Comparative study of the molecular mechanism of action of the synthetic progestins, Medroxyprogesterone acetate and Norethisterone acetate

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives (norethisterone enanthate (NET-EN); norethisterone acetate (NET-A)), are used by millions of women as contraceptives and in hormone replacement therapy (HRT). Although both progestins are widely used, very little is known about their mechanism of action at the molecular level. In this thesis, the differential regulation of gene expression and molecular mechanism of action via different steroid receptors by these synthetic progestons, as compared to progesterone (Prog) was investigated in human cell lines. In the first part of the study, the effect of Prog, MPA and NET-A on the expression of endogenous cytokine genes was investigated in two epithelial cell lines of the human female genital tract, Ect1/E6E7 (an ectocervical cell line) and Vk2/E6E7 (a vaginal cell line). Quantitative realtime RT-PCR (QPCR) showed ligand-specific and cell-specific regulation of the interleukin (IL)-6, IL-8 and RANTES (Regulated-upon-Activation, Normal T cell Expressed and Secreted) genes with Prog, MPA and NET-A. Moreover, the repression of the TNFα-induced RANTES gene by MPA in the Ect1/E6E7 cell line was found to be mediated by the androgen receptor (AR). The second part of the study focused on elucidating the androgenic activities of these two progestins, in comparison to Prog. Competitive binding in whole cells revealed that Prog, MPA and NET-A have a similar binding affinity for the hAR as the natural androgen dihydrotestosterone (DHT). Both transactivation and transrepression transcriptional assays demonstrate that, unlike Prog, MPA and NET-A are efficacious AR agonists, with activities comparable to DHT. Using a mammalian two-hydrid assay, it was shown that MPA and NET-A exert their androgenic actions by different mechanisms. NET-A, like DHT and other well-characterised androgens, induces the ligand-dependent interaction between the NH_2- and COOH-terminal domains (N/C-interaction) of the AR independent of promoter-context, while MPA does this in a promoter-dependent manner. In the third part of this study, competitive binding revealed that MPA and NET-A have a similar binding affinity to each other, but about a 100-fold lower affinity than Prog for the human mineralocorticoid receptor (hMR), while RU486 has an even lower affinity for the hMR. Promoter-reporter assays showed that MPA, NET-A and RU486 are all antagonists of the hMR, but unlike Prog, they have weak antagonistic activity. However, on the endogenous MR-regulated Orm-1 (α-glycolytic protein or orosomucoid-1) gene expressed in a rat cardiomyocyte cell line, NET-A and RU486, but not MPA, has similar antagonistic activity as Prog. This study is the first to show that, NET-A and RU486, but not MPA, can dissociate between transrepression and transactivation via the hMR. Taken together, these results show that natural Prog and the synthetic progestins, MPA and NET-A display differential promoter-, cell- and receptor-specific effects on gene expression. Furthermore they may have important implications for cervicovaginal immune function, cardiovascular and other physiological functions.

OPSOMMING

Medroksieprogesteroon asetaat (MPA), noretisteroon (NET) en derivate daarvan (noretisteroon enantaat (NET-EN); noretisteroon asetaat (NET-A), word deur miljoene vroue gebruik as voorbehoedmiddels en vir hormoon vervangingsterapie (HVT). Tenspyte daarvan dat beide hierdie progestiene algemeen gebruik word, is min bekend oor hulle meganisme van werking op molekulêre vlak. In hierdie proefskrif word die differensiële regulering van geenuitdrukking asook die molekulêre meganisme van werking deur middel van steroïedreseptore van beide hierdie sintetiese progestiene, ondersoek, en vergelyk met progesteroon (Prog), in menslike sellyne. In die eerste deel van die studie is die effek van Prog, MPA en NET-A op die uitdrukking van endogene sitokinien gene ondersoek in twee epiteel sellyne van die menslike vroulike geslagskanaal, Ect1/E6E7 ('n ektoservikale sellyn) en Vk2/E6E7 ('n vaginale sellyn). Kwantitatiewe intydse RT-PKR het ligand-spesifieke en sel-spesifieke regulering van interleukien (IL)-6, IL-8 en RANTES (Regulering-na-Aktivering, Normale T-sel Uitgedrukte en Afgeskei) gene getoon met Prog, MPA en NET-A. Verder is gevind dat die onderdrukking van die TNF-α-geïnduseerde RANTES geen deur MPA in die Ect1/E6E7 sellyn bemiddel word deur die androgeen reseptor (AR). Die tweede deel van die studie het gefokus op die toeligting van die androgeniese aktiwiteit van die twee progestiene in vergelyking met Prog. Kompeterende binding in volselle het getoon dat Prog, MPA en NET-A 'n soortelyke bindings affiniteit vir die menslike AR as die natuurlike androgeen dehidrotestosteroon (DHT) vir die menslike AR het. Beide transaktiverings en transonderdrukkings transkripsionele analieses toon dat, anders as Prog, MPA en NET-A effektiewe AR agoniste is met aktiwiteite wat vergelykbaar is met die van DHT. Deur die gebruik van 'n soogdier twee-hibried toets, kon gewys word dat MPA en NET-A hul androgeniese effekte uitoefen deur verskillende meganismes. NET-A, soos DHT en ander goed gekarakteriseerde androgene, induseer die ligand-afhanklike interaksie tussen die NH_2- en COOH-terminale domeine (N/C-interaksie) van die AR, onafhanklik van die promoter-konteks. MPA, aan die ander kant, doen dit op 'n promoter-afhanklike manier. In die derde deel van die studie het kompeterende binding getoon dat MPA en NET-A soortelyke relatiewe bindings affiniteite vir die menslike mineralokortikoïed reseptor (hMR) het, maar dat hierdie affiniteit ongeveer 100-voud laer is as die van Prog en dat die affiniteit van RU486 vir hMR selfs nog laer is. Promoter-rapporteerder toetse het getoon dat MPA, NET-A en RU486 almal antagoniste van die hMR is, maar anders as Prog, is hierdie 'n swak antagonistiese aktiwiteit. Nietemin, op die endogene MR-gereguleerde Orm-1 (α-glikolitiese proteïen of orosomukoïed-1) geen, uitgedruk in 'n rot kardiomiosiet sellyn, het NET-A en RU486, maar nie MPA nie, 'n soortgelyke antagonistiese aktiwiteit as Prog. Hierdie studie is die eerste om te wys dat NET-A en RU486, maar nie MPA nie, kan onderskei tussen transrepressie en transaktivering deur middel van die hMR. Samevattend toon die resultate dat natuurlike Prog en die sintetiese progestiene, MPA en NET-A, 'n differentiële promoter-, sel- en reseptor-spesifieke effek op geenuitdrukking het. Verder mag die resultate belangrike implikasies vir servikovaginale immuunfunksie, asook kardiovaskulêre en ander fisiologiese funksies, inhou.

