiolabelled Dex of 20 nM (from homologous displacement curves) were used in the calculation of Ki values (see section 2.9). The fractional occupancy was calculated with the equation given in Figure 1.6.

Test compound	K_i (nM)	Fractional occupancy (%) at 100 nM	Fractional occupancy (%) at 10 µM
Dexamethasone	3.0	97.09	99.97
Cortisol	19.8	83.47	99.80
Prednisolone	9.1	91.66	99.91
Progesterone	36.1	73.48	99.64
MPA	2.4	97.65	99.98
NET-A	186.1	34.95	98.17
Aldosterone	138.0	42.02	98.64
RU486	1.2	98.81	99.99
D06	2419.0	3.97	80.52
AL438	7.5	93.02	99.93
Compound A	n/a	n/a	n/a
UDCA	n/a	n/a	n/a

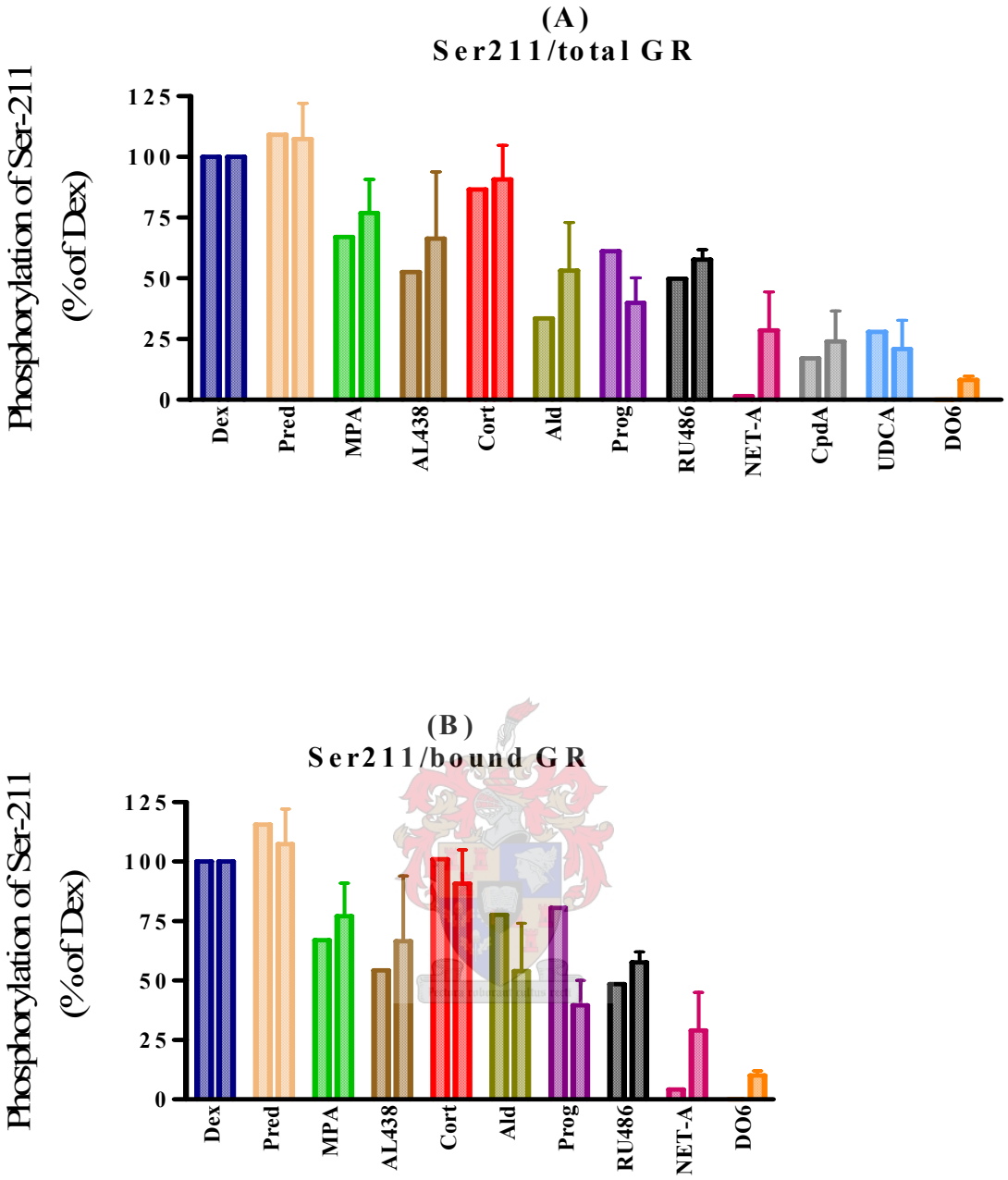


Figure 3.12. The phosphorylation pattern at Ser211 of the human GR by the panel of test compounds in COS-1 cells. The amount of phosphorylation induced by different test compounds at Ser211 on the human GR was quantified and normalised against total GR loaded, Thereafter the results were expressed relative to total GR (A) or fractional occupancy (B) of the test compounds for the receptor. The basal phosphorylation (EtOH control) was subtracted from each test compound. The phosphorylation induced by Dex was set as 100 % and the phosphorylation induced by the other test compounds was calculated relative to Dex. Two concentrations of test compounds were investigated, 100 nM (first bar for each test compound) and 10 µM (second bar). The results with 100 nM test compounds are from one experiment while the results with 10 µM test compounds are from three independent experiments. CpdA and UDCA are not able to fully displace [^{3}H]-Dex from GR so no binding affinity has been determined for these two test compounds, therefore no results are plotted for CpdA and UDCA induced phosphorylation normalised with bound GR.

The potent agonists, Dex, Pred and Cort, induced the highest levels of Ser211 GR phosphorylation for both 100 nM and 10 μM (Figure 3.12). Treatment with Ald leads to substantial phosphorylation of GR at Ser211 although levels were lower than for other full agonists, despite the fact that Ald showed full agonist activity in transactivation assays. The partial/full agonists MPA and AL438 are equally potent in phosphorylation as compared to Ald. Treatment with the partial weak agonist, Prog and antagonist RU486, resulted in considerable phosphorylation, though lower than the full and strong partial agonists, whereas NET-A, CpdA, UDCA and D06 induced minimal phosphorylation at both 100 nM and 10 μM test compound, when compared to untreated cells. The rank order seen in phosphorylation induced at Ser211 by the different ligands is essentially maintained at both 10 μM and 100 nM. Interestingly, however, there are subtle differences, especially for ligands with lower affinity for the receptor, such as Ald, MPA, Prog and NET-A. Ald and Prog induce strong phosphorylation at 100 nM (75 %) and only about 50 % at 10 μM. MPA, however, induces less phosphorylation than both Ald and Prog at 100 nM, but significantly more at 10 μM (75 %). Both NET-A and D06 induce low levels of phosphorylation at 100 nM, but NET-A induces significantly more phosphorylation at 10 μM compared to D06. The significance of these differences between 100 nM and 10 μM for the test compounds is uncertain as only one experiment has been performed with 100 nM test compound. These 100 nM experiments need to be repeated and verified.

To investigate if a correlation exists between phosphorylation at Ser211 and transactivation potency and efficacy by the panel of test compounds, correlation analyses were performed in GraphPad Prism software (Figures 3.13 and 3.14). The analyses for 10 μM of test compounds indicate a strong correlation between the extent of phosphorylation at Ser211 induced by all test compounds, for both potency ($r^2 = 0.85$, $p < 0.0001$) and efficacy ($r^2 = 0.78$, $p < 0.0002$) (Figure 3.13). A slightly weaker correlation is observed for 100 nM of test compounds, where the correlation with potency is $r^2 = 0.61$ ($p < 0.0078$) and for efficacy is $r^2 = 0.67$ ($p < 0.0038$) (Figure 3.14). Note that since only one experiment was performed at 100nM, as apposed to three experiments at 10 μM, the results for the 100 nM correlation are most likely less accurate. However, they are informative in comparing overall trends (Table 3.6-3.8). The correlation analysis performed after excluding certain subgroups did not substantially change the correlation coefficient, supporting the conclusion that a good correlation exists for all the test compounds regardless of type of biological activity.

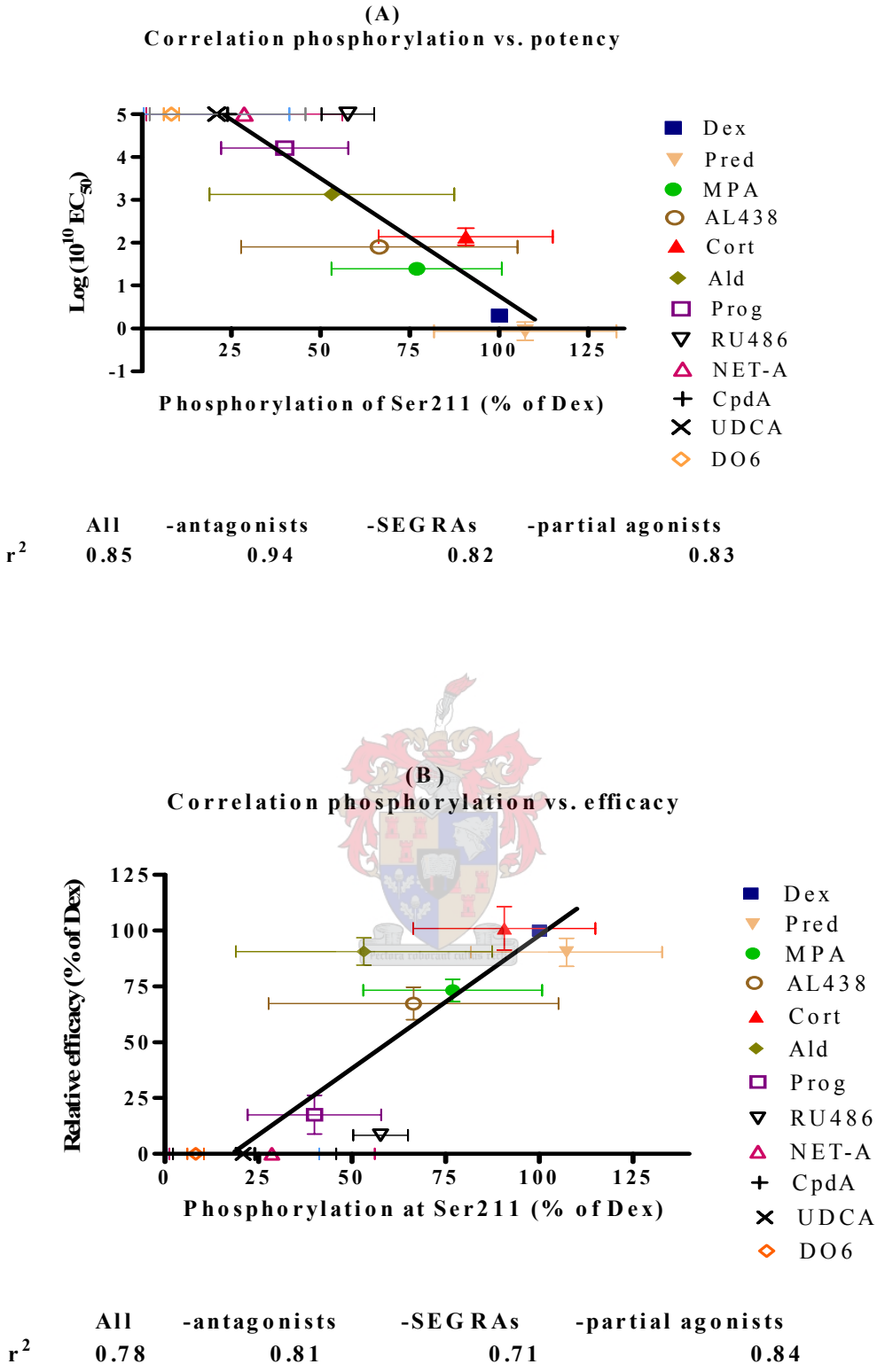
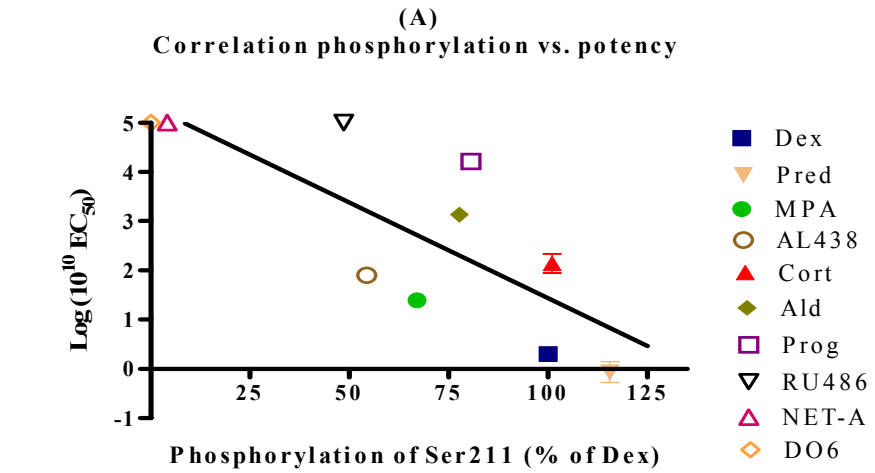
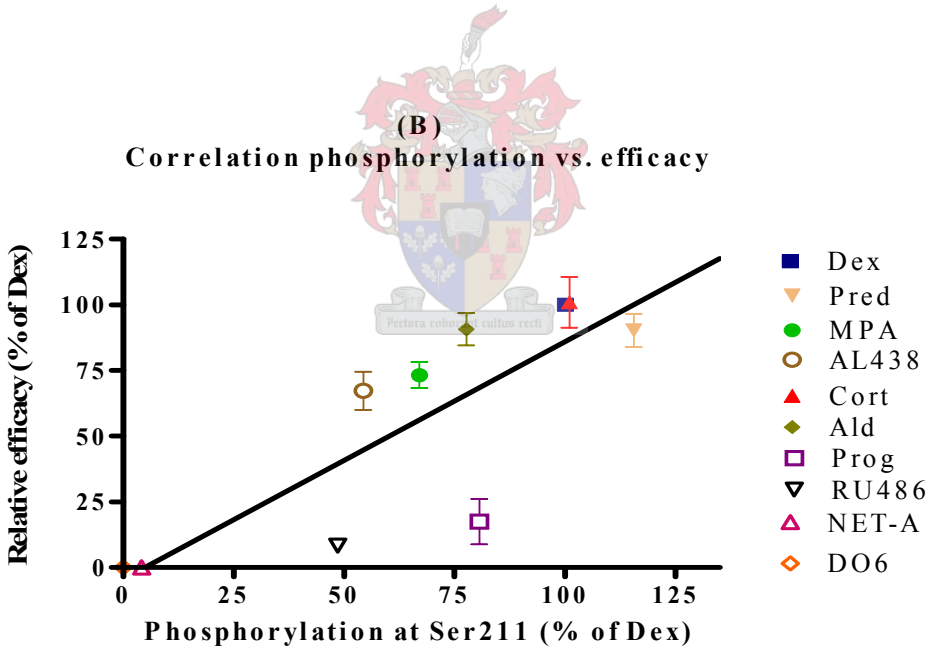


Figure 3.13. Correlation between phosphorylation at Ser211 and potency and efficacy for transactivation for the panel of test compounds at 10 µM in COS-1 cells. The graphs show data from phosphorylation studies at Ser211 with 10 µM performed in COS-1 cells (three independent experiments) and data from transactivation studies in COS-1 cells (three independent experiments) correlated for 12 different test compounds. Phosphorylation is correlated with potency (A) and relative efficacy (B) for transactivation.



	All	-antagonists	-SEGRAs	-partial agonists
r^2	0.61	0.52	0.65	0.74



	All	-antagonists	-SEGRAs	-partial agonists
r^2	0.67	0.60	0.72	0.82

Figure 3.14. Correlation between phosphorylation at Ser211 and potency and efficacy for transactivation for the panel of test compounds at 100 nM in COS-1 cells. The graphs show data from phosphorylation studies at Ser211 at 100 nM performed in COS-1 cells (one experiment) and data from transactivation studies in COS-1 cells (three independent experiments) correlated for 12 different test compounds. Phosphorylation is correlated with potency (A) and relative efficacy (B) for transactivation.

3.2.2 Effects in U2OS-hGR cells

Phosphorylation studies were also performed in U2OS-hGR cells stably transfected with human GR as the previous work by Wang et al. was done in this cell line (Wang *et al.*, 2002). The same panel of test compounds was investigated for their ability to induce phosphorylation at Ser211. The U2OS-hGR cells were stimulated with 100 nM (one experiment) or 10 µM (three independent experiments) of each test compound for 1 hour (Figure 3.15).

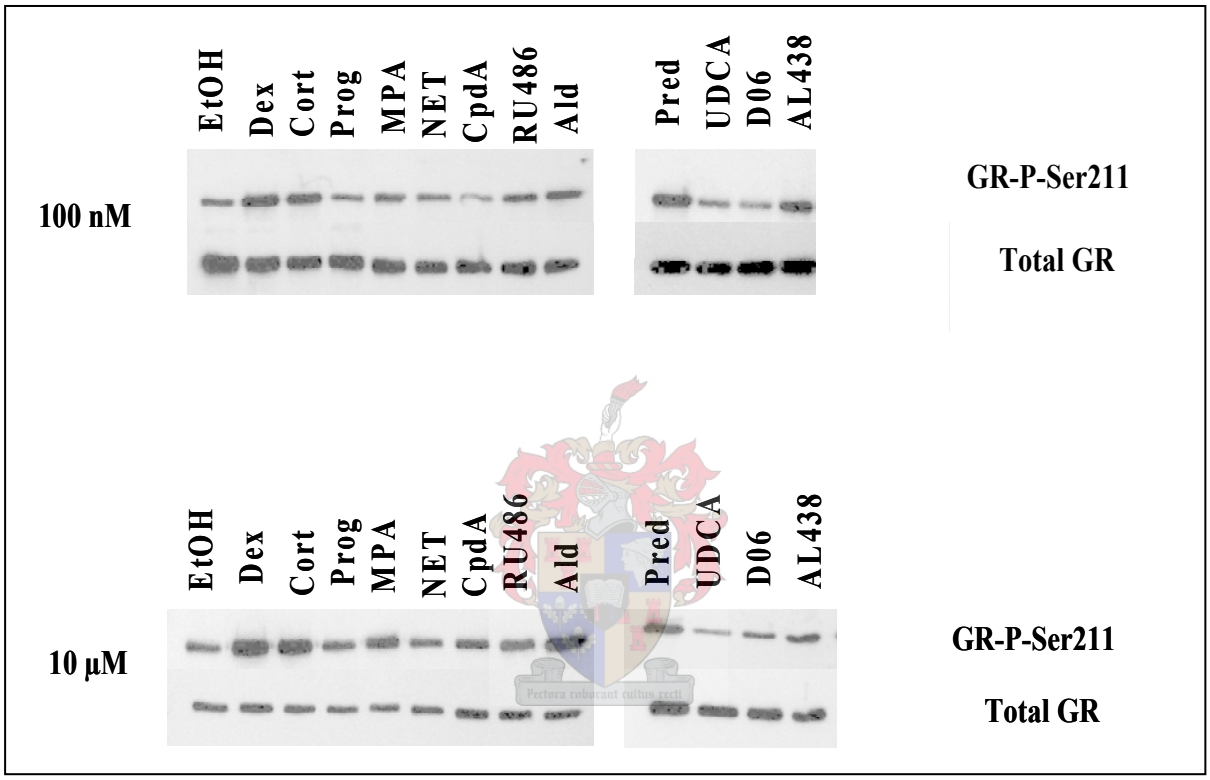


Figure 3.15. The effect of the panel of test compounds on GR phosphorylation at Ser211 in U2OS-hGR cells. U2OS-hGR cells were stimulated with 100 nM or 10 µM of each test compound for 1 hour. Whole cell extracts were prepared and equal amounts of protein (20 µg) from each sample was analysed by Western blot and probed with anti-phospho-Ser211 antibody and total GR antibody. The data shown are from a single experiment representative of at least three independent experiments for 10 µM test compound.

The Western blots were quantified and the amount of phosphorylation induced at Ser211 was normalised to the total GR level, as a loading control. Then the normalised data were expressed either as GR phosphorylated at Ser211 as a percentage of total GR (ligand bound plus unbound) or GR phosphorylated at Ser211 as a percentage of ligand-bound GR only (Figure 3.16). The highest induction of phosphorylation at Ser211 is seen with Dex, Pred, Cort and Ald, followed by MPA, AL438 and RU486. Prog, NET-A, CpdA, UDCA and D06 all induce minimal phosphorylation at both 100 nM and 10 µM (Figure 3.16). Interestingly, MPA induces only about 60 % phosphorylation as compared to Dex, despite full agonist

activity in the U20S-hGR transactivation assays, whereas RU486 induces about 50 % phosphorylation of the receptor at Ser211, despite minimal agonist activity in the U20S-hGR transactivation assays. When comparing the results for 10 μM and 100 nM in U20S-hGR cells, it can be seen that the rank order for the test compounds is similar, but subtle differences are apparent, especially for ligands with lower affinity for the receptor, such as NET-A and D06. NET-A induces strong phosphorylation at 100 nM compared to 10 μM. D06 induces relatively high levels of phosphorylation (~ 40 %) at 10 μM, as compared to Dex, despite no agonist activity in the transactivation assays. As mentioned, only one experiment was performed at 100 nM with the test compounds and also one experiment was performed with D06 at 10 μM, so these experiments need to be repeated and verified.

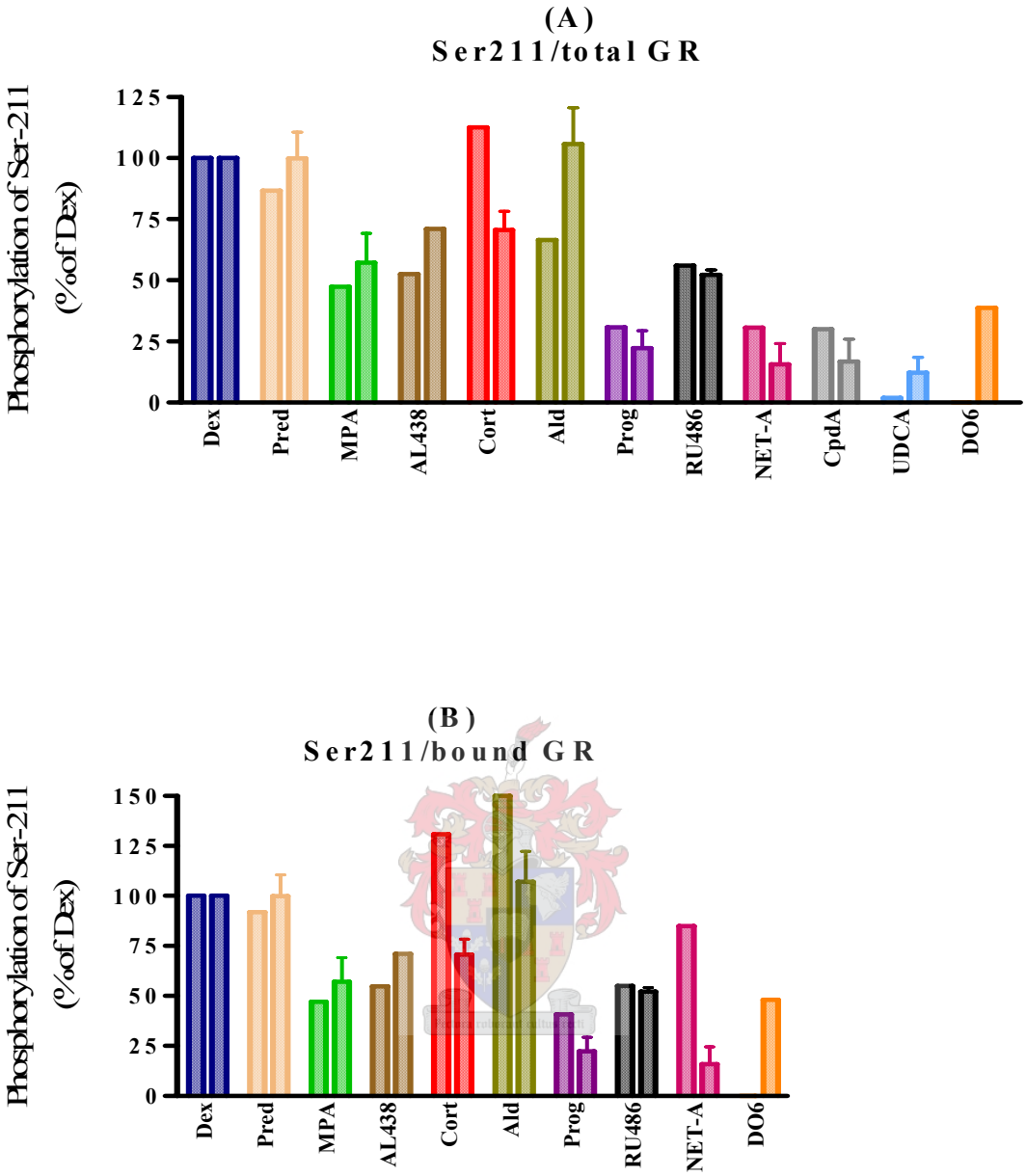
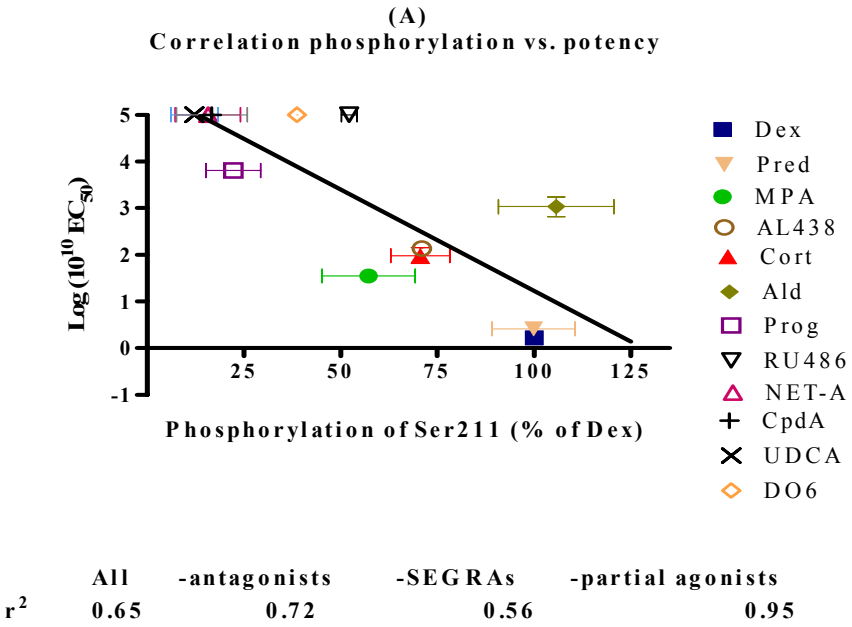


Figure 3.16. The phosphorylation pattern at Ser211 of the human GR induced by the panel of test compounds in U2OS-hGR cells. The amount of phosphorylation induced by different test compounds at Ser211 on the human GR in U2OS-hGR cells was quantified and normalised for total GR, and results were expressed relative to total GR (bound and unbound) (A) or to only bound-GR (B). The basal phosphorylation (EtOH control) was subtracted from each test compound. The phosphorylation induced by Dex was set as 100 % and the phosphorylation induced by the other test compounds was calculated relative to Dex. Two concentrations of test compounds were investigated, 100 nM (first bar for each test compound) and 10 µM (second bar). The results with 100 nM test compounds are from one experiment while the results with the 10 µM test compounds are from three independent experiments, except for AL438 and D06 which are from one experiment CpdA and UDCA are not able to fully displace [^{3} H]-Dex from GR so no binding affinity has been determined for these two test compounds, therefore no results are plotted for CpdA and UDCA induced phosphorylation normalised with bound GR.