To my family…

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AF-1	activation function-1
AF-2	activation function-2
Ald	aldosterone
AP-1	Activator Protein-1
AR	androgen receptor
ARE(s)	androgen response element(s)
ATCC	American Type Culture Collection
BMD	bone mineral density
bp	base-pair
CBG	corticosteroid-binding globulin
cDNA	complementary DNA
CEE / MPA	conjugated equine estrogen / medroxyprogesterone acetate
CHD	coronary heart disease
Cort	cortisol
C-terminal	carboxy-(COOH-) terminal
CVD	cardiovascular disease
DBD	DNA-binding domain
DEPC	diethylpyrocarbonate
Dex	Dexamethasone
DHT	dihydrotestosterone

DMEM	Dulbecco's Modified Eagle Medium
DMPA	depot medroxyprogesterone acetate
DHP	5α-dihydroprogesterone
DNA	deoxyribonucleic acid
E_2	17β-estradiol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
ES	endometrial stromal
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
FSH	follicle-stimulating hormone
GCs	glucocorticoids
GR	glucocorticoid receptor
GRE(s)	glucocorticoid response element
HESCs	human endometrial stromal cells
HIV	human immunodeficiency virus
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPLC	high-performance liquid chromatography
HPV	human papillomavirus
HRE(s)	hormone response element(s)
HRT	hormone replacement therapy
Hsp(s)	heat-shock protein(s)

HSV-1	herpes simplex virus-1
HSV-2	herpes simplex virus-2
HT/HRT	hormone replacement therapy
5-HT	5-hydroxytryptamine
HUVECs	human umbilical vein endothelial cells
IL-1	interleukin -1
IL-2	interleukin -2
IL-6	interleukin -6
IL-8	interleukin -8
IgG	immunoglobulin G
iNOS	nitric oxide synthase
JNK	Jun N-terminal kinase
LH	luteinizing hormone
LNCaP	lymph node carcinoma of the prostate
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MEK	MAPK kinase
MIB	mibolerone
MR	mineralocorticoid receptor
M-MLV	Moloney Murine Leukemia Virus
MMTV	mouse mammary tumor virus
MPA	medroxyprogesterone acetate
MRE(s)	mineralocorticoid response element(s)
mRNA	messenger ribonucleic acid
N-CoR	nuclear receptor co-repressor

NET	norethisterone / norethindrone
NET-A	norethisterone / norethindrone acetate
NET-EN	norethisterone / norethindrone enanthate
NFκB	nuclear factor kappa-B
NOS	nitric oxide synthase
nGRE	negative glucocorticoid response element
NTD	amino (NH_2)-teminal domain
N/C-interaction	interaction between the N- and C-terminal domains
OHF	hydroxyflutamide
Orm-1	α-acidic glycoprotein or oricomucosoid
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PHA	phytohemaglutinin
PMA	phorbol-myristate acetate
PR	progesterone receptor
Prog	progesterone
PTHrP	parathyroid hormone related peptide
RANTES	Regulated-upon-Activation, normal T cell Expressed and Secreted
R1881	methyltrienolone
R5020	promegestone
RT-PCR	reverse transcription-polymerase chain reaction
RU486	mifepristone

SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SHBG	sex hormone binding globulin
SHIV	simian-human immunodeficiency virus
SRC-1	steroid receptor co-activator-1
STI	sexually transmitted infection
THP	3α,5α-tetrahydroprogesterone
TNF-α	tumor necrosis factor-alpha
TPA	tetradecanoyl phorbol acetate
WHI	Women's Health Initiative
WHIMS	Women's Health Initiative Memory Study
WHO	World Health Organization
WISDOM	Women's International Study of Long Duration Oestrogen after the Menopause

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THESIS OUTLINE

This thesis consists of five chapters. Chapters 1, 2, 3 and 4 are written up in manuscript format. Chapters 2, 3 and 4 include a brief introduction to the specific aims of the particular studies, report and discuss the undertaken experiments and the results obtained. These chapters will shortly be submitted for publication. The references for all the chapters are included in one section following Chapter 5.

1. Chapter 1: **Literature review**. This chapter gives a detailed overview of the relevant knowledge currently available in the literature, with a particular focus on directly comparing the molecular mechanism of action of medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN)/norethisterone acetate (NET-A). The review was written by the candidate. Nicolette Verhoog also works on the progestin project, and contributed in the exchange of information and ideas. Dominique Koubovec previously reviewed the topic in her PhD thesis and this material was used as a guideline for the current review.
2. Chapter 2: **Differential regulation of endogenous pro-inflammatory cytokine genes by MPA and NET-A in cell lines of the female genital tract.** This chapter contains the results of a study investigating and comparing the regulation of endogenous cytokine genes by Prog, MPA and NET-A in Ect1/E6E7 (human ectocervical) and Vk2/E6E7 (human vaginal) cell lines. All experiments were performed by the candidate, except for the maintenance of the cell lines, which was performed by Carmen Langeveldt.

3. Chapter 3: **A comparative study of the androgenic properties of progesterone and the synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A).** This chapter includes the results of a study into the molecular mechanism of action of MPA and NET-A, as compared to Prog, via the hAR in the COS-1 cell line. All experiments were performed by the candidate, except for the maintenance of the COS-1 monkey kidney cell line, which was performed by Carmen Langeveldt.
4. Chapter 4: **Investigating the anti-mineralocorticoid properties of synthetic progestins used in hormone replacement therapy.** This chapter reports on the findings of a study investigating whether MPA and NET-A, like Prog, can act via the MR. This study was performed in COS-1 (monkey kidney) as well as H9C2 cell lines (rat cardiomyocytes). All experiments were performed by the candidate, except for the maintenance of the cell lines, which was performed by Carmen Langeveldt.
5. Chapter 5: **Conclusions and Future Perspectives.** In this final chapter, the results of the overall study are discussed and conclusions drawn in the context of the larger body of work.

The appendices (A-D), found at the back of the thesis, include data not shown, but referred to as supplementary material within the manuscripts, as well as additional results not included in the chapters and definitions.

The literature review presented in Chapter 1 will be submitted to Endocrine Reviews. The manuscript presented in Chapter 2 will be submitted to Contraception, while the manuscripts presented in Chapter 3 and Chapter 4 will be submitted to the Journal of Steroid Biochemistry and Molecular Biology and Molecular and Cellular Endocrinology, respectively.

Consistent with manuscript format, the collective term “we” and “our” is often used in the thesis. However, all the experimental work was performed by the candidate, barring the figure in Appendix B5 which was performed by Tamzin Tanner (MSc thesis) previously from the Hapgood laboratory.

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Chapter 1

Reviewing the molecular mechanism of action of medroxyprogesterone acetate and norethisterone enanthate/acetate

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Abstract

Medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives (norethisterone enanthate (NET-EN); norethisterone acetate (NET-A)), are used by millions of women as contraceptives and in hormone replacement therapy (HRT). In addition, both MPA and NET-acetate (NET-A) are used in cancer therapy and in the treatment of gynaecological disorders such as endometriosis and premenstrual dysphoria. Although both progestins are widely used, very little is known about their mechanism of action at the molecular level. The importance of investigating these mechanisms, as compared to those of progesterone (Prog), has recently been highlighted by clinical evidence showing that MPA increases the risk of the development of breast cancer and coronary heart disease in HRT users. In addition, use of MPA as a contraceptive has also been shown to increase viral shedding, which raises concern as to its impact on the spread of viral diseases. There are currently no reviews in the literature other than one of our own, comparing the mechanism of action of MPA and NET-A or NET-EN (Hapgood *et al.,* 2004). Here we review the physiological effects of these two progestins, as well as their regulation of and/or binding to serum-binding proteins and steroidogenic enzymes. In addition, as it is known that both MPA and NET can bind not only to the progesterone receptor, but also to the glucocorticoid, androgen, mineralocorticoid, and possibly the estrogen receptors, it is plausible that MPA and NET exert therapeutic actions as well as side-effects via some of these receptors. We thus also review the molecular mechanism of action of both MPA and NET via each of the above steroid receptors on various target genes.

1.1 Introduction

Progestins are a class of synthetically developed compounds. Their development was based on similarity of biological actions to that of the endogenous ovarian hormone progesterone, which plays a pivotal role in female reproduction. These progestins have many therapeutic applications in female reproductive medicine, and are used instead of progesterone because of their longer biological half-life (Speroff, 1996). A wide variety of progestins are available, that in addition to their common progestogenic effects, exhibit a range of biological effects that differ not only from each other, but also from that of progesterone (Schindler *et al.,* 2003). The synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN), are two highly effective injectable progestogen-only contraceptives that have been available in many countries for over 40 years (Westhoff, 2003), including South Africa. In fact, in South Africa, MPA and NET-EN are the most commonly used contraceptives (Draper *et al.,* 2006). These progestins are not only used as contraceptive agents, but also in hormone replacement therapy (HRT) (also referred to as hormone therapy (HT)), as well as a number of other non-contraceptive therapies.