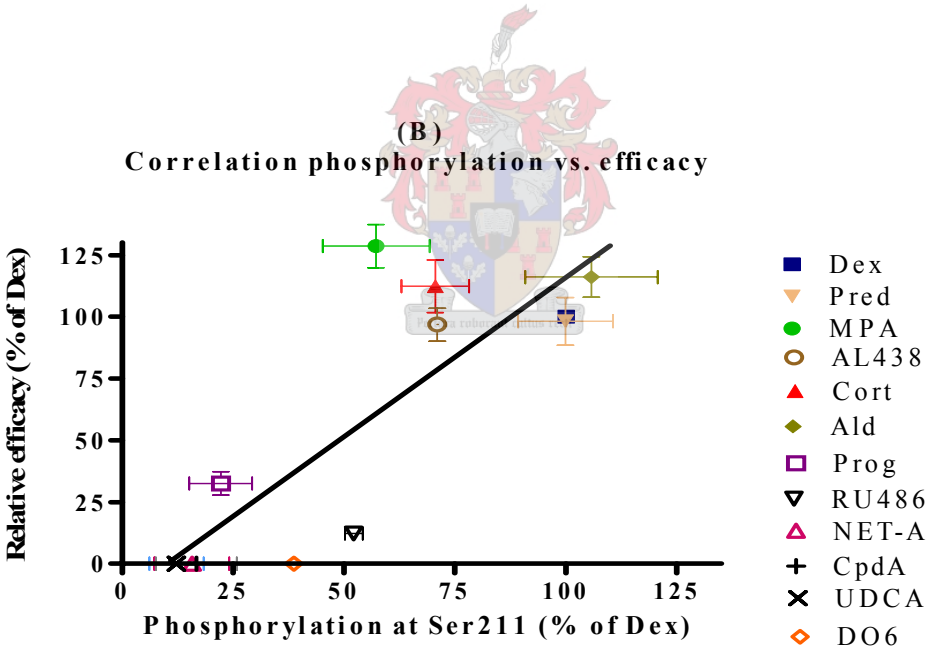
Similar to COS-1 cells, correlation analyses were performed in GraphPad Prism software to investigate a possible correlation between phosphorylation at Ser211 and transactivation potency and efficacy by a panel of test compounds in U2OS-hGR cells (Figure 3.17 and 3.18). The analyses again show a strong correlation between the extent of phosphorylation at Ser211 induced by all test compounds and both the potency ($r^2 = 0.65$, $p < 0.0015$) and efficacy ($r^2 = 0.68$, $p < 0.0009$) in GR-mediated transactivation for all the test compounds at 10 μM (Figure 3.17). The correlation analyses with certain subgroups of the test compounds were similar to what was observed in the COS-1 cells. The removal of subgroups of test compounds does not substantially change the analyses, suggesting that the correlation holds for all the test compounds.

In contrast, the correlation between phosphorylation and transactivation potency ($r^2 = 0.16$, $p < 0.247$) and efficacy ($r^2 = 0.32$, $p < 0.089$) is not significant for 100 nM test compound, normalised to bound-GR (Figure 3.18). The reasons for this poor correlation at 100 nM test compounds in U20S-hGR cells are unknown, but could possibly be due to experimental error (only one experiment was performed), although this is unlikely since a similar single experiment in COS-1 cells at 100 nM gave a much better correlation.

A more thorough analysis of the possible contribution of cell-specific effects towards the correlation analyses was performed by comparing the correlation data and transactivation data for all the experiments in both cell lines (Table 3.6-3.8).



	All	-antagonists	-SEGRAs	-partial agonists
r^2	0.65	0.72	0.56	0.95



	All	-antagonists	-SEGRAs	-partial agonists
r^2	0.68	0.75	0.58	0.77

Figure 3.17. Correlation between phosphorylation at Ser211 and potency and efficacy for transactivation for the panel of test compounds at 10 µM in U2OS-hGR cells. The graphs show data from phosphorylation studies at Ser211 with 10 µM performed in U2OS-hGR cells (three independent experiments) and data from transactivation studies in U2OS-hGR cells (three independent experiments) correlated for 12 different test compounds. Phosphorylation is correlated with potency (A) and relative efficacy (B) for transactivation.

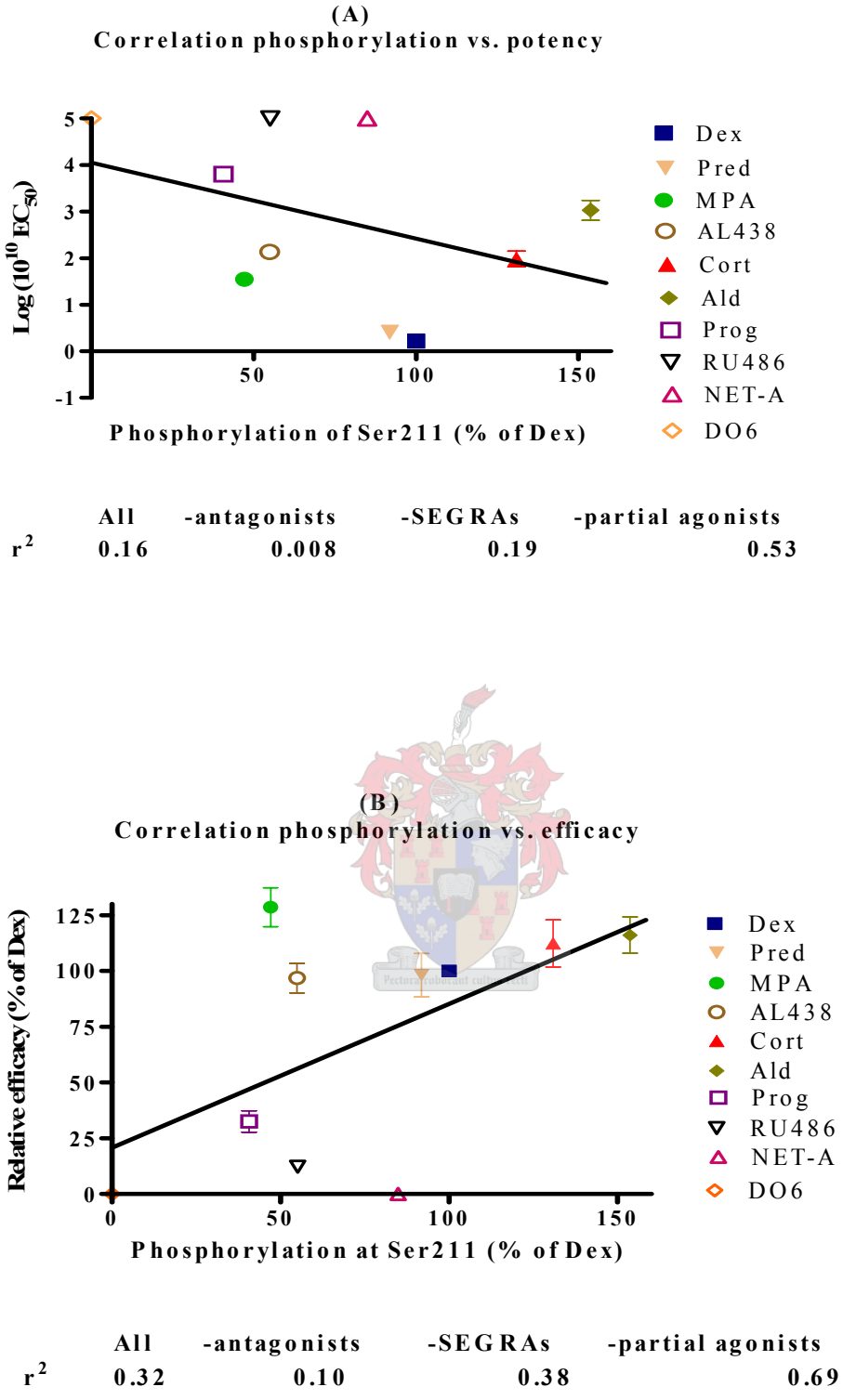


Figure 3.18. Correlation between phosphorylation at Ser211 and potency and efficacy for transactivation for the panel of test compounds at 100 nM in U2OS-hGR cells. The graphs show data from phosphorylation studies at Ser211 with 100 nM performed in U2OS-hGR cells (one experiment) and data from transactivation studies in U2OS-hGR cells (three independent experiments) correlated for 12 different test compounds. Phosphorylation of bound GR is correlated with potency (A) and relative efficacy (B) for transactivation.

3.2.3 Summary of phosphorylation, transactivation and correlation analyses

The following tables (3.6-3.8) summarise the phosphorylation data obtained at two concentrations, the transactivation data from a synthetic promoter-reporter construct and the correlation analyses performed within the two different cell systems.

Table 3.6. The ability to induce phosphorylation at Ser211 of GR by 12 test compounds as compared to Dex, which is set as 100 %. Two concentrations were investigated, 100 nM normalised to bound-GR and 10 µM normalised to total GR in COS-1 and U2OS-hGR cells. The basal phosphorylation (EtOH) control is subtracted. n/d: not determined

	COS-1 cells (% of Dex)		**U2OS-hGR cells (% of Dex)**	
Test compound	**100 nM**	**10 µM**	**100 nM**	**10 µM**
Dexamethasone	100	100	100	100
Cortisol	101	91	131	71
Prednisolone	116	107	92	100
Progesterone	81	40	41	22
MPA	67	77	47	57
NET-A	4	29	85	16
Aldosterone	78	53	154	106
RU486	49	58	55	52
D06	0	8	0	39
Compound A	n/d	24	n/d	17
AL438	54	66	55	71
UDCA	n/d	21	n/d	12

Table 3.7 Functional properties of 12 test compounds in GR-mediated transactivation in two cell systems. n/a: no activity, n/d: not determined.

	COS-1 cells		U2OS-hGR cells	
Test compound	**EC_{50} (nM)**	**Efficacy (% of Dex)**	**EC_{50} (nM)**	**Efficacy (% of Dex)**
Dexamethasone	0.228 ± 0.12	100	0.18 ± 0.09	100
Cortisol	16.77 ± 12.2	101.0 ± 16.8	10.9 ± 5.95	112 ± 18.4
Prednisolone	0.104 ± 0.06	90.32 ± 11.0	0.28 ± 0.13	98.2 ± 16.8
Progesterone	1688 ± 498	17.56 ± 15.0	700 ± 350	32.6 ± 8.19
MPA	2.501 ± 0.42	73.25 ± 7.08	3.63 ± 1.10	127 ± 17.5
NET-A	n/a	n/a	n/a	n/a
Aldosterone	138.5 ± 31.6	90.65 ± 10.6	139 ± 126	116 ± 14.1
RU486	n/d	8.385 ± 1.56	n/d	12.3 ± 1.88
D06	n/a	n/a	n/a	n/a
Compound A	n/a	n/a	n/a	n/a
AL438	8.559 ± 3.35	67.35 ± 12.6	14.6 ± 5.44	96.9 ± 11.5
UDCA	n/a	n/a	n/a	n/a

Table 3.8. Summary of the correlation analyses between potency and efficacy in transactivation and phosphorylation induced at Ser211 at two concentrations in two different cell systems.

	COS-1 cells				U2OS-hGR cells			
	100 nM		**10 µM**		**100 nM**		**10 µM**	
	Potency	Efficacy	Potency	Efficacy	Potency	Efficacy	Potency	Efficacy
All test compounds	0.61	0.67	0.85	0.78	0.16	0.32	0.65	0.68
- antagonists	0.52	0.60	0.94	0.81	0.008	0.10	0.72	0.75
- SEGRAs	0.65	0.72	0.82	0.71	0.19	0.38	0.56	0.58
- partial agonists	0.74	0.82	0.83	0.87	0.53	0.69	0.95	0.77

The present study shows that Dex, Cort and Pred induce strong phosphorylation at Ser211 and all behave like full agonists with similar potencies in GR-mediated transactivation in both cell lines.

Although RU486 induces a phosphorylation of about 50 %, it behaves like a partial agonist, not an antagonist, in transactivation assays in both cell systems. On the other hand, D06 induces virtually no phosphorylation in the COS-1 cells but significant phosphorylation at 10

μM in the U2OS-hGR cells even though it has no agonistic activity in transactivation via GR. The data for D06 in these experiments are limited, as only one experiment was performed with D06 at 10 μM in the U2OS-hGR cells, due to limited amounts available of this ligand. The correlation analyses show a stronger correlation when the antagonist subgroup is removed at 10 μM, but not at 100 nM in both cell systems. This inconclusive result suggests that more "true" antagonists should be included in further investigations to confirm whether antagonistic activity of a ligand correlates with phosphorylation at Ser211.

The SEGRAs investigated in these experiments have different transactivation abilities and induce different extents of phosphorylation. CpdA and UDCA induce minimal phosphorylation and have no agonistic activity in transactivation in both cell lines. AL438 induces a phosphorylation of about 60 % in both cell systems, which is reflected well in its transactivation ability both in the COS-1 cells and in the U2OS-hGR cells. The correlation analyses done without the SEGRA subgroup show a slightly stronger correlation at 100 nM, but not 10 μM, in both cell lines. The data for CpdA and UDCA are not included in the 100 nM correlations as no K_i has been determined for these compounds, so only AL438 is included in these correlations. However, overall, the correlation between phosphorylation at Ser211 and transactivation appears to correlate well for this group of compounds.

The subgroup least consistent with the hypothesis are the partial agonists. In all the correlations analyses (except for 10 μM potency in COS-1 cells) where the partial agonist subgroup are excluded, the correlations are substantially greater, suggesting that the hypothesis does not hold as well for partial agonists. For example, Prog and MPA induce stronger phosphorylation in the COS-1 cells compared to the U2OS-hGR cells, although, the agonist activity of Prog and MPA in transactivation is higher in the U2OS-hGR cells compared to the COS-1 cells. In addition, NET-A which has no agonistic activity for transactivation in either cell line, induces about 85 % phosphorylation at 100 nM in the U2OS-hGR cells. However, as mentioned, these data are from a single experiment that needs to be repeated. The reason for this behaviour by the partial agonists could be due to differential conformational changes induced in the receptor, which will be discussed in Chapter 5.

3.2.4 Studies with phosphorylation mutant

The correlation between phosphorylation at Ser211 of GR and transactivation potency and efficacy for 10 μM test compounds in both COS-1 cells and U2OS-hGR cells raises the question as to the precise role of phosphorylation at Ser211 in transactivation. One hypothesis is that phosphorylation *per se* is needed for the full maximal transactivation activity for all the ligands, and that the absence of the phosphate group at Ser211 would result in a loss or reduction of maximal activity. It is also possible that the extent of phosphorylation at Ser211 is responsible for the differential effects on gene transcription with different ligands. Should this be so, and if the presence of the phosphate group *per se* is required, one would expect that all the ligands would exhibit the same maximal transcriptional activity via a mutated GR that cannot be phosphorylated at Ser211. Another hypothesis is that phosphorylation may be a consequence of a conformational change in the receptor induced by a particular ligand, where phosphorylation itself is not required for transcriptional effects. If such a conformation is maintained in a mutant receptor which cannot be phosphorylated at Ser211, then one would expect no difference in the transactivation activity of a wildtype GR versus a Ser211 mutated GR, and that ligands retain their differential effects via this mutant GR.

These hypotheses were tested using a Ser211 site mutant receptor (S211A), which abrogates receptor phosphorylation at that specific site. Transactivation studies in COS-1 cells were performed with the mutant receptor on the same promoter-reporter construct used in the transactivation assay with the wildtype receptor (section 3.1.2). The results show that the mutant lacking a phosphorylatable amino acid at position 211 can transactivate when stimulated with all the test compounds in the same relative manner as the wildtype GR. The potency and the relative efficacy for transactivation is similar to the mutant receptor for all the test compounds relative to the Dex-response as compared to the results obtained in section 3.1.2 with the wildtype receptor (Figure 3.19 and Table 3.9).