Progestins are chemically derived from parent compounds such as testosterone resulting in the 19-nortestosterone derivatives or from progesterone, resulting in the 17-hydroxy (17-OH) progesterone derivatives and 19-norprogesterone derivatives. MPA (6α-methyl-17-acetoxy pregn-4-ene-3, 20-dione) (also referred to as Depo-Provera®, depot medroxyprogesterone acetate (DMPA) or Petogen®, the latter locally manufactured in South Africa) is a 17-OH progesterone derivative (21-carbon series steroid) containing the pregnane nucleus, while NET-EN (17α-ethynyl-17β-

heptanoyloxy-4-estren-3-one) (also referred to as either norethindrone enanthate, norethisterone enanthate or Nur-Isterate®) is a 19-nortestosterone derivative (19-carbon series steroid) containing the androstane nucleus (structures depicted in Figure 1). Due to the aforementioned structures, MPA is often referred to as a true progestin, while NET-EN, which retains its androgenic activity, is referred to as an androgenic progestin (Darney, 1995).

As with most drugs, a number of side-effects, some more severe than others, have been reported with the clinical use of MPA and NET. Interestingly, the World Health Organization (WHO) classifies MPA and NET-EN in the same category of medical eligibility, and makes no distinction between the two with regard to their side-effects or contra-indications (WHO, 2004). Similarly, Haider and Darney (2007) recently reported that NET-EN has the same mechanism of action as MPA in terms of contraceptive action and efficacy, with the same advantages and disadvantages. The advantages refered to the convenient and effective contraceptive method, the fact that it can be used by women with contra-indications to estrogen, as well as some therapeutic benefits, while the disadvantages refer to the side-effect profile. Similarly, the Cochrane comparative review on the contraceptive effectivity, reversibility and side-effects of MPA and NET-EN, also reported that MPA and NET-EN are similar, barring the slower return to fertility with MPA (Draper *et al.,* 2006). While clinical studies have not identified significant differences between the side-effect profile of MPA and NET in patients, given the enormous spectrum of possible side-effects, and the specific circumstances under which these may manifest, it is possible that differences may yet be identified, especially given the differences in their activity recently identified at a cellular level (Koubovec *et al.,* 2005; Sasagawa *et al*., 2008).

Interestingly, these studies observed differences in mechanism of action not only between MPA and NET, but also as compared to Prog. These observed differences in activity are a matter of concern, especially since these progestins are usually reported to act in a similar manner. A number of factors may account for the differential effects of MPA vs. NET, such as their differences in molecular structure, metabolism, bio-availibility, and binding affinities to different steroid receptors or receptor isoforms (Stanczyk *et al.,* 2003; Schindler *et al.,* 2003). It is thus clear that additional comparative studies between these two progestins, relative to each other and Prog, are needed at the cellular level. The objective of the present review is thus to highlight the differences between MPA and NET-EN/NET-A[1], as compared to Prog, in terms of (1) therapeutic applications, observed side-effects and physiological effects, (2) their interaction with serum proteins, and (3) their mechanism of action via different steroid receptors.

[1] NET-EN is a derivative of NET used in injectable contraception; NET-A is the acetate ester of NET and is used in oral contraception or HRT; the derivatives, NET-EN and NET-A, have to be metabolically converted to NET in order to become biologically active. At times, NET will be used generically to include all derivatives.

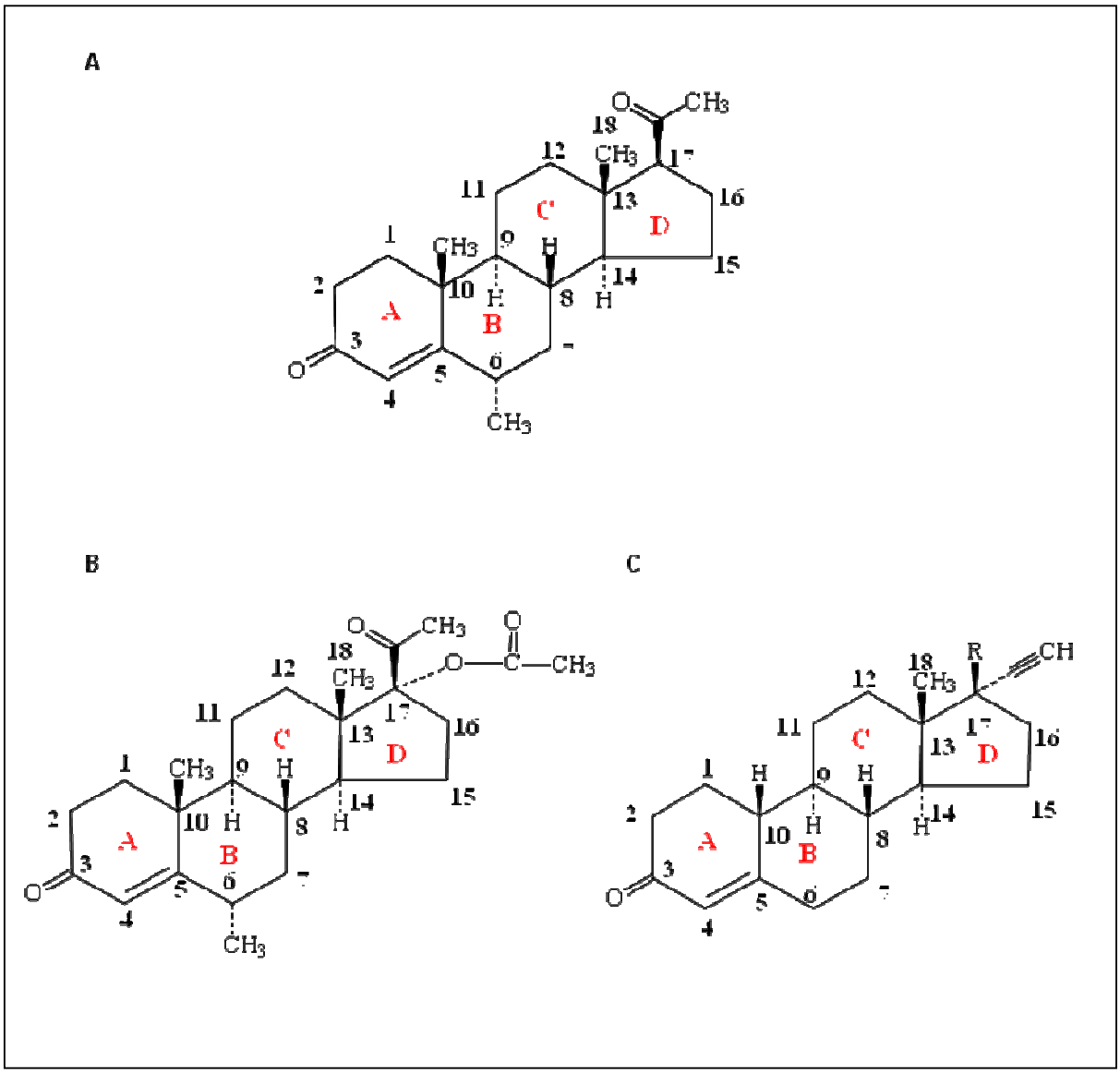

Figure 1. The chemical structures of (A) progesterone (Prog), (B) medroxyprogesterone acetate (MPA) and (C) norethisterone (NET) (R=OH), norethisterone acetate (NET-A) (R=$OCOCH_3$) and norethisterone enanthate (NET-EN) [R=$OCO(CH_2)_5CH_3$]. Note that 'norethisterone' is the same as 'norethindrone'. Basic ring structures are composed of 17 carbon arranged in four rings conventionally denoted by the letters A, B, C and D (adapted from Hapgood *et al.,* 2004).