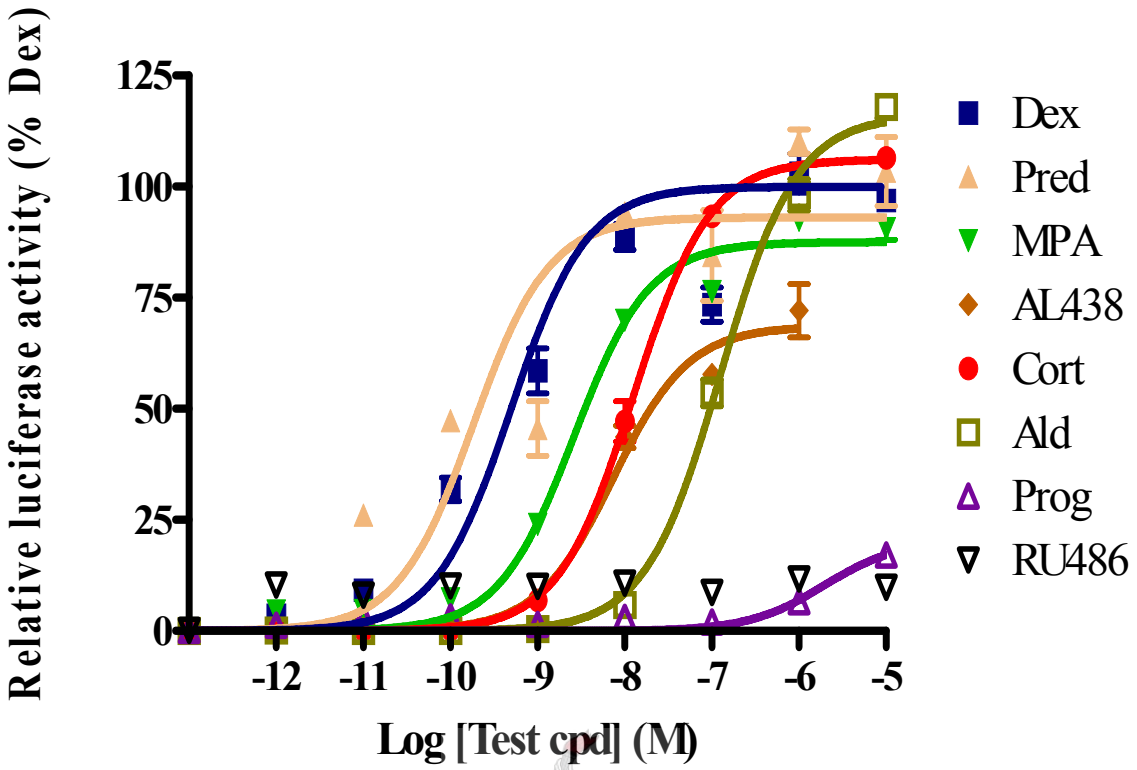
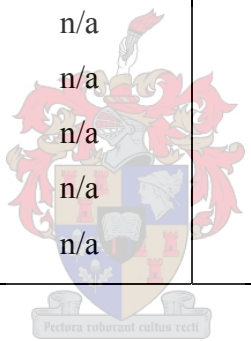


Figure 3.19. The effect of the panel of test compounds on GR-dependent transactivation with a S211A mutant receptor in COS-1 cells. COS-1 cells were plated in 24-well plates at a density of 2.5 X 10^4 and transfected with 0.125 µg pCMV-HA-hGR$_{S211A}$ and 0.047 µg TAT-luciferase promoter-reporter construct (pTAT-GRE-E1b-luc) and 0.075 µg β-galactosidase reporter construct (pSVβ-gal) per well and treated with the indicated concentrations of test compounds. After 16 h, luciferase activity was assayed. The luciferase values were normalised to β-galactosidase activity. Dose-response curves for all test compounds with the mutant receptor were performed once with each value in triplicates plotted as means ± SEM, expressed as relative luciferase activity (Dex set as 100 %).

Table 3.9. Functional properties of the test compounds on GR-dependent transactivation with a S211A mutant receptor in COS-1 cells. Dose-response curves for all the compounds with the mutant receptor were performed in one experiment. The EC_{50} and relative efficacy were determined by GraphPad Prism software. n/a: no ability to activate transcription. The data for the wildtype receptor are from section 3.1.2.

Test compounds	EC_{50} (nM) mutant receptor	EC_{50} (nM) wildtype receptor	Relative efficacy to Dex (%) mutant receptor	Relative efficacy to Dex (%) wildtype receptor
Dexamethasone	0.498	0.228 ± 0.12	100.0	100.0
Prednisolone	0.184	0.104 ± 0.06	93.10	90.32 ± 11.0
MPA	2.618	2.501 ± 0.42	87.54	73.25 ± 7.08
AL438	7.378	8.559 ± 3.35	68.59	67.35 ± 12.6
Cortisol	12.74	16.77 ± 12.2	106.1	101.0 ± 16.8
Aldosterone	127.2	138.5 ± 31.6	116.0	90.65 ± 10.6
Progesterone	1930	1688 ± 498	20.42	17.56 ± 15.0
RU486	n/a	n/a	10.21	8.385 ± 1.56
DO6	n/a	n/a	n/a	n/a
CpdA	n/a	n/a	n/a	n/a
UDCA	n/a	n/a	n/a	n/a
NET-A	n/a	n/a	n/a	n/a



However, it was noticed in the previous experiment that the absolute luciferase readings with the mutant receptor were significantly lower than with wildtype receptor transactivation assays. Therefore, parallel transactivation experiments were performed with wildtype receptor and mutant receptor. Again, the potency for Dex was similar between the wildtype and the mutant receptor, however, a significant drop in efficacy was observed between the two receptors as the mutant GR only has an efficacy of 60 % as compared to the wildtype GR (Figure 3.20). Similar results were seen for some of the other test compounds when comparing the efficacy between the wildtype and mutant receptor, e.g. the Pred-response with mutant receptor has an efficacy of 60 % compared to the Pred-response with wildtype receptor (Figure 3.21). The loss in efficacy that is observed with the mutant receptor is not due to differences in expression of the two constructs, as can be seen in Figure 3.10, where the expression of the mutant receptor is equal to the wildtype receptor (probed with total GR antibody).

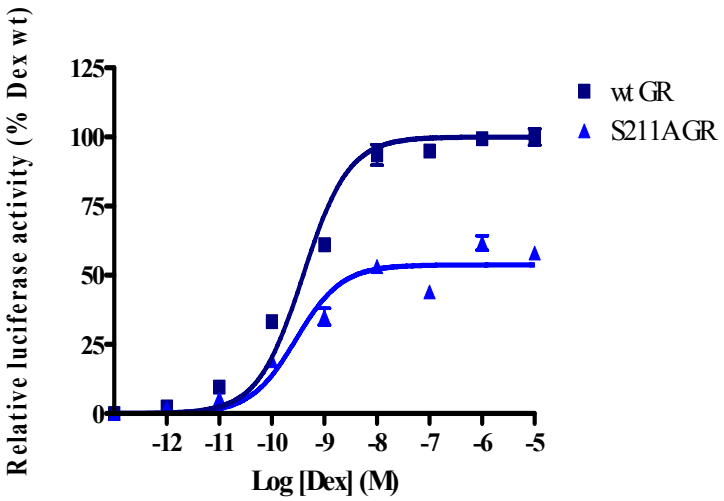


Figure 3.20. Transactivation of wildtype and mutant receptor by Dex in COS-1 cells. COS-1 cells were plated in 24-well plates at a density of 2.5 X 10^4 and transfected with 0.125 µg pCMV-HA-hGR or pCMV-HA-hGR_{S211A} and 0.047 µg TAT-luciferase promoter-reporter construct (pTAT-GRE-E1b-luc) and 0.075 µg β-galactosidase reporter construct (pSVβ-gal) per well and treated with the indicated concentrations of test compounds. After 16 h, the cells were lysed and analysed for luciferase activity. Data shown are from one experiment, expressed as relative luciferase activity, where maximal induction by Dex with the wildtype receptor is set as 100 %. Each value plotted is the mean ± SEM of triplicates.

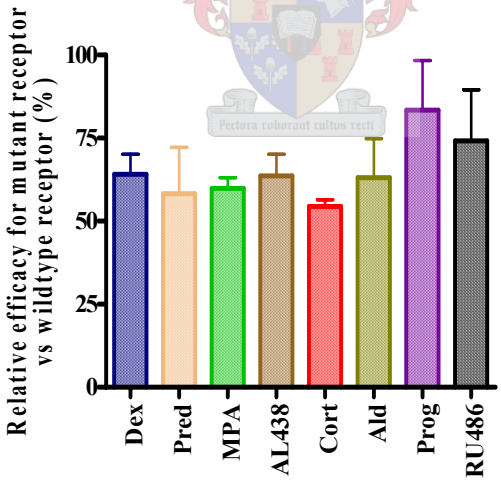


Figure 3.21. The relative efficacy in transactivation for the mutant S211A receptor compared to wildtype receptor for test compounds in COS-1 cells. Three independent transactivation experiments (each in triplicates) with the wildtype and mutant receptor in parallel were performed with 10 µM test compounds. Relative efficacy for mutant versus wildtype receptor is plotted for each compound, i.e. by dividing the normalised (for β-galactosidase) total luciferase activity due to each compound with mutant receptor by that obtained with wildtype receptor, and expressing the value as a percentage. Background luciferase activity obtained with no test compound (ethanol only) was subtracted from the luciferase activity to obtain total activity.

Therefore, the results show that phosphorylation at Ser211 is not essential for transactivation but it is required for full efficacy for all the agonists investigated. In addition, phosphorylation at Ser211 is not responsible for discriminating between the potencies and efficacies for different ligands, as the same relative potency and efficacy between the test compounds are maintained when the site is mutated (Table 3.9). Given the correlation data between phosphorylation and transactivation potencies and efficacies, it is however still possible that the levels of phosphorylation at Ser 211 reflect different conformational changes of the bound GR, induced by the different ligands, possibly resulting in differential recruitment of cofactors, such that these changes are maintained in the mutated receptor.

Several groups have previously investigated the effects of individual phosphorylation sites on the transactivational ability of GR and it was discovered that the effect of phosphorylation was promoter-specific (Almlöf *et al.*, 1995; Mason and Housley, 1993; Webster *et al.*, 1997). Two studies have shown no loss of efficacy in transactivation of a MMTV promoter-reporter construct in COS-1 cells with a mutated mouse GR on the Ser220 site (homologous to the human Ser211 site) (Mason and Housley, 1993; Webster *et al.*, 1997) and another study showed no loss of efficacy in transactivation of a 1 X GRE-lacZ reporter gene in yeast cells with mutated human GR on the Ser211 site (Almlöf *et al.*, 1995). However, Webster et al. also investigated transactivation on a 2 X GRE-TATA-CAT (GRE from the TAT gene promoter) in COS-1 cells and a significant loss (35 % efficacy as compared to wildtype) in transactivation efficacy was observed (Webster *et al.*, 1997). In the present study a similar 2 X GRE (from the TAT gene promoter) promoter-reporter construct was used and a significant drop in efficacy (60 % efficacy as compared to wildtype) is observed, in accordance with the literature. Webster et al. did not examine if the mutation at the Ser211 site had any effect on the potency of transactivation or the selectivity of different ligands.

As only a partial loss in efficacy is observed when the Ser211 site is mutated, it is possible that other phosphorylation sites in the AF-1 domain are also involved in the maximal response in transcriptional activation and that phosphorylation will occur on alternative residues when the primary site has been mutated. There is evidence of interdependency between the Ser203, Ser211 and Ser226 phosphorylation sites (reviewed in Ismaili and Garabedian, 2004). Mutation of the Ser203 site leads to less phosphorylation at Ser211 but a stronger phosphorylation of Ser226 and a mutation of the Ser226 site leads to stronger phosphorylation of the Ser203 site, suggesting that the lack of ability to phosphorylate at one site leads to

hyperphosphorylation of other sites (reviewed in Ismaili and Garabedian, 2004). This idea is supported by transactivation studies with phosphorylation mutant receptors. No loss in efficacy in transactivation was observed with the mouse GR when single phosphorylation sites where mutated, even a triple mutant of S212/S220/S234A (homologous to human Ser203, Ser211 and Ser226) showed no loss in efficacy. However, when the five phosphorylation sites that are conserved between the mouse and the human GR were mutated, a 22 % decrease in efficacy was observed on a MMTV promoter-reporter construct in COS-1 cells (Mason and Housley, 1993). In contrast, no loss in efficacy in transactivation with either single site mutations or all five phosphorylation sites mutated were observed with the human GR in yeast cells on a 1 X GRE-lacZ promoter-reporter construct (probably due to promoter-specific effects) (Almlöf *et al.*, 1995). Similarly, the loss in efficacy in transactivation on the 2 X GRE-CAT promoter-reporter construct by the mouse GR in COS-1 cells was not any different between single mutation or multiple phosphorylation mutations (Webster *et al.*, 1997). Possibly, the promoter complexity of the MMTV promoter reporter construct where the presence of other interacting proteins is required for transactivation (Guido *et al.*, 1996) can make up for the loss of phosphorylation of the receptor, compared to on a more simple GRE promoter, like the TAT promoter. These studies did not, however, investigate the role of phosphorylation on differential ligand effects.

The present study shows a strong correlation between transcriptional ability of a TAT-GRE promoter-reporter construct and phosphorylation at Ser211. The loss of efficacy from the promoter when the phosphorylation site is mutated, certainly shows that the phosphorylation at the Ser211 site is directly required for full transcriptional activation. The results also suggest that GR conformational changes may be required for ligand selectivity, from this specific promoter, rather than a requirement for the presence of the phosphate group *per se*.

CHAPTER 4

Results and discussion

CpdA binding to the glucocorticoid receptor

4.1 Background

A part of this thesis was a further investigation into the properties of CpdA, since this is a compound that is extensively studied in our group. It has been shown that CpdA dissociates between GR-mediated transactivation and transrepression (described in 1.9.4.1), placing it in the interesting group of SEGRA compounds. However, it has not been established that CpdA binds directly to GR. UDCA has recently been shown to exhibit no direct binding to GR, but still it behaves like a full agonist in GR-mediated transrepression with weak agonist activity in transactivation (Weitzel *et al.*, 2005; Miura *et al.*, 2001), making it a SEGRA like CpdA. All the other test compounds in the panel are classified as GR ligands as they are able to fully displace [^{3}H]-Dex from GR in a concentration-dependent manner.