1.2 Therapeutic applications

1.2.1 Contraception

1.2.1.1 Female contraception

When used as injectable contraceptives in women, both MPA and NET-EN formulations are administered as intramuscular injections, however, they differ in dosage and frequency of administration. MPA is administered as a 150 mg aqueous suspension every three months (Mishell, 1996), whereas NET-EN is administered as a 200 mg oily suspension every two months (Garza-Flores *et al.,* 1991). Interestingly, a new formulation of MPA, at a 30% lower dose (104 mg), has recently been approved in the United States for subcutaneous administration every 3 months (Jain *et al.,* 2004), and is referred to as Depo-Sub Q.

After injection, MPA is fairly stable and is itself the active contraceptive compound (Speroff, 1996), whereas NET-EN and NET-A are hydrolysed to norethindrone/norethisterone (NET) and other metabolites, which together have contraceptive action (Stanczyk and Roy, 1990). Women receiving the 150 mg intramuscular injection of MPA, typically have serum concentrations of about 2.6-3.9 nM for the duration of contraceptive treatment (Mathrubutham and Fotherby, 1981; Mishell, 1996), while the 200 mg dose of NET-EN has been reported to result in serum concentrations of about 1.5-59 nM (Fotherby *et al.,* 1983).

MPA mediates its contraceptive action by inhibiting the secretion of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), thereby preventing follicular maturation and ovulation (Mishell, 1996; Kaunitz, 2000; Greydanus *et al.,* 2001). MPA also alters the endometrial lining and reduces

glycogen secretion, thus preventing a blastocyst from entering the endometrial cavity (Mishell, 1996). In addition, MPA thickens the cervical mucus, which interferes with sperm penetration into the uterus (Greydanus *et al.,* 2001). Although NET-EN has also been shown to block ovulation, (Bhowmik and Mukherjea, 1988), its primary contraceptive action involves altering the content of cervical mucus thus preventing sperm movement into the uterine cavity (Bhowmik and Mukherjea, 1987). MPA and NET-EN therefore have multiple sites of action, and are thus both highly effective contraceptive agents in women.

Despite the effectiveness of MPA and NET-EN in preventing pregnancy, there are several side-effects associated with their use. The side-effect profile of both these progestins includes irregular bleeding, amenorrhea, breast tenderness, headaches, weight gain, acne and vaginal discharge (Kaunitz, 2000; Greydanus *et al.,* 2001; Benagiano *et al.,* 1978; Darney, 1995; Tyler, 1970; Schwallie, 1976; El-Mahgoub and Karim, 1972; Westhoff, 2003; Haider and Darney, 2007; Spencer *et al.,* 2009). Interestingly, in a community-based cross-sectional household survey to determine perceived side-effects of MPA and NET-EN in KwaZulu-Natal in South Africa, many women reported vaginal wetness as a side-effect (Smit *et al.,* 2002). It is unclear whether this vaginal wetness is the same as the previously reported side-effect of vaginal discharge. In addition, side-effects such as dizziness, fatigue, bloating of the abdomen or breasts, behavioural changes, reduced libido and decreased bone mineral density (BMD) have also been reported for MPA (Kaunitz, 2000; Greydanus *et al.,* 2001). Although the side-effect profile of NET-EN is not as well defined as that of MPA, it is assumed to be similar to, but less severe than MPA, with a more rapid return to fertility after termination of treatment (Benagiano *et al.,* 1978; Fraser and

Weisberg 1982; Koetsawang, 1991, Draper *et al.*, 2006). This difference in time before return to fertility has been challenged by Bigrigg and co-workers (1999), as they report that there is in fact, no statistically significant delay in return to fertility by MPA users.

1.2.1.2 Male contraception

Male contraception involves the administration of synthetic analogues of testosterone, which functions as a contraceptive by suppressing the secretion of the gonadotropins, LH and FSH, from the pituitary (Amory and Bremner, 1998; Morse *et al.,* 1973). The suppression of LH and FSH deprives the testes of the stimulatory signals required for spermatogenesis, leading to decreased sperm counts and reversible infertility in most men. However, the administration of testosterone derivatives alone does not completely suppress sperm production in all men (Amory and Bremner, 1998; McLachlan *et al.,* 2002). For this reason, recent research has been investigating the use of testosterone analogues in combination with progestins. Testosterone esters, combined with injections of MPA or NET-EN, show severe suppression of spermatogenesis, due to the synergistic suppression of gonadotropin levels (Kamischke *et al.,* 2000a; Kamischke *et al.,* 2000b; Turner *et al.,* 2003; reviewed by Nieschlag *et al.,* 2003; Gu *et al.,* 2004, reviewed by Amory, 2008). Thus, combined treatment with testosterone and progestins, holds promise for an effective male contraceptive. However, as observed in women, the use of synthetic progestins causes certain side-effects in men. For example, the use of NET-EN in male contraception has been shown to decrease the levels of high-density lipoprotein (HDL) and lipoprotein, which may lead to negative effects on the cardiovascular system (Zitzmann *et al.,* 2002). Current research in male hormonal contraception, as

with female contraception, is thus focusing on producing an effective contraceptive agent, while minimizing side-effects.

1.2.2 Hormone replacement therapy

Hormone replacement therapy (HRT/HT) is commonly prescribed to alleviate symptoms experienced by women after menopause. These symptoms, including hot flushes, urogenital atrophy, bone loss and vaginal dryness, are due to a decrease in estrogen levels (Hickey *et al.,* 2005; Greendale, 1999). HRT includes administration of either estrogen alone, or estrogen combined with a progestin, such as MPA or NET-EN (Greendale, 1999). The latter treatment is used for menopausal women with an intact uterus so as to counteract the proliferative effects of estrogen on the uterine epithelium, thereby preventing estrogen-induced endometrial hyperplasia (Gambrell Jr *et al.,* 1980; Taitel and Kafrissen, 1995; Brunelli *et al.,* 1996; Palacios *et al.,* 2006). The progestin may be administered either continuously (every day) or sequentially (for a part of each month). It has been reported that, in the longterm, continuous therapy may be more protective against endometrial hyperplasia than sequential therapy (reviewed by Lethaby *et al.,* 2004).

Whether MPA or NET is used as the progestin of choice for HRT differs internationally. In the United States, the most commonly used progestin is MPA, generally combined with conjugated equine estrogens (CEE) in formulations for oral administration (Newcomb *et al.,* 2002). Similarly, in France, MPA or cyproterone acetate is mainly used (Fournier *et al.,* 2005). In contrast, only a small percentage of women in the United Kingdom and Northern Europe use MPA (≤ 20%) (Beral, 2003; Magnusson *et al.,* 1999; Jernstrom *et al.,* 2003; Stahlberg *et al.,* 2004), while the

majority use NET-A or other 19-nortestosterone-derivatives (Campagnoli *et al.,* 2005; Fournier *et al.,* 2005).

Originally, the dose of MPA employed in HRT was a sequential dosage of 10 mg/day for about 11 days per month, but subsequently the dose has been reduced to 2.5 to 5 mg/day (Brunelli *et al.,* 1996; Van de Weijer, 2007; Archer and Pickar, 2000). HRT doses of NET range from about 0.35 to 2.1 mg/day (Taitel and Kafrissen, 1995). Women receiving the Activelle HRT regime (0.5 mg NET-A, 1 mg estradiol) are reported to have peak serum concentrations of NET ranging between 3.64 and 17.7 nM (Activelle package insert reg. no. 33/21.8.2/0532, Novo Nordisk Inc.), while serum levels of MPA for HRT range between 0.02 and 0.2 nM (Ghatge *et al.,* 2005).

Side-effects of MPA and NET used in HRT include changes in the levels of lipids and lipoproteins, as well as adverse effects on vasomotion, which may increase cardiovascular risk in postmenopausal women (Sitruk-Ware, 2000). In addition, MPA and NET have been implicated in increased risk of breast cancer development (Riis *et al.,* 2002; Stahlberg *et al.,* 2003). Although these side-effects have long been recognized, it had always been assumed that the benefits of HRT for postmenopausal women outweighed the risks. However, the Women's Health Initiative (WHI) trial in the USA of a combined estrogen and progestin (MPA) HRT regime in healthy postmenopausal women highlighted several side-effects such as increased risk of breast cancer, coronary heart disease (CHD), venous thromboembolism, stroke and dementia (Rossouw *et al.,* 2002). In addition, data by investigators working on the same trial (estrogen plus MPA) also suggested an increase in the risk for ovarian cancer (Anderson *et al.,* 2003). These side-effects

were deemed so severe that the trial was terminated two years earlier than planned. This caused much confusion and alarm amongst HRT users, and as a result many postmenopausal women have stopped using HRT (Ettinger *et al.,* 2004). It is noteworthy that a similar trial on the use of estrogen alone also indicated an increased risk of stroke, but no increase in breast cancer risk or cardiovascular disease (CVD) (Anderson *et al.,* 2004), thus implicating the MPA component in breast cancer and CVD side-effects. The Women's International Study of Long Duration Oestrogen after the Menopause (WISDOM) investigation (Vickers *et al.,* 2007) was prematurely stopped following publication of the results of the WHI study. Their results were consistent with the WHI study in indicating increased cardiovascular and thromboembolic risk when HRT (estrogen plus MPA) was started a considerable time after menopause (Vickers *et al.,* 2007). The 'Million Women Study' found that both MPA and NET substantially increased the risk of breast cancer in long-term HRT users (Beral; 2003). Taken together, results from these studies indicate that usage of MPA may result in increased risk of breast cancer. In addition, all studies except the Million Women Study which did not investigate cardiovascular effects, indicate that MPA may have increased risk of CVD. NET usage has also been implicated, in the Million Women Study, in an increase of breast cancer risk.

The controversy surrounding the risk/benefit ratio of progestins in HRT has resulted in a trend towards prescription of HRT with lower doses of progestin, and different routes of administration such as gels, sprays, vaginal rings, intrauterine systems or transdermal patches (Nath and Sitruk-Ware, 2009). In this way, the potentially harmful effects of estrogen on the endometrium are still counteracted by MPA, while the progestin-induced side-effects on the breast and heart may possibly be

minimized (Sitruk-Ware, 2007). However, the safety of these new systems (low dosage, parenterally administered) vs. the old (higher dosage, oral therapy) needs to be evaluated.

1.2.3 Other applications

In addition to the use of MPA and NET-EN/NET-A/NET in contraception and HRT, they are also used in a number of other therapeutic applications. MPA is used in the treatment of gynaecological disorders such as dysmenorrhea, menorrhagia (excessively heavy menstrual bleeding), ovulatory pain, pain associated with ovarian disease, premenstrual dysphoria, perimenopausal symptoms (Kaunitz, 1998) and endometriosis, a complex disorder causing pelvic pain and infertility (Irahara *et al.,* 2001; Harrison and Barry-Kinsella, 2000; Muneyyirci-Delale and Karacan, 1998; Vercellini *et al.,* 2003). NET can also be used in the treatment of endometriosis (Vercellini *et al.,* 2003), and the dosage used for both MPA and NET is about 50-100 mg/day (Harrison and Barry-Kinsella, 2000; Telimaa *et al.,* 1989). MPA has also been associated with haematological improvement in women with sickle cell disease (Grimes, 1999), as well as reduced seizure frequency in women with seizure disorders (Kaunitz, 2000). A recent study, however, observed increased seizure occurrences with the use of CEE/MPA in HRT, suggesting that MPA may not be the optimal progestin for HRT in postmenopausal women with epilepsy (Harden *et al.,* 2006).

In cancer therapy, MPA is used at very high doses (Etienne *et al.,* 1992; Yamashita *et al.,* 1996), typically between 500 and 1500 mg/day taken orally for about 12 weeks (Blossey *et al.,* 1984). At these high cancer therapy doses, serum levels of MPA are

approximately 0.14-1.7 µM (Thigpen *et al.,* 1999; Focan *et al.,* 2001), and potent glucocorticoid-like side-effects such as inhibition of adrenal function (Blossey *et al.,* 1984; Papaleo *et al.,* 1984; Lang *et al.,* 1990) and immunosuppression (Yamashita *et al.,* 1996; Mallmann *et al.,* 1990; Scambia *et al.,* 1988), have been observed.

Finally, MPA is also prescribed for mentally handicapped women who have menstrual hygiene problems (Elkins *et al.,* 1986). Furthermore it is also used in the treatment of deviant sexual behaviors in men such as pedophilia, exhibitionism, transvestism, and voyeurism (Kravitz *et al.,* 1995; Bradford, 1999). Although NET is not as widely used as MPA, with the exception of use for HRT and contraception, it has been used in the treatment of acne (Zouboulis and Piquero-Martin, 2003) and in treating gastrointestinal symptoms of women with colorectal endometriosis (Ferrero *et al.,* 2009).

1.3 Physiological effects of MPA and NET-EN/NET-A/NET

1.3.1 Reproduction

Hypothalamic gonadotropin-releasing hormone (GnRH) is the key hormone responsible for regulating reproduction. It is secreted by the hypothalamus and travels via the blood to the anterior pituitary where it binds to the GnRH receptor on the cell surface of gonadotrope cells. Intracellular signal transduction pathways are subsequently activated, stimulating the synthesis and release of the gonadotropins, LH and FSH. These gonadotropins then enter the systemic circulation to regulate gonadal function, including steroidogenesis and gametogenesis. MPA mediates its contraceptive action by inhibiting the secretion of LH and FSH, thereby preventing

follicular maturation and preventing ovulation (Mishell, 1996; Kaunitz, 2000). The inhibition of the gonadotropins results in suppression of ovarian estradiol production.

Although the effect of MPA and NET on GnRH synthesis and release has not yet to our knowledge been determined, a few studies have investigated the effects of MPA and to a lesser extent NET, on LH, FSH and steroid hormone levels, in an attempt to fully understand the contraceptive mechanism of action of these two progestins. An early study showed that MPA and NET-EN inhibits the mid-cycle surge of FSH and LH, but that the release of these gonadotropins continues at luteal phase levels (Mishell *et al.,* 1977; Franchimont *et al.,* 1970). Another early study measured the peripheral blood levels of LH, FSH, and estradiol after intramuscular injection of a contraceptive dose of MPA in normal women (Jeppsson and Johansson, 1976). The levels of all three hormones remained in the range of the early follicular phase of a normal menstrual cycle (low levels) and ovulation was suppressed due to suppression of the LH peak. Interestingly, no suppression of basal LH and FSH levels was reported in any of the women, an effect which likely contributes to the lack of menopausal-like symptoms in women receiving contraceptive doses of MPA. In another study using contraceptive doses, normal menstruating women showed a decline in plasma levels of estradiol, progesterone and 17α-hydroxyprogesterone to early follicular phase levels sixteen days after MPA administration (Aedo *et al.,* 1981). In addition, no LH surge or ovulation was detected in patients receiving 1 mg of NET over a 5-day period in *in vitro* fertilisation studies (Letterie, 2000).

For male contraception, MPA or NET-EN, combined with testosterone esters, show severe suppression of spermatogenesis, due to the synergistic suppression of

gonadotropin (LH and FSH) levels (Kamischke *et al.,* 2000; Turner *et al.,* 2003; reviewed by Nieschlag *et al.,* 2003; Gu *et al.,* 2004; reviewed by Amory 2008). Similarly, it has been shown that MPA effectively suppresses spermatogenesis by inhibiting testosterone and gonadotropin production in rats (Lobl *et al.,* 1983; Flickinger, 1977).