CpdA is suggested to occupy the LBD of GR similar to other steroidal GR ligands (De Bosscher *et al.*, 2005). In classical competitive binding assays, a typical [^{3}H]-Dex displacement curve is obtained with unlabelled Dex or other ligands for GR (example with Dex in Figure 4.1) because both the radiolabelled ligand and the competitor bind reversibly to the same binding site at the receptor. All ligands that bind to the same site at the receptor would be expected to displace all radiolabelled ligand to the same extent. A one-site competitive binding curve is equivalent to a standard sigmoidal curve. Initial competitive binding studies with CpdA done previously in the lab have indicated that CpdA is only able to partially displace [^{3}H]-Dex from GR at saturating concentrations in COS-1 cells and L929 cells (Figure 4.1 shows a representative experiment in COS-1 cells, performed by Prof. Hapgood, personal communication), but with a relative high binding affinity. CpdA displays an atypical binding characteristic compared to typical [^{3}H]-Dex displacement curves obtained with Dex, displacing a maximum of 42 % of the specific binding of [^{3}H]-Dex to GR in COS-1 cells while in the L929 cells CpdA is able to displace up to 81 % (personal communication Dr. Louw). In COS-1 cells, the approximate IC_{50} of CpdA is 0.08 nM compared to an IC_{50} of

12.0 nM for Dex, while in L929 cells the IC_{50} of Dex is 25.9 nM and 6.4 nM for CpdA (De Bosscher *et al.*, 2005).

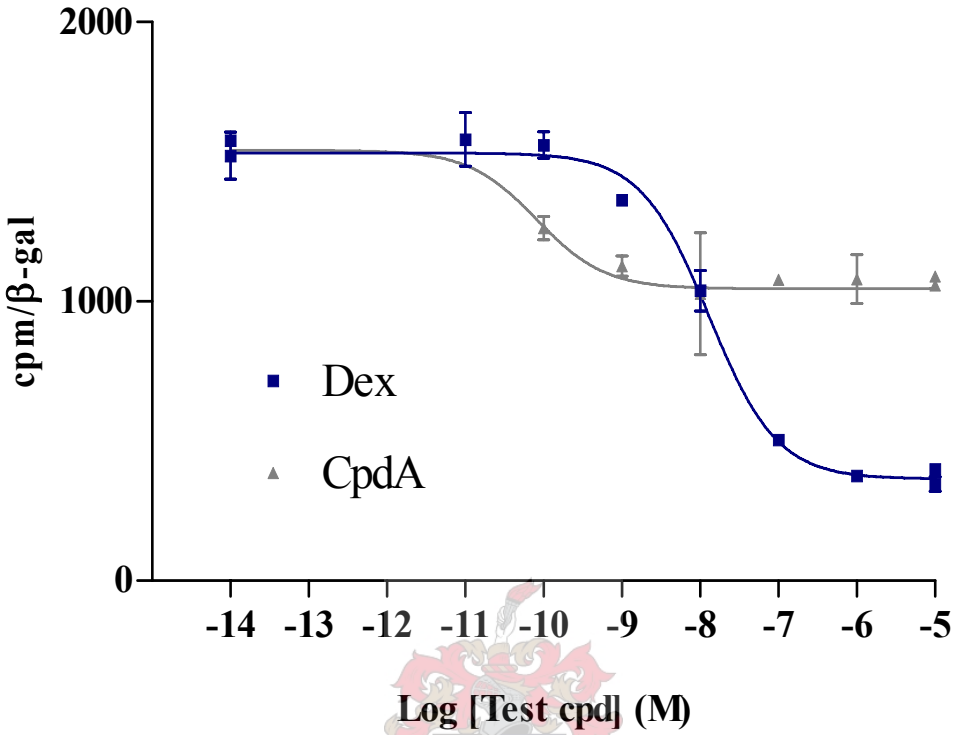


Figure 4.1. Dex and CpdA compete with [^{3}H]-Dex for binding to the rat GR. COS-1 cells, transiently transfected with pSVGR1 and pSVβ-gal expression vectors, were incubated with 10 nM [^{3}H]-Dex in the absence and presence of varying concentrations of Dex or CpdA for one hour. Results shown are total specific counts (Non-specific counts are 370) normalised to β-galactosidase and are typical of two independent experiments, where each condition was performed in triplicates (± SEM).

CpdA can form an aziridine that can react with nucleophilic groups on the receptor forming covalent bonds that could potentially lead to covalent modification of GR (Louw *et al.*, 1997). The covalently modified receptor might no longer be able to bind [^{3}H] Dex to the same extent, so the small displacement that is seen with CpdA might reflect the removal of a pool of modified receptors from the equilibrium. However, reversibility experiments have shown that binding of CpdA to GR is not covalent and is reversible (De Bosscher *et al.*, 2005). CpdA also stabilises the receptor upon interaction, investigated with limited proteolysis of the liganded-receptor. CpdA protects the receptor better than solvent and produces the same patterns compared to Dex (De Bosscher *et al.*, 2005), indicating a possible competition for the receptor molecule. Another indication that CpdA binds to GR is, that like Dex, CpdA induces nuclear translocation of the receptor (De Bosscher *et al.*, 2005). However, UDCA has also

been shown to promote nuclear translocation and DNA-binding of the receptor, despite recent evidence that it does not bind to the GR (Weitzel *et al.*, 2005). Thus it is still unclear whether CpdA binds directly to the GR.

The atypical binding curve obtained for CpdA (Figure 4.1), where only partial displacement of [^{3}H]-Dex from GR is achieved, might indicate that CpdA does not bind to the LBD as do other GR ligands or possibly that CpdA differentially binds to certain subpopulations of the receptor. There are several subpopulations of the receptor present in a cell. For example, there are six different isoforms of the receptor (see section 1.4.4-7). In addition, some receptors might be bound to the Hsp complex resulting in an active receptor capable of binding to a ligand and some receptors may be unbound to the Hsp complex resulting in GR defective in ligand binding (Kovacs *et al.*, 2005). Some receptors are monomers and at a high receptor concentration possibly homodimer formation occurs in the absence of ligand (Cho *et al.*, 2005b) and CpdA may have differential binding affinity for homodimers versus monomers. CpdA might also have differential binding affinities towards receptors that are differentially phosphorylated (Ismaili and Garabedian, 2004). If CpdA only recognises one specific subpopulation of the receptor, this might explain the atypical binding data seen for CpdA.

The hGR is transcribed and translated into several isoforms and the most common is the hGRα, which is the transcriptional active receptor of 777 amino acids. However, two translational isoforms of hGRα are produced by alternative translation initiation, termed GRα-A and GRα-B. GRα-A is transcribed from the initial start codon (Met1) while the GRα-B is transcribed from an internal start codon (Met27). Hence, the GRα-B is 27 amino acids shorter in the N-terminal end of the receptor. When looking at different human cell lines with endogenous GR, the expression level of the A-isoform is prominent, while the relative expression of the B-isoform varies more in some cell lines than others (Yudt and Cidlowski, 2001). If CpdA could differentiate between the two isoforms, and both were present in a particular cell, then the displacement of [^{3}H]-Dex by CpdA would not be saturable. A possible explanation for the differences in % total displacement of [^{3}H]-Dex by CpdA in COS-1 versus L929s cells could be the presence of different relative levels of the two isoforms in the cells. When hGRα is transfected and expressed in COS-1 cells, the expression of the A- and the B-isoform is approximately equal (50:50 %) (Yudt and Cidlowski, 2001), which would be consistent with the 42 % displacement of [^{3}H]-Dex by CpdA observed in COS-1 cells (Figure 4.1), if CpdA was able to recognise only one of the isoforms.

4.2 Results and discussion

4.2.1 Investigation of binding to human GRα A- and B-isoform

To examine if CpdA differentially binds to the A- or B-isoform of the hGRα, competitive whole-cell binding assays were performed (Figure 4.2). The binding experiments were carried out in COS-1 cells that are deficient in endogenous GR (Figure 3.7), transfected with the hGRα wildtype or the hGRα A- or B-isoform constructs. The hGRα A-isoform construct is mutated at start codon Met27 while the B-isoform is mutated at Met1 to produce only one single isoforms from the constructs (Yudt and Cidlowski, 2001).

Figure 4.2 shows CpdA competition for [^{3}H]-Dex binding to GR. As expected, CpdA is not able to fully compete for the [^{3}H]-Dex binding to the wildtype receptor and it does not discriminate between the A- or the B-isoform either as CpdA only partially displaces [^{3}H]-Dex in a similar fashion for the two isoforms. CpdA (10 μM) reduced [^{3}H]-Dex binding to GR isoforms by approximately 40 %. Six other known GR ligands were added to the experiment to see if these ligands could possibly differentiate between the GR isoforms. None of the ligands differentiated between the A- or B-isoform. NET-A displaces 95 % of [^{3}H]-Dex at a concentration of 10 μM however there is no detectable difference between the A- and the B-isoform.

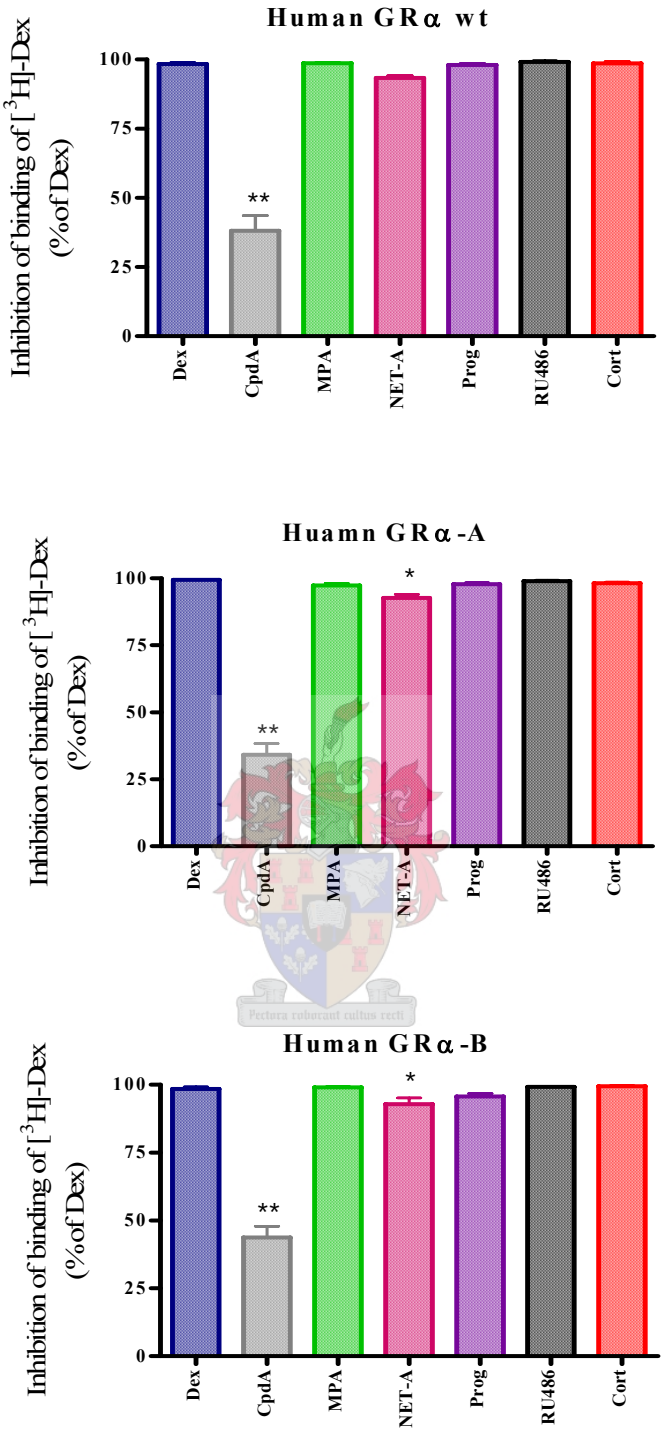


Figure 4.2. CpdA competition for [^{3}H]-Dex binding to the human GRα wildtype, GRα A- and B-isoforms. Ligand-binding studies were performed in COS-1 cells transfected with human GRα wildtype, GRα A- and B-isoforms using a concentration of 5 nM [^{3}H]-Dex for all constructs. The concentration of unlabelled test compounds used was 10 μM. The non-specific binding was subtracted (non-specific counts were about 5 %). The specific binding data were normalised and the results are expressed as a percentage of the inhibition of [^{3}H]-Dex binding by unlabelled Dex set as 100 %. The graphs show the mean ± SEM from the results of three independent experiments. * P<0.05, relative to Dex, ** P<0.01, relative to Dex.

4.2.2 Investigation of binding to human GRα phosphorylation mutants

As CpdA did not appear to discriminate between the A- and B-isoforms of GRα, other possibilities were explored. It was therefore decided to look at three different GR constructs that are mutated at specific phosphorylation sites, the Ser203, Ser211 and Ser226. It could be possible that CpdA only recognises GR molecules that are phosphorylated on a certain site. The serine residues are phosphorylated at a basal level and become more hyper-phosphorylated in the presence of hormone (reviewed in Ismaili and Garabedian, 2004).

Again, competitive whole-cell binding assays were performed, as described above. This time the COS-1 cells were transfected with the hGRα wildtype and three mutant constructs where the serine residues at 203, 211 and 226 were mutated to alanine (Wang *et al.*, 2002). Figure 4.3 shows that neither CpdA nor any of the other test compounds differentiated between GR phosphorylation mutants and wildtype GR. CpdA is only able to displace about 40 % of [^{3}H]-Dex from GR phosphorylation mutants as compared to wildtype receptor. The displacement of [^{3}H]-Dex from the Ser211 mutant receptor by CpdA is slightly higher than from the other mutant receptors (Figure 4.3) but it is not significantly different from the displacement obtained by CpdA with the wildtype GR (data not shown). Similarly, NET-A displaces about 95 % of [^{3}H]-Dex from the wildtype receptor and the other mutant constructs.

The results in this study clearly show that CpdA does not discriminate between any of the subpopulations of GR that were investigated in these experiments. Saturating concentrations of CpdA displace the same percentage of [^{3}H]-Dex from the wildtype receptor as from the A- and B-isoforms and the phosphorylation mutants. Thus, from this study, it was possible to discount the hypothesis that these different receptor forms are involved in the binding of CpdA. However, it is possible that CpdA does differentiate between other, as yet untested, subpopulations of the receptor. GR can exist as monomers and homodimers and possibly CpdA binds to only one of these subpopulations. Dimerisation of the receptor is often required in GR-mediated transactivation but not transrepression. As CpdA is only effective in transrepression via GR, it is possible that CpdA only binds to monomer receptor molecules and this may be investigated with GR dim mutants (Reichardt *et al.*, 2001). At high receptor concentrations, cooperative ligand binding occurs and more dimers are formed independently of ligand-binding with an increased affinity for Dex (Cho *et al.*, 2005b). If CpdA only binds to monomer receptor molecules, it may be possible to see differences in binding assays performed with low and high GR concentrations.