MPA and to a much lesser extent NET, has been shown to influence expression of a number of genes involved in reproductive functions. Examples of such genes include tissue factor (TF) (Krikun *et al.,* 2000), decidual cell-expressed plasminogen activator inhibitor-1 (PAI-1) (Lockwood, 2001), transforming growth factor-β (TGF-β), (Arici *et al.,* 1996b), vascular endothelial growth factor (VEGF) (Sugino *et al.,* 2001), *c-fos* and prolactin (PRL) (Reis *et al.,* 1999), and the metalloproteinases (MMPs) (Bruner-Tran *et al.,* 2006). Tissue factor, a cell membrane-bound glycoprotein, is responsible for the initiation of hemostasis during implantation and placentation, and is associated with decidualisation (differentiation) in the uterus. Decidualisation is an adaptation of the uterus to enable implantation of the embryo, and may occur as a result of hormonal contraception. MPA at 100 nM was shown to significantly enhance TF gene transcription in human endometrial stromal cells (HESCs) (the progenitors of decidual cells) (Krikun *et al.,* 2000). Furthermore, decidual cell-expressed PAI-1 plays a role in preventing haemorrhage during human pregnancy implantation. MPA, in the absence and presence of estradiol, was shown to enhance PAI-1 expression in HESCs (Schatz and Lockwood, 1993; Schatz *et al.,* 1995; Lockwood, 2001). It is noteworthy that estradiol is ineffective alone, but enhances the MPA-mediated effects.

Another MPA-regulated gene involved in endometrial functions is transforming growth factor beta (TGF-β), which is believed to play a role in the predecidualisation of HESCs and in the completion of decidualisation after blastocyst implantation. Treatment of cultured HESCs with 1 nM MPA resulted in reduced levels of TGF-β3 mRNA, and a small increase in TGF-β1 mRNA levels (Arici *et al.,* 1996b). In contrast, in endometrial samples from women having received higher doses of MPA (10 mg/day), TGF-β3 expression was enhanced, with no observable change in TGF-β1 levels (Reis *et al.,* 2002). On the other hand, in a comparative study of MPA and NET in MCF-7 breast cancer cell lines, MPA did not affect TGF-β2 and TGF-β3 mRNA levels, whereas a highly significant decrease was observed with NET (Jeng and Jordan, 1991). Collectively, the data by Reis *et al.* (2002) and Jeng and Jordan (1991), suggest that MPA acts in a cell-specific manner, and moreover suggest that MPA and TGF-β3 may together mediate endometrial differentiation.

VEGF and its receptors play important roles in implantation and maintenance of pregnancy. Expression of VEGF and one of its receptors, kinase insert domain-containing region (KDR), was significantly increased by MPA (1 μM) and estrogen (10 nM) in human endometrial stromal cells isolated from proliferative phase endometrium (Sugino *et al.,* 2001). Another study in human endometrium showed that MPA inhibited *c-fos* gene expression, and enhanced the expression of PRL (Reis *et al.,* 1999). The authors suggested that inducing similar *c-fos* and PRL expression levels to those in secretory endometrium may be the mechanism by which MPA exerts its anti-proliferative effects. In addition, MPA, NET-A and Prog were shown to differentially regulate pro-matrix metalloproteinase (pro-MMP)-3 and pro-MMP-7 protein expression in stromal cells isolated from normal endometrial

tissue donors and endometriosis patients, in the absence and presence of the pro-inflammatory cytokine IL-1α (Bruner-Tran *et al.,* 2006). MMP expression is crucial for endometrial growth and remodeling, but failure to suppress MMPs may impair implantation and promote the development of endometriosis (Bruner *et al.,* 1997; Osteen *et al.,* 2005). MPA and Prog suppressed pro-MMP-3 and pro-MMP-7 in both healthy tissue donors and endometriosis patients regardless of IL-1α challenge, while NET-A could do so only in normal cells and in the absence of IL-1α challenge. The fact that NET could not suppress these MMP's in the presence of IL-1α induced inflammation, or in endometriosis (inflammatory disease) patients (Podgaec *et al.,* 2007), suggests that NET is a weaker anti-inflammatory agent as compared to MPA and natural Prog, and thus may not be an optimal treatment for women with endometriosis.

1.3.2 Adrenal function and steroidogenesis

Surprisingly little research appears to have been carried out in humans on the effects of MPA on adrenal function, and to our knowledge, there is only one report on the effects of NET (Amatayakul *et al.,* 1988).

Jones and co-workers (1974) reported lowered baseline plasma cortisol levels in contraceptive users of MPA. Similarly, a later study also showed that administration of a single dose of MPA resulted in a slight but significant reduction of cortisol in normal menstruating women (Aedo *et al.,* 1981). However, healthy, non-lactating Thai women who received long-term MPA and NET treatment were found to have no significant change in adrenal function as measured by cortisol levels (Amatayakul *et al.,* 1988).

At higher doses (up to 1500 mg orally per day), MPA has been shown to cause significant inhibition of adrenal function (Hellman *et al.,* 1976; Blossey *et al.,* 1984; Papaleo *et al.,* 1984; Lang *et al.,* 1990), which may be attributed to its glucocorticoid activity (van Veelen *et al.,* 1985). Furthermore, a study evaluating the adrenal function of postmenopausal breast cancer patients treated with MPA (300 mg), reported no difference in adrenocorticotropic hormone (ACTH) levels, but significantly lower cortisol, androstenedione and dehydroepiandrosterone sulphate (DHEA-S) levels, when compared to a control group (van Veelen *et al.,* 1984). Androstenedione is the main precursor of estrogens in postmenopausal women and this reduction in its levels could be the cause of hypoestrogenism induced by MPA. Earlier studies, however, observed reduction in both ACTH and cortisol levels by MPA (Matthews *et al.,* 1970; Hellman *et al.,* 1976). In addition, MPA used in the treatment of abnormal sexual behaviors in males (100 to 1000 mg weekly by intramuscular injection) significantly reduced mean serum concentrations of total testosterone and cortisol (Guay, 2008).

MPA directly inhibits multiple steps in human sex steroid biosynthesis. Studies using cultured rodent Leydig cells and testicular homogenates showed that MPA (1 μM) inhibited the activities of three key enzymes involved in steroidogenesis: 17α-hydroxylase (P450c17), 3β-hydroxysteroid dehydrogenase/D5-D4-isomerase (3βHSD) and 17β-hydroxysteroid dehydrogenase (17βHSD) (Barbieri and Ryan, 1980). These enzymes are responsible for the synthesis of estradiol and Prog, in the ovary, in response to LH and FSH. Prog is synthesized from pregnenolone by the 3βHSD, while estradiol is synthesized from testosterone by aromatase, or from estrone by 17β-HSD. Furthermore, Prog is a precursor for testosterone via the action

of P450c17 and 17β-HSD. In a study evaluating the effect of MPA as a substrate for, or inhibitor of the enzymes mediating the early steps common to both human adrenal and gonadal steroidogenesis, namely cholesterol side-chain cleavage enzyme (P450scc), 17α-hydroxylase/17,20-lyase (P450c17) and type II 3βHSD (3βHSDII), MPA showed no effect on P450c17 or P450scc, whereas it competitively inhibited 3βHSDII (Lee *et al.,*1999). Since 3βHSDI shares 93.5% amino acid identity with 3βHSDII (Rhéaume *et al.,* 1991), it is likely that 3βHSDI may also be inhibited by MPA. Since MPA is structurally similar to 17-hydroxyprogesterone, the mechanism by which MPA inhibits 3βHSD is likely to be product inhibition.

In summary, most studies on the effect of MPA on adrenal function focus on high doses such as those used in cancer therapy. More research on the effects of lower doses of MPA, and particularly of NET, on adrenal function is thus necessary for a better understanding of side-effects of contraceptive and HRT doses.

1.3.3 Skeletal function

Bone mineral density (BMD) is an important indicator of skeletal health in postmenopausal women. A number of studies have reported that long-term contraceptive use of MPA has a negative effect on BMD (refs), although the mechanism is poorly understood. However, it has been postulated that this occurs as a consequence of estrogen deficiency, which is induced by MPA due to inhibition of secretion of the pituitary gonadotropins by MPA (Jeppsson *et al.,* 1982). Indeed, in a study by Cundy *et al.* (2003), it was shown that supplemental estrogen therapy arrested MPA-related bone loss in premenopausal women treated for a minimum of two years with MPA and with a below average baseline lumbar spine BMD. Similarly,

in another randomized trial comparing estrogen supplementation with placebo among adolescents, increases in BMD were observed in the group receiving estrogen supplements, and decreases in the placebo group (Cromer *et al.,* 2005). Notably, the decrease in bone density tends to be most significant in women who start MPA use at an early age, and in those whose duration of use exceeds 15 years (Cundy *et al.,* 1991; Cromer *et al.,* 1996; Cundy *et al.,* 1998; Paiva *et al.,* 1998; Gbolade *et al.,* 1998; Scholes *et al.,* 1999; Tang *et al.,* 1999; Cundy *et al.,* 2003; Cromer *et al.,* 2005). These findings are significant as the peak bone mass attained during adolescence is one of the primary determinants of osteoporosis risk in post-menopausal women. Thus, the main reason for concern for women using MPA in adolescence is the potential risk for future osteoporosis and osteoporotic fractures.

A substantial number of studies evaluating the potential association between MPA usage and changes in BMD, indicate decreases in BMD among MPA users (Cundy *et al.,* 1998; Cundy *et al.,* 1994; Scholes *et al.,* 2002; Scholes *et al.,* 2004; Berenson *et al.,* 2004; Clark *et al.,* 2004; Busen *et al.,* 2003; Cromer *et al.,* 1996; Lara-Torre *et al.,* 2004; Cromer *et al*., 2004; Scholes *et al.,* 2005). In contrast, a few studies have showed positive effects of MPA on BMD. For example, premenopausal women with amenorrhea or abnormal menstrual cycles treated with MPA (10 mg/day for 10 days per month) were shown to have improved spinal bone density (Prior *et al.,* 1994). However, a study investigating the effect of MPA (20 mg/day) on BMD in postmenopausal women showed that MPA therapy could not arrest spinal bone loss. However, when postmenopausal women were treated with MPA (10 mg/day) combined with estrogen (0.3 mg/ day), bone loss was reduced (Gallagher *et al.,* 1991). Similarly, results from the Postmenopausal Estrogen/Progestin Interventions

trial (PEPI) showed that postmenopausal women receiving estrogens (0.625 mg/day) in combination with MPA (10 mg/day for 12 days/month) exhibited an increase in bone mass (Writing Group for the PEPI trial, 1996). In addition, results from the Women's Health Initiative trial demonstrated that conjugated equine estrogen (CEE) (0.625 mg/day) plus MPA (2.5 mg/day) increased bone BMD and reduced the risk of fracture in postmenopausal women (Cauley *et al.,* 2003). Furthermore, a cross-sectional study among postmenopausal women showed that the mean BMD of past users of MPA (median length of use ~3 years), was comparable to non-users (Orr-Walker *et al.,* 1998), indicating that bone loss occurring with MPA use is reversible. Similarly, a study following up on adolescent users of MPA following discontinuation, showed a significant increase in BMD (Scholes *et al.,* 2005). Consistent with these latter two studies, three prospective studies indicated that BMD tended towards baseline values following MPA discontinuation in women of all ages (Clark *et al.,* 2004; Clark *et al.,* 2006; Kaunitz *et al.,* 2006). In addition, recovery in BMD was seen as early as 24 weeks after cessation of therapy, and the BMD in past MPA users was similar to that in nonusers, 2-3 years after discontinuation of contraceptive injections (Rosenberg *et al.,* 2007). Taken together, most studies have found that women lose BMD while using MPA, but regain it after discontinuation of MPA use.

Studies on the effects of NET on BMD are limited. Oral administration of contraceptive doses of NET (0.35 mg/day) was reported to protect against loss of bone mass in breast-feeding women (Caird *et al.,* 1994). In addition, clinical studies have shown that an oral contraceptive containing 20-35 μg/day of ethinyl estradiol in combination with NET resulted in the optimal bone-sparing effect in premenopausal women (DeCherney, 1996). Similarly, women receiving a NET-containing oral

contraceptive pill showed a 2.33% gain in BMD (Berenson *et al.,* 2001). Furthermore, the effect of NET in HRT has also been investigated and appears to be controversial. NET-A has been reported to have positive effects on postmenopausal bone metabolism, and has been shown to increase bone mass more than alendronate, an effective candidate for both the prevention and treatment of osteoporosis (Riis *et al.,* 2002). Similarly, in the review by Taitel and Kafrissen (1995), a number of studies reported increased bone mass and BMD in postmenopausal women treated with estradiol and NET-A. In addition, NET (5 mg/day) for 4 months was also shown to prevent bone loss in postmenopausal osteoporosis by decreasing bone turnover (Horowitz *et al.,* 1993). Conversely, another study was unable to show a consistent increase in markers of bone formation in postmenopausal women treated with 5 mg/day NET-A for 9 weeks (Onobrakpeya *et al.,* 2001), indicating that NETA does not have short-term anabolic effects on bone.

In contrast to the effects seen with the majority of studies on the oral contraceptive formulation of NET, limited data with injectable NET-EN indicate a negative effect on BMD in adolescent (Beksinska *et al.,* 2007; Beksinska *et al.,* 2009) and adult users (Rosenburg *et al.,* 2007). Consistent with the reversal of negative BMD effects following the discontinuation of injectable MPA use, the recovery of BMD was also seen when usage of injectable NET was stopped (Rosenburg *et al.,* 2007; Beksinska *et al.,* 2009). However, according to Sarfati and de Vernejoul (2009), bone loss does not occur with injectable NET, and NET exerts anabolic effects on bone. These authors also report that bone formation with NET may reflect peripheral conversion to ethinylestradiol or direct androgenic effects on bone tissue. In addition, Ishida *et al.* (2008) reported that postmenopausal women who have been diagnosed with

osteoporosis, should preferably use NET-A, rather than MPA, in HRT so as to prevent the possibility of fractures. It has been speculated that the positive effect of NET-A on BMD may be attributed to its androgenic activity (Sarfati and de Vernejoul, 2009), and/or its lack of glucocorticoid activity (Ishida *et al.,* 2008). The latter would be consistent with the proposal that bone loss associated with MPA, at contraceptive doses or higher, is due to its glucocorticoid activity (Ishida and Heersche, 2002).

In summary, studies to date provide sufficient evidence to support a link between MPA usage and reduction of BMD, although the benefits of MPA as a contraceptive may outweigh the risk of decreased BMD. Whether NET has such negative effects on BMD is controversial. Nevertheless, concern still remains for adolescent users, as maximal BMD is obtained during adolescence. Further studies are thus needed to establish whether, after stopping the use of MPA and NET as injectable contraceptives, BMD remains at lower levels long-term, and how the risk of future fractures is affected. Additional research is also needed to address the role of MPA vs. NET on bone metabolism in HRT users. Finally, many factors, such as dosage, age of onset of usage, duration of usage, peak BMD at adolescence, and even the level of exercise, may contribute to the effect of the progestins on BMD. Thus, these factors need to be considered in future research assessing the impact of MPA vs. NET on BMD.