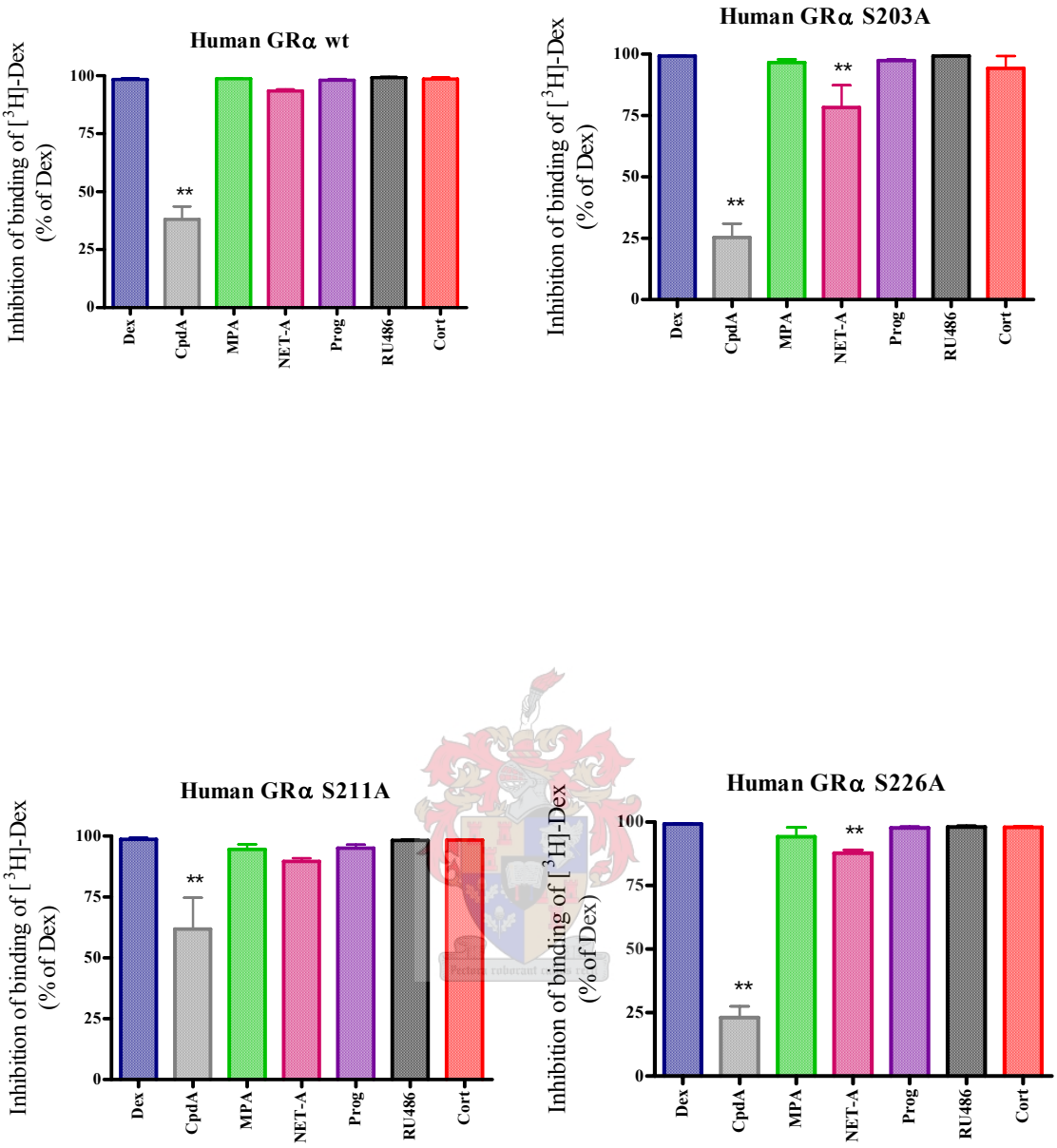


Figure 4.3. CpdA competition for [^{3}H]-Dex binding to the human GRα wild type, GRS203A, GRS211A and GRS226A mutants. Ligand-binding studies were performed in COS-1 cells using a concentration of 5 nM [^{3}H]-Dex for all constructs. The concentration of unlabeled test compounds used was 10 μM. The results are expressed as a percentage of the inhibition of [^{3}H]-Dex by unlabelled Dex set as 100 %. The graphs show the mean ± SEM from the results of three independent experiments. ** P<0.01, relative to Dex.

CHAPTER 5

Conclusions and future perspectives

5.1 Correlation between phosphorylation and transcriptional activity

In 2002, Wang *et al*. hypothesised that the ligand-induced phosphorylation at Ser211 of GR correlated with the agonist activity of the ligand in GR-mediated transactivation (Wang *et al.*, 2002). In this study, five different GR ligands were tested at one concentration (100 nM) in U2OS-hGR cells and no quantification of the levels of phosphorylation of liganded-GR was presented. Thus, the main aim of this thesis was to examine this hypothesis in more detail and by more quantitative methods. Experiments were performed to test whether a correlation exists between the extent of phosphorylation induced at Ser211 on liganded-GR by a larger panel of ligands, for both the potency and efficacy of the ligand, in both GR-mediated transactivation as well as transrepression and in two different cell lines. Twelve very different compounds have been tested for their ability to induce phosphorylation at Ser211 of liganded-GR in both COS-1 cells transiently transfected with human GR as well as in U2OS-hGR cells stably transfected with human GR. The transactivation studies were performed in three independent experiments and the phosphorylation studies were performed at two different concentrations: 100 nM (one experiment) and 10 μM (three independent experiments) in both cell lines, in order to give a good basis for correlation analyses. Two concentrations of test compound were tested as differences in phosphorylation at Ser211 might not be apparent at saturating concentrations, but only at subsaturating concentrations.

When comparing the results obtained by Wang *et al*. to those obtained in this thesis for agonists and antagonists in the U20S-hGR cells, both similarities and differences can be found. Wang *et al*. found that Dex, Pred and fluocinolone induced phosphorylation at Ser211 to the same extent while RU486 induced minimal and ZK299 induced the least (Wang *et al.*, 2002). In this study, Dex and Pred strongly induce phosphorylation at Ser211 while RU486 is able to induce phosphorylation levels of 50 % of that induced by Dex (Table 3.6). Interestingly, RU486 induces a slightly greater relative phosphorylation level than reported by Wang *et al*., showing that under the conditions of this study, RU486-induced phosphorylation is greater than would be expected from its poor agonist activity.

When considering the results obtained in both cell systems for the full panel of ligands, this study shows that there exists a strong and significant correlation between both potency and efficacy in transactivation and phosphorylation induced at Ser211 at saturating ligand concentrations, suggesting that under these conditions the level of phosphorylation induced at Ser211 by a ligand is independent of the cell system investigated. The correlation also holds for all test compounds at 100 nM in COS-1 cells but not in the U2OS-hGR cells. Furthermore, the correlation is stronger for phosphorylation induced at saturating concentrations (10 μM) compared to subsaturating concentrations (100 nM) in the COS-1 cells (Table 3.8). This suggests that cell-specific differences play an important role at subsaturating ligand concentrations, and that in the same cell, differences in the correlation are more apparent at subsaturating concentrations. The reason for this is probably due to deviations from the correlation by the partial agonists in particular, as discussed in more detail below. However, it should be noted that since the 10 μM results derive from three independent experiments but the 100 nM was only performed once, further experiments should be conducted with 100 nM to verify the trends observed at this concentration in this study.

Focusing on the transactivation results for the compounds at the extremes of the spectrum, i.e. the full agonists and the complete antagonist D06, revealed a very good correlation in general for both cell systems and at both saturating and subsaturating ligand concentrations. However, the picture for the antagonists was less clear if one includes RU486 in the analysis. As mentioned above, RU486 appeared to deviate from the correlation. RU486 was grouped as an antagonist in the correlation analyses in this study. However, since it behaves like a weak partial agonist in transactivation in both cell systems, it may be more appropriate to group it as a partial agonist. As such the results with RU486 would be more consistent with the trend observed for the partial agonists, which appears to deviate to the greatest extent from the correlation analyses. D06 is a GR-specific antagonist and it shows no agonist activity in GR-mediated transactivation in either cell line tested, therefore it can be considered the only antagonist investigated in this study. In most experiments, D06 induced very little phosphorylation at Ser211 correlating with its lack of agonist activity, except for in the single experiment at 10 μM in the U2OS-hGR cells. Thus, one drawback of the experiments presented in this study is the limited number of antagonists. There are several reports in the literature of the existence of other GR antagonists, such as ZK299 (Snyder *et al.*, 1989). However, these have been developed by pharmaceutical companies, and despite several efforts, our laboratory has as yet been unable to obtain any of these compounds. Therefore,

further investigations should be conducted with D06 and other GR antagonists to confirm the trend observed in this study that antagonists induce very little phosphorylation at Ser211.

The SEGRA subgroup of test compounds shows a good overall correlation between phosphorylation at Ser211 and transactivation in both cell systems and with both concentrations investigated. Removing the SEGRA subgroup from the correlation analyses does not change the general picture and thus the hypothesis holds for these test compounds. The ability of CpdA to induce phosphorylation at Ser211 has previously been investigated by De Bosscher *et al.* who showed minimal phosphorylation induced by CpdA at 10 μM and no agonist activity in transactivation in A549 cells (De Bosscher *et al.,* 2005). This result has been confirmed in this study as CpdA induces minimal phosphorylation at Ser211 with no agonist activity in both COS-1 and U2OS-hGR cells. This further supports the finding that the level of phosphorylation induced at Ser211 by CpdA is independent of the cell system investigated.

If one focuses on the transactivation results obtained with the partial agonists in both cell systems, it is apparent that the correlations are stronger when excluding the partial agonists, suggesting that the hypothesis does not hold for this particular subgroup. Differences in the relative rank orders for Prog and NET-A are observed in COS-1 versus U2OS-hGR cells at 100 nM compared to 10 μM (Table 3.6). There also exist some cell-specific differences in phosphorylation induced by MPA and Ald. MPA induces stronger phosphorylation in COS-1 cells, relative to Dex, at both concentrations despite a higher transactivation efficacy in the U2OS-hGR cells. Ald switches from middle rank order in induction of phosphorylation in the COS-1 cells to being the strongest inducer in the U2OS-hGR cells at both concentrations and an increase in transactivation potential is also observed for Ald in the U2OS-hGR cells (Table 3.6 and 3.7). This inconsistent behaviour of the partial agonists has an impact on the correlation analyses. Possibly, the conformational change induced in the receptor by a partial agonist causes differential behaviour with regards to phosphorylation and transactivation in a cell-specific manner. The differential conformational change could lead to selective recruitment of kinases responsible for phosphorylation or coactivators responsible for transactivation in the two cell lines, which will be discussed in more detail later.

Regarding the results for transrepression, only preliminary observations can be discussed, since these transrepression experiments were not completed and no proper correlation

analyses could be performed. However, the data obtained for GR-mediated transrepression for the limited panel of test compounds in this study suggest that a good correlation between the extent of phosphorylation induced at Ser211 by a ligand and the potency and efficacy of a ligand in transrepression does exist. Dex and Cort both behave like agonists in transrepression with high potency and both induce strong phosphorylation at Ser211. RU486 induces 50 % phosphorylation at Ser211 compared to Dex. The potency and efficacy (73 % of Dex) of RU486 in transrepression is high and similar to Dex, unlike the activity seen in transactivation, suggesting that for transrepression, the correlation for RU486 is much greater than for transactivation. MPA has a low potency but a high relative efficacy of 80 % in transrepression and MPA induces about 75 % phosphorylation at Ser211 compared to Dex. In this cell system, Prog has no agonist activity in transrepression and induces low levels of phosphorylation compared to Dex. NET-A and CpdA induce phosphorylation at Ser211 just above basal levels, consistent with the lack of agonist activity in transrepression in this study. In general, these preliminary results suggest that the correlation between phosphorylation and efficacy for transrepression is good and may be better than for transactivation. However, further experiments are necessary to confirm the possible correlation.

The hypothesis that there will also be a correlation between phosphorylation and transrepression, like with transactivation, is supported by available literature. Webster *et al*. investigated whether phosphorylation of the mouse GR is important in down-regulation of the GR gene itself by Northern analysis in transfected COS-1 cells (Webster *et al.*, 1997). A hormone-bound GR interacts with intragenic cis elements in the GR cDNA, which results in a decrease in transcription of the human GR gene (Burnstein *et al.,* 1994). The wildtype receptor caused a decrease of 50 % in the GR mRNA while the S220A (homolog to human Ser211 site) mutant receptor only caused a decrease of 30 % in the receptor mRNA levels after treatment with Dex. Mutation of three or more phosphorylation sites caused no change in the mRNA level, suggesting that phosphorylation of the receptor is important in gene transrepression (Webster *et al.*, 1997). The induction of the endogenous TAT gene and an MMTV synthetic promoter-reporter construct by GR is cell cycle-dependent. Transactivation only occurs in the G_1 and S phases, while it subsides in the G_2 and M phases, despite that phosphorylation of GR is more prominent in the G_2 phase (reviewed in Ismaili and Garabedian, 2004). However, GR-mediated transrepression was not affected in the G_2 phase, indicating that phosphorylation of the receptor might be required for gene repression (Hsu and DeFranco, 1995). Thus, these studies indicate that GR-mediated gene repression may be

dependent on the phosphorylation status of the receptor, supporting the trend observed in this study.