1.3.4 Brain function

The role of Prog or synthetic progestins in the brain has recently been under much scrutiny, after a number of studies reported differences in neurological effects of Prog and MPA in cell culture models (Nilsen and Brinton, 2002a; Nilsen and Brinton,

2002b; Nilsen and Brinton, 2003). Both Prog and MPA can cross the blood-brain barrier (Lanthier and Patwardhan, 1986; Skatrud *et al.,* 1978), while Prog is also synthesized in the brain *de novo* (Baulieu *et al.,* 2001). Two clinical trials showed that MPA abrogated beneficial effects of longterm estrogen replacement therapy on cognitive function (Ohkura *et al.,* 1995; Rice *et al.,* 2000). Similarly, analysis from the Women's Health Initiative Memory Study (WHIMS) clinical trial reported that estrogen plus progestin (MPA) therapy did not improve cognitive function in older women, and resulted in a doubling of the diagnosis of Alzheimer's disease (Rapp *et al.,* 2003). This finding could possibly be explained by the results of the study by Nilsen and Brinton (2002a), which suggest that MPA may counteract the beneficial effects of estrogen on cognitive function and prevention of Alzheimer's disease.The authors showed that 17β-estradiol and Prog, but not MPA, protect hippocampal neurons against glutamate neurotoxicity. In addition, unlike MPA, progesterone results in nuclear translocation of mitogen-activated protein kinase (MAPK) in hippocampal neurons, which appears to be linked to the neuroprotective profile of progesterone (Nilsen and Brinton, 2003). In addition, a study in rats reports that Prog, but not MPA, is neuroprotective *in vivo* (Ciriza *et al.,* 2006). In contrast to MPA, Prog is metabolised to 5α-dihydroprogesterone (DHP), which is subsequently reduced to 3α, 5α-tetrahydroprogesterone (THP), in the nervous system. The authors show that the observed neuroprotective effects of Prog are due to these metabolites. Prog can also be metabolised to allopregnenalone (AP) (3α-5α–tetrahydroprogesterone), which is a potent modulator of the $GABA_A$ receptor, and also plays a role in neuroprotection (Hosie *et al.,* 2006). Most progestins used in HRT, like MPA and NET, contain the typical steroidal backbone (Figure 1). The potential thus exists for MPA and NET to be metabolised into neuroactive steroids that may modulate the $GABA_A$ receptor.

Winneker *et al.* (2003) used rat behavioural models to assess the potential $GABA_A$ receptor activity of MPA and NET. Both these progestins were shown to produce some anxiolytic-like effects, supporting the concept of their metabolism into neuroactive steroids (Pluchino *et al.,* 2009). However, a recent study indicated that unlike Prog, MPA could not downregulate the mRNA expression of the α4 subunit of $GABA_A$ receptors in the CA1 hippocampus of female rats (Pazol *et al.,* 2009). Changes in the α4 subunit expression in the CA1 hippocampus have important implications for the regulation of anxiety (Shen *et al.,* 2005). Further clinical studies are needed to establish the eventual functional consequences of the differential regulation of gene expression by Prog, MPA and NET.

In summary, the above data indicates opposing effects of Prog and MPA on neuroprotection. Thus it is evident that MPA cannot be used as a simple substitute for Prog in the brain. Indeed, the WHIMS clinical trial found that estrogen combined with MPA showed an increased risk for dementia in women aged 65 and older (Shumaker *et al.,* 2003). On the other hand, a recent study in primary cultures of cortical neurons indicates that Prog and MPA exert similar protective effects on neurons, and that the effects depend strictly on the previous hormonal status of the neurons and timing of exposure (Mannella *et al.,* 2009). In addition, the authors showed that the activation of nitric oxide synthase (NOS) is critical for the neuroprotective effects by Prog and MPA. Finally, there is paucity in the research on the effects of NET on neuroendocrine function, which needs to be addressed considering current knowledge that the type of progestin is likely to be critical for achieving therapeutic benefits, preventing neurodegenerative disease and sustaining cognitive function throughout the menopausal years. Clearly, additional research is

needed to explore the neurobiology of progesterone vs. different synthetic progestins.

1.3.5 Reproductive cancers

1.3.5.1 Breast cancer

There is evidence in the literature to suggest that the use of MPA as a contraceptive does not increase breast cancer risk (reviewed by Kaunitz, 1996; Dillis and Schreiman, 2003). Based on comparative mammograms of two patients while using injectable MPA vs. after discontinuation, Dillis and Schreiman (2003) suggested that MPA has a suppressive effect on breast density in women receiving contraceptive injections. In contrast, recent clinical evidence suggests that MPA in HRT use increases the risk of breast cancer (2003) suggested a suppressive effect of MPA on breast density in women receiving contraceptive injections. In contrast, recent clinical evidence suggests that MPA in HRT use increases the risk of breast cancer (Rossouw *et al.,* 2002; Beral, 2003). This raises the question of whether the addition of MPA, or NET, to estrogen replacement therapy is more harmful than beneficial. Indeed, although the use of NET in HRT has been implicated in increased risk of breast cancer in clinical studies (Beral, 2003; Colditz, 2005), it has also been reported to significantly inhibit breast cancer cell proliferation *in vitro* (Seeger *et al.,* 2003). Thus reports on the effects of progestins at a cellular level do not concur with studies conducted at a systemic level.

The above-mentioned trials confirmed results from numerous observational studies demonstrating that MPA, and NET are associated with an increase in breast cancer risk (de Lignières, 2002; Newcomb *et al.,* 2002; Lee *et al.,* 2005; Ross *et al.,* 2000;

Ewertz *et al.,* 2005). In the study by Newcomb and co-workers (2002), postmenopausal women using estrogen-only HRT showed a significant increase in breast cancer risk, and this risk was increased by the addition of MPA. Furthermore, Ross *et al.* (2000) compared the risk for breast cancer in women on combined estrogen and MPA therapy, to women on estrogen alone therapy, and demonstrated that the use of MPA increased the risk for breast cancer compared with estrogen alone. Observations from a population-based cohort study (Ewertz *et al.,* 2005), and a meta-analysis of 61 studies (Lee *et al.,* 2005), showed that both MPA and NET were associated with increased risk of breast cancer, with increased duration of use of MPA and NET in HRT.

In a study in the MCF-7 breast cancer cell line, MPA was shown to have no effect on TGF-β2 and TGF-β3 mRNA levels, whereas a dramatic decrease was observed with NET (Jeng and Jordan, 1991). This effect seen with NET was accompanied by cell growth stimulation, which suggested that the differential regulation of TGF-β expression by NET might be partly responsible for the growth stimulation induced by NET. These results are consistent with a role for NET in contraception that might facilitate the development of breast cancer. In a study in a normal human breast epithelial cell line (MCF10A), the effects of Prog, MPA and NET on proliferation and apoptosis were investigated. The results showed that MPA significantly increased the growth factor-induced stimulation of MCF10A cells, while Prog and NET had no effect, thus implying an enhanced proliferative effect by MPA (Krämer *et al.,* 2006). In a human cancerous breast cancer cell line (HCC1500) however, MPA and NET enhanced the initial growth factor-induced proliferative effect, while Prog had no effect in these cells. Collectively, these data indicate differences between Prog, MPA

and NET in terms of breast cancer risk in the same *in vitro* model, and moreover raises the concern that MPA may increase the mitotic rate of normal epithelial breast (Seeger and Mueck, 2008).

MPA and NET may also enhance the conversion of weaker endogenous estrogens into more potent estrogens (Coldham *et al.,* 1990; Seeger *et al.,* 2000; Campagnoli *et al.,* 2005; Xu *et al.,* 2007), which could potentially contribute to their carcinogenic effects. For example, Seeger *et al.* (2000) showed that NET-A, used by postmenopausal women for HRT, may promote the formation of the genotoxic estrogen metabolite 16-hydroxyestrone. In human breast cancer cell lines, MPA but not NET was shown to stimulate the conversion of inactive estrone sulfate into active estrone by stimulating sulfatase, as well as increasing 17-beta-hydroxysteroid reductase activity (Xu *et al.,* 2007). Other studies demonstrated an increase in the activity of 17-beta-hydroxysteroid reductase by both MPA and NET (Campagnoli *et al.,* 2005; Coldham *et al.,* 1990). Increased activity of this enzyme may increase the intracellular production of more potent estrogens, and possibly increase breast cancer risk.

MPA has also been implicated in breast cancer cell migration and invasion (Birrell *et al.,* 2007; Fu *et al.,* 2008]. In