The mechanism whereby different test compounds are able to induce different extents of phosphorylation for the same degree of receptor occupancy is poorly understood. This may be explained by different conformational changes induced in the receptor when bound to different ligands. Due to the different conformational changes in the receptor, the different ligand-bound GRs might recruit differential amounts of the kinase responsible for the phosphorylation at Ser211, thereby causing ligand-specific degrees of phosphorylation at this site. Recently, Bruna *et al*. showed that the GR-JNK interaction is ligand-dependent, as Dex-bound GR promotes interaction with JNK while RU486-bound GR fails to induce binding with JNK (Bruna *et al.*, 2003). JNK has been shown to be responsible for phosphorylation at Ser226 (Itoh *et al.*, 2002) and p38 has been shown to be responsible for phosphorylation at Ser211 in the human GR (Miller *et al.*, 2005). It is thus possible that ligand-bound GR may differentially recruit p38, where the extent of recruitment may correlate with biological activity of the ligand. The recruitment of p38 could in the future be tested by GST pull-down assays, bioluminescence resonance energy transfer (BRET) assays, avidin-biotin complex DNA (ABCD) assays or mammalian two hybrid assays. Phosphorylation itself could also cause a conformational change in the receptor structure, which might modulate the function of the receptor. This has been shown for proteins other than steroid receptors. For example, the phosphorylation of the Hsp30 and HMG proteins induces conformational changes and this is important for the regulation of the activity of these proteins (Fernando *et al.*, 2003; Wisniewski *et al.,* 1999). However, the results obtained in the present study with the phosphorylation mutant receptor indicate that the conformational change induced in the receptor by a ligand is more likely to be responsible for the differential transactivational effects of the different ligands, and not presence of the phosphate group itself at Ser211, as the phosphorylation mutant receptor-ligand complexes maintained their relative potencies and efficacies in transactivation. The conformational change induced in the receptor may cause differential recruitment of both p38 and coactivators, but the recruitment of coactivators by a specific ligand-bound GR may be what determines the ligand-specific transactivational activity of the liganded-GR complex.

There exists, however, some evidence that receptor phosphorylation in itself is involved and important in the recruitment of coactivators. It has previously been shown that the extent of

phosphorylation of some transcription factors regulates the extent of their ability to affect gene expression via regulating cofactor recruitment. For example, increased phosphorylation of the vitamin D receptor (VDR) leads to increased interaction with the VDR-interacting protein (DRIP) coactivator complex (Barletta *et al.*, 2002). Garabedian's group has suggested that recruitment of the DRIP coactivator complex to GR also depends on the phosphorylation status of the receptor (reviewed in Ismaili and Garabedian, 2004). If the phosphorylation status of the receptor determines interaction with coactivators as proposed by Garabedian's group (reviewed in Ismaili and Garabedian, 2004), it may be that the recruitment of only some coactivators, in particular those interacting with the AF-1 domain in the N-terminus of the GR, will also correlate with transactivational activity of a ligand.

There is accumulating evidence from the literature that ligand-specific conformations of bound-GR do regulate the extent of recruitment of some cofactors. Dex-bound GR interacts with GRIP1 and TIF-2 to the greatest extent. Prog-bound GR interacts at an intermediate level with the coactivators and RU486-bound GR interacts the least (Cho *et al.,* 2005a), correlating with the agonist activity of these ligands. The same applies to corepressors where Dex-bound GR interacts less with NCoR than RU486-bound GR, as examined by mammalian two-hybrid assays (Wang *et al.,* 2004b). Thus, the agonist activity of a GR ligand may be determined by the binding affinity of the ligand-receptor complex to certain coactivators. This hypothesis however needs to be further tested for a larger panel of ligands, in order to establish whether it holds for several antagonists, partial agonists and SEGRAs, for GRIP1, TIF-2 and other cofactors. Several methods can be used to test the hypothesis that different ligands cause different conformational changes in the receptor and there is already some evidence in the literature that ligand-selective conformational changes occur in GR and other steroid receptors. It has been shown by crystallography that full agonists, like Cort and Dex, induce similar conformational changes in GR (Bledsoe *et al.*, 2002; Von Langen *et al.*, 2005) while RU486 induces a different change in the receptor (Kauppi *et al.*, 2003). Similar models have been proposed for other steroid receptors. Prog (Williams and Sigler, 1998) and RU486 (Robin-Jagerschmidt *et al.*, 2000) have been shown to induce different conformational changes in PR and estradiol and tamoxifen in ER (Brzozowski *et al.*, 1997). However, all these studies are based on crystal structures and may not be physiologically relevant. Another method that can be used is limited proteolysis of liganded-GR complexes. The different conformational changes in the receptor induced by different ligands can be observed as different ligands show distinctive proteolysis patterns (Hellal-Levy *et al.*, 1999), although the

significance of this method is unclear as it is performed *in vitro*. Circular dichronism and steady-state fluorescence have been used to show a similar mechanism for a GPCR, the serotonin 5-HT (4 a) receptor (Baneres *et al.,* 2005). Different conformational changes occurred in the second extracellular loop when the receptor was activated by either an antagonist, a partial or full agonist or an inverse agonist (Baneres *et al.,* 2005). Another powerful method that can be used to study conformational changes in a receptor when bound to a ligand is NMR, which has been used to show that there is a distinct conformational change in the ER when bound to estradiol (Luck *et al.,* 2000). In the future, a combination between further investigations into the cofactor recruitment by mammalian two-hybrid assays and studies on conformational changes induced in the receptor by NMR by a variety of GR ligands would be of great interest and could possibly explain ligand-selective transcriptional regulation. Phosphorylation deficient receptor mutants can also be used in the mammalian two-hybrid assays to investigate if phosphorylation at specific sites *per se* is required for the recruitment of cofactors to the AF-1 domain in the N-terminus. In particular, the recruitment of NCoR is of interest as Wang *et al.* have shown that the N-terminus is required for binding of NCoR to GR (Wang *et al.,* 2005). The effect of phosphorylation itself on structural changes in the receptor can be investigated by electron paramagnetic resonance (EPR) spectroscopy, as it is more sensitive than NMR. A conformational change induced by phosphorylation in the smooth muscle myosin and ERK2 was recently investigated by this method (Nelson *et al.,* 2005; Hoofnagle *et al.,* 2004).

5.2 Transcriptional activity of the panel of test compounds

The potency and efficacy in transactivation for the panel of test compounds were determined mainly for the usage of these data in the correlation analyses between the behaviour of the liganded-GR at specific steps in the GR transcriptional regulation pathway. As the transactivation was investigated in several different cell lines with the same promoter-reporter construct, many interesting aspects of cell-specific differences for the test compounds became apparent. Some aspects that will be discussed in this section are the differences in rank order and relative potency and efficacy compared to Dex for the test compounds in different cell systems. The switch from antagonist to partial agonist activity or from partial agonist to full agonist activity that is observed for some test compounds is especially intriguing. Previous results by others have shown that this phenomenon can be either linked directly to receptor

levels in the cell system or the cellular environment, like the concentration of cofactors available (see section 1.8).

The agonist activity was determined for twelve test compounds in GR-mediated transactivation of a GRE-TAT promoter-reporter construct, in three different cell systems. These were A549 cells containing endogenous GR, COS-1 cells transiently transfected with a pCMV-HA-hGR expression vector and U2OS-hGR cells, stably transfected with the human GR. From these results summarised in Table 5.1, several insights on cell-specific effects on the same promoter-reporter construct for the panel of test compounds are apparent.

Table 5.1 Agonist properties for 12 test compounds in transactivation mediated via GR.

Test compound	A549	COS-1	U2OS-hGR
Dexamethasone	Full agonist	Full agonist	Full agonist
Cortisol	Full agonist	Full agonist	Full agonist
Prednisolone	Not tested	Full agonist	Full agonist
Progesterone	No agonist activity	Weak partial agonist	Partial agonist
MPA	Partial agonist	Partial/full agonist	Full agonist
NET	No agonist activity	No agonist activity	No agonist activity
Aldosterone	Not tested	Full agonist	Full agonist
RU486	No agonist activity	Weak partial agonist	Weak partial agonist
D06	Not tested	No agonist activity	No agonist activity
Compound A	No agonist activity	No agonist activity	No agonist activity
AL438	Not tested	Partial/full agonist	Full agonist
UDCA	Not tested	No agonist activity	No agonist activity

It has previously been shown for some ligands and in some cells (see section 1.8) that the absolute EC_{50} value (not the relative EC_{50}) for an agonist-GR complex is not constant for a given gene, but that it differs between different cell systems and even within the same cell (reviewed in Simons, 2003). This phenomenon is clearly shown in this study for a panel of ligands in the three cell systems. Several factors have been proposed to modulate the EC_{50} value of an agonist complex (reviewed in Simons, 2003) and these will be discussed within the context of what was observed in this particular study. Several studies have shown that the receptor concentration can shift the EC_{50} value for induction of genes by agonists to lower concentrations (Szapary *et al.*, 1996; Szapary *et al.*, 1999; Zhao *et al.*, 2003). This can clearly

be seen in this study as well. When transiently transfecting COS-1 cells with varying amounts of GR, the EC_{50} value for Dex decreases by 4-fold when the receptor concentration is increased by 10-fold (Figure 3.4). The difference in EC_{50} value is also apparent between the A549 cells and the two other cell lines. For example, in the A549 cells, Dex has an EC_{50} of 2.77 ± 0.63 nM. When comparing the potency of Dex in the A549 cells with the potency in the two other cell systems, about a 13-fold higher potency for Dex is observed in the COS-1 (EC_{50} = 0.228 ± 0.12 nM) and in the U2OS-hGR cells (EC_{50} = 0.18 ± 0.09 nM). Cort and MPA show a slightly lower difference in potency (5-fold) between the A549 cells and the two other cell lines. This most likely depends on the receptor concentration, as the Western blot analysis in Figure 3.7 shows a relatively low expression of endogenous GR in the A549 cells and relatively high expression of GR in transiently transfected COS-1 cells and stably transfected U2OS-hGR cells. Thus, the low potency for the ligands observed in the A549 cells, compared to the other cell lines, is probably due to low expression of the receptor. Slight differences in potency are also observed between the COS-1 and U2OS-hGR cells for the test compounds. Cort and Prog show a slight increase in potency (1.5- and 2.5-fold, respectively) while MPA and AL438 show a slight decrease in potency (1.5- and 1.7-fold, respectively) in the U2OS-hGR cells compared to COS-1 cells. Dex and Ald show no significant difference in potency between the two cell lines. These minor changes in EC_{50} values may not be significant and the log scale in the dose-response curves makes it difficult to draw a conclusion. The relative potency for Pred compared to Dex, however, changes significantly as Pred is more potent than Dex in the COS-1 cells (relative potency of 225.5 ± 114 %) as compared to in the U2OS-hGR cells (relative potency of 77.8 ± 54.9 %). This dramatic difference in potency for Pred could theoretically reflect varying levels of endogenous PR in the two cell systems, with more PR present in the U2OS-hGR cells. Insignificant levels of endogenous PR are present in the COS-1 cells as Pred has no transactivation activity in untransfected COS-1 cells and the effect observed by Pred is mediated via GR (Figure 3.3). The levels of endogenous PR in the U2OS-hGR cells have not been determined. However, if significant levels of PR were present in the U2OS-hGR cell line, it would be expected that the potency of other PR ligands, such as MPA and Prog, would also change significantly. However this is not the case. Thus, it is unlikely that endogenous PR in the U2OS-hGR cells is responsible for the difference in potency observed for Pred between the two cell lines.

The receptor level present in a cell is only one determinant of the EC_{50} value for an agonist-GR complex. However, other factors have also been proposed that are able to modulate the EC_{50} value. Several studies have shown that the level of coactivators and corepressors present in a cell system can shift the EC_{50} value of an agonist-GR complex, similar to receptor concentrations. It has been hypothesised that the conformational change induced in the receptor by an agonist will have a higher affinity for coactivators than corepressors (but both cofactors will bind) (Wang *et al.*, 2004). The observation that elevated concentrations of several p160 coactivators, like SRC-1, TIF2 and GRIP1 (Chen *et al.*, 2000; Cho *et al.*, 2005a; He *et al.*, 2002) and of corepressors, like NCoR or SMRT (Szapary *et al.*, 1999), in transiently transfected cells modify the EC_{50} values of agonist-GR complexes, supports this hypothesis. Also other coactivators or comodulators with HAT activity can play a role in GR-mediated induction of gene expression. Studies have shown that elevated levels of CBP (Szapary *et al.*, 1999), p/CAF (He *et al.*, 2002) or the tissue-specific coactivator GT198 (Ko *et al.*, 2002b) can lower the EC_{50} value of an agonist-GR complex. Therefore, the difference in relative potency compared to Dex for Pred between the COS-1 cells and the U2OS-hGR cells may be attributed to a different cofactor environment in the cellular milieu. This could also explain the difference observed for AL438 between the low potency that has been reported previously in Hep2G cells (EC_{50} of 800 nM) (Coghlan *et al.*, 2003) and the high potency in COS-1 (EC_{50} of 8.559 ± 3.35 nM) and U2OS-hGR cells (EC_{50} of 14.6 ± 5.44 nM) in this study on a similar promoter-reporter construct.

The studies of factors that affect the EC_{50} values for agonist-GR complexes also led to the observation that an increase in agonist activity (or relative maximum efficacy) of some antagonists and partial agonists, but not for full agonists and full antagonists, were modulated by many of the same factors as for the EC_{50} value (reviewed in Simons, 2003). The data (Table 5.1) in this thesis support and underline the importance of the cellular environment when investigating proposed ligands for GR. Prog and RU486 switch from no agonist activity in the A549 cells to a weak partial agonist in the COS-1 cells and to a partial and weak partial agonist, respectively, in the U2OS-hGR cells. AL438 switches from a partial/full agonist in the COS-1 cells to a full agonist in the U2OS-hGR cells. MPA also switches from weak partial agonist activity in the A549 cells (relative efficacy of 35.0 ± 5.65 %) to partial agonist activity in the COS-1 cells (relative efficacy of 73.25 ± 7.08 %) to full agonist activity in the U2OS-hGR cells (relative efficacy of 127 ± 17.5 %). To illustrate this effect, the dose-

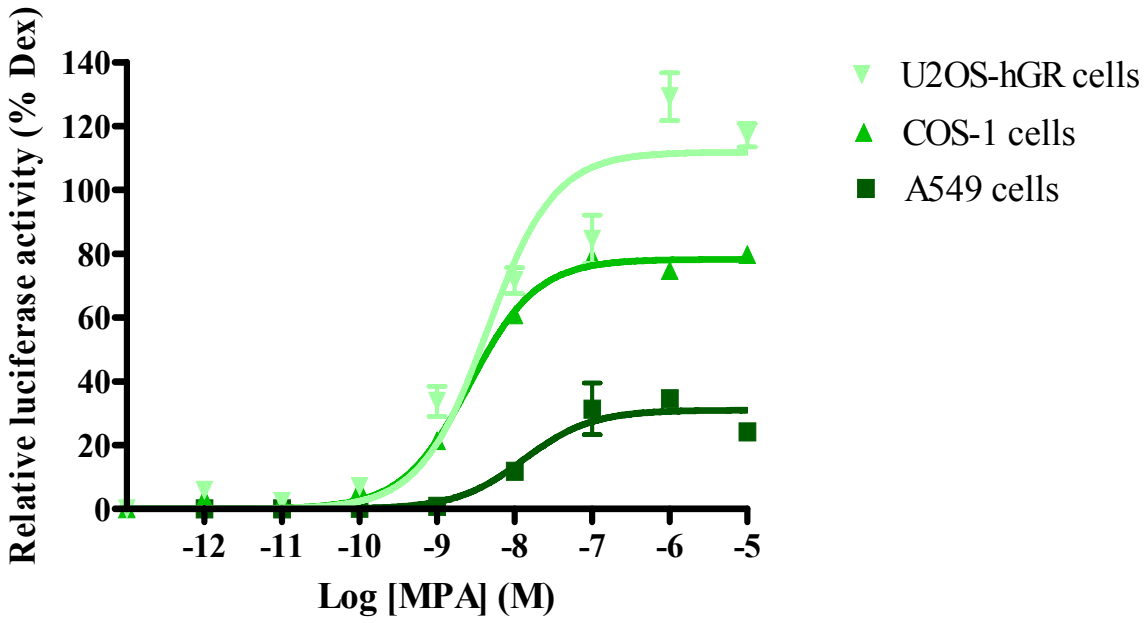


Figure 5.1. The effect of MPA on GR-dependent transactivation in three different cell systems. All cell lines were transfected with a pTAT-GRE-E1b-luc promoter-reporter construct and COS-1 cells with pCMV-HA-hGR. The following day, the cells were treated with various concentrations of MPA and 16 h later, the cells were harvested for luciferase assay. Data shown are representative of two (A549 cells) or three (COS-1 and U2OS-hGR cells) independent experiments, expressed as relative luciferase activity and triplicates are plotted as mean ± SEM.

response curves for MPA in GR-mediated transactivation in the three cell systems investigated are shown in Figure 5.1. Thus, these results are consistent with the hypothesis that partial agonists can have different relative maximum efficacies on the same gene promoter in different cellular environments.

It has been shown for many partial agonists that increased maximal agonist activity is dependent on receptor and cofactor concentrations present in the cell. Increased expression of the receptor and coactivators GRIP1, TIF2 and SRC-1 increase the partial agonist activity of GCs (Szapary *et al.,* 1999). MPA has been shown by others to switch from partial agonist to full agonist activity, and RU486 and Prog from an antagonist to a partial agonist in several studies, depending on receptor and cofactor concentration in the system (Chen *et al.*, 2000; Bamberger *et al.,* 1999; Zhao *et al.,* 2003; He *et al.,* 2002; Cho *et al.,* 2005a; Wang *et al.,* 2004b). This can also clearly be seen in the model systems tested in this study. The differences in levels of partial agonist activity of Prog, RU486 and MPA in A549 cells versus in COS-1 cells can be explained by the differences in receptor concentration present in the

cell systems, as suggested by the Western analysis (Figure 3.7), where the A459 cells have low levels while the COS-1 cells have high levels of GR.

However, the switch observed for MPA and AL438 between the COS-1 and U2OS-hGR cells cannot be explained by receptor density as the COS-1 cells have similar receptor concentration to the U2OS-hGR cells. The cell-specific effects seen with these partial agonists might be due to differential levels of expressed cofactors in the cell systems or due to cell-specific cofactors in the U2OS-hGR cells that preferentially interact with a particular liganded-GR complex, increasing the activity of that particular ligand. Coghlan *et al.* have already shown that AL438 is unable to recruit PGC-1, but is fully capable of binding to GRIP1, as compared to Pred, which binds both coactivators efficiently (Coghlan *et al.,* 2003). This differential cofactor recruitment by AL438 may apply to MPA as well and might cause the difference in efficacy observed between the two cell systems. Therefore, the differential effects seen between different ligands on the same gene in the same cell might not necessarily just be differential interaction with the same cofactor, as proposed in several studies (He *et al.,* 2002; Cho *et al.*, 2005a; Wang *et al.*, 2004b), but also the recruitment of different cofactors, as proposed by Coghlan *et al.* (Coghlan *et al.,* 2003).

The mechanisms of partial agonist activity and especially, the switch in agonist activity for some partial agonists and antagonists are not well understood. Several hypotheses have been proposed but no definite evidence exists to explain this phenomenon. Structural features of the ligand and the conformational change induced in the LBD of the receptor by the ligand are important, possibly leading to differential interactions with cofactors and coregulators, as discussed earlier. However, why increased concentrations of the receptor result in an increased relative agonist activity of a partial agonist, relative to Dex, is still unknown. Cho *et al.* have found that at high GR concentrations cooperative binding of ligands to receptor dimers occurs while at low GR concentrations mostly monomeric species of GR are present and therefore the binding of ligands is non-cooperative (Cho *et al.,* 2005b). It was proposed that the monomers present at low GR concentrations would have an N- to C-terminal intramolecular association while at high GR concentrations intermolecular association occurs between the receptor molecules causing the formation of homodimers. It was found that a specific cofactor, Ubc9, could modulate the agonist activity of a partial agonist only at high GR concentrations, possibly because the binding site for this cofactor lies within the N-terminal domain and is only available when dimers are formed, which is mediated through the

C-terminal (Cho *et al.,* 2005b). Hence, it may be that Prog, RU486 and MPA, unlike agonists like Dex, recruit specific cofactors that only bind to the GR at high receptor concentrations, causing an increase in their agonist activity in cell systems with high receptor density.

The ability of various factors, such as the receptor and cofactor concentrations present in a cellular environment, to modulate the response of a ligand in gene regulation has significant physiological implications. Although GR is expressed in almost all tissues and cells, the expression levels vary considerably between tissues or cell types (Miller *et al.*, 1998). The expression of various GR isoforms (see section 1.4.4-1.4.7) also differs between tissues and as these isoforms have different activities in transcriptional regulation, this may lead to differential gene induction among cell types. Differential levels of phosphorylated GR are also present depending on cell type, which can lead to differential GC sensitivity and regulation of GR target genes (Lee *et al.,* 2005). Even though, the circulating concentration of an endogenous hormone can be assumed to be the same throughout the human body, the hormone will induce differential gene expression for the same gene in different cells and/or for different genes within a single cell. This variation is likely to be beneficial as it could provide differential gene induction in different cellular environments by the same concentration of circulating steroid. Therefore, the variation in potency and efficacy of GCs depending on receptor and cofactor concentration in GR-mediated transcriptional regulation has significant clinical implications (reviewed in Simons, 2003). In endocrine therapy, antagonists are used for the suppression of elevated levels of endogenous steroids, like in Cushing's syndrome (Cadepond *et al.*, 1997). These compounds often produce undesirable side-effects due to the suppression of all genes induced by the steroid they antagonise. Therefore, antagonists which display partial agonist activity for most responsive genes and antagonise designated genes may be more appropriate (Chen *et al.*, 2000; He *et al.*, 2002; Szapary *et al.*, 1999). This selective blockage of clinically relevant target genes would reduce the number of undesirable side-effects. A partial agonist, like MPA, can also act as a partial antagonist. Therefore, the density of the receptor present in a cell could determine the activity of MPA. In cells with low levels of GR, MPA will have some partial agonist activity but at the same time be able to antagonise endogenous GC-dependent transcriptional regulation leaving the overall response reduced. In cells with high levels of GR, MPA will have an effect on GR-mediated transcriptional regulation but will not change the overall effect of the endogenous GCs. GCs are potent inhibitors of NF-κB proinflammatory gene expression and in human endothelial cells, hydrocortisone prevents NF-κB nuclear translocation and DNA

binding. However, MPA reduces the effect of hydrocortisone (at physiological concentrations), acting as a partial antagonist, resulting in increased proinflammatory gene expression. In contrast, Prog shows no effect, reflecting the affinity of MPA and Prog for GR (Simoncini *et al.,* 2004).

The classification of the GR ligands in section 1.9 was based on previous studies by others with these ligands. It is important to note that the classification of a ligand to a particular class is an operational definition, in the sense that ligands display cell-specific and promoter-specific properties. Thus for some of the ligands in this study, the biological activities in the systems used were different to that expected from the classification used in the Introduction. Although the correlation analyses were performed using the classification groupings for the ligands as outlined in the Introduction, regrouping some of the ligands may have been more appropriate. Only two studies have previously investigated the glucocorticoid properties of NET-A (Koubovec *et al.,* 2005; Schoonen *et al.*, 2000). NET-A was classified as a dissociative GC for GR in work recently published by our lab where NET-A shows 23 % efficacy in transrepression compared to Dex with no agonist activity in transactivation in HEK293 cells on the same promoter-reporter constructs as used in this study (Koubovec *et al.*, 2005). However, in this study, NET-A displays no agonist activity for transactivation in either cell line tested and no agonist activity for transrepression in COS-1 cells. Thus, according to the definition in Chapter 1, NET-A could be classified as an antagonist in these model systems. Ald acts as a full agonist in the present study, with a relative efficacy compared to Dex of 90.65 ± 10.6 % and 116 ± 14.1 % in the COS-1 cells and U2OS-hGR cells, respectively. Ald was classified as a partial agonist in section 1.9.2, however, full agonist activity has been observed previously in CV-1 cells (Grossmann *et al.*, 2004). RU486 was classified as an antagonist but RU486 behaves like a weak partial agonist for transactivation in both the COS-1 and U2OS-hGR cells with a relative efficacy compared to Dex of 8.385 ± 1.56 % and 12.3 ± 1.88 %, respectively, and like a full agonist in transrepression in COS-1 cells. CpdA has no agonist activity in either GR-mediated transactivation or transrepression in this study, and as for NET-A, CpdA could thus be classified as an antagonist in these systems. CpdA was classified as a SEGRA in section 1.9.3 as others have earlier observed significant agonistic properties in transrepression, but no activity in transactivation, for CpdA (De Bosscher *et al.,* 2005; personal communication Prof. Hapgood and Dr. Louw). Because of these observations, the correlation analyses data were discussed and analyzed for the entire panel of test compounds primarily. However, the

correlation analyses were also performed without possible subgroupings to pick out any general trends or unusual properties of specific ligands. There are clearly cell-specific differences for some of the individual compounds in both phosphorylation and transactivation, but the overall trend is that the correlation between phosphorylation at Ser211 and transactivation holds.

One of the goals of this study was to determine the agonistic properties of all the test compounds in GR-mediated transrepression and to test if a correlation exists between phosphorylation at Ser211 and transrepression, as with transactivation. However, it proved to be difficult to establish the transrepression assay in COS-1 cells and a further investigation of this problem was not within the scope of this thesis. These parameters will be investigated further in the future by others, using different promoter reporter constructs and different inducers such as PMA. In addition, a different cell line, like the U2OS-hGR cells will be investigated. Further insights into a possible correlation between phosphorylation and transrepression will therefore have to wait until more transrepression data have been obtained for the all the test compounds.

It would also be interesting to directly investigate the antagonistic activity in GR-mediated transcriptional regulation by the test compounds and to correlate this with the phosphorylation studies as well. However, it can be predicted that any compound that has a lower efficacy (partial agonist) than another will antagonise the effect of the other compound with higher efficacy (full agonist) if the experimental design allows it to. Antagonism could be achieved if the concentration of a high-affinity partial agonist is similar to the concentration of the ligand it is competing off the receptor. Similarly, antagonism could be achieved if the concentration of a low-affinity ligand is high and the concentration of the ligand it is competing of the receptor is low. For example, RU486 and MPA, will most likely antagonise Dex-mediated transactivation in the A549 cells at low concentrations as the binding affinity for GR is high (Koubovec *et al.*, 2005; Wagner *et al.*, 1999) and the relative efficacy in transactivation of RU486 (0 %) and MPA (35.0 ± 5.65 %) is low in this system. Prog and NET-A will only antagonise Dex-mediated transactivation at high concentrations with low Dex concentration, as the relative binding affinity for GR is low for these two compounds (Koubovec *et al.*, 2005). Thus, great care must be taken when designing the antagonistic experiments for them to have some physiological relevance.

CpdA has previously shown atypical binding properties for GR as described in Chapter 4 and it is necessary to conduct much more research to understand the possible binding of CpdA to GR and how this compound mediates its effects via the receptor. One possibility to further investigate CpdA binding to GR, is to use [^{3}H]-CpdA. This would be an important tool to examine the direct binding of the compound to the receptor and it could explain why the binding of CpdA to GR differs from Dex. Structure analysis and crystallography would also be helpful in understanding how the compound possibly binds to the LBD of the receptor. Therefore, a further investigation into the binding properties of CpdA would be of great interest and it can possibly give some valuable insights into the mechanism of dissociated glucocorticoid activity.

5.3 Concluding remarks

The data presented in this thesis show that the phosphorylation induced at Ser211 of GR by a ligand correlates in general with the potency and efficacy of the ligand in transactivation. The correlation is significant at saturating concentrations in both cell systems investigated. The correlation is weaker at subsaturating concentrations, due to either experimental error (single experiment) or differential induction of phosphorylation by partial agonists at subsaturating concentrations. When the partial agonist subgroup is removed from the correlation analyses, the correlation is stronger, indicating that the hypothesis does not hold for this particular subgroup of test compounds. The preliminary observations with the transrepression data suggest that a correlation might exist between phosphorylation at Ser211 and the potency and efficacy in transrepression.

Experiments with a mutant (S211A) receptor show that the presence of the phosphate group at Ser211 is important for the full maximal response, but not for the ligand-specific relative potency and efficacy in transactivation. The phosphorylation at Ser211 is, therefore, unlikely to be responsible for ligand-selective effects in GR-mediated transcriptional regulation. Thus, other steps in the GR transcriptional pathway must also be essential in determining the ligand-selective responses. As discussed, it is believed that the conformational change induced in the receptor by a ligand determines the interactions between the ligand-receptor complex and various coregulators important for the transcriptional regulation by GR. The phosphorylation at Ser211 is most probably an indirect effect of the conformational change induced in the receptor by the ligand. However, phosphorylation at Ser211 has a direct effect on the full

maximal response of a ligand in transactivation and the mechanism for this effect is still an unanswered question. Possibly, phosphorylation at Ser211 is somehow involved in the recruitment of cofactors. Further investigations into these issues, the cofactor recruitment by a liganded-GR complex and the conformational change induced by a ligand in the receptor, might help us to understand which other factors, besides phosphorylation at Ser211, determine the physiological response of a GR ligand.

This study also investigated the binding properties of CpdA to GR. The results concluded that CpdA does not differentiate between certain GR subpopulations investigated, but CpdA may still differentiate between other GR subpopulations, like monomer and dimers. Further binding studies with CpdA to GR are necessary to understand how CpdA mediates its effects through the receptor.

This thesis has looked at one specific step in the GR-regulation pathway believed to be involved in ligand-selective effects in gene transcription. Other steps will be investigated in the future in our laboratory in order to determine the mechanisms behind ligand-specific physiological responses. By understanding the fundamental mechanisms and the determinants of the physiological response of a liganded-GR, it might be possible in the future to design synthetic glucocorticoids with much better therapeutic results and fewer side effects.

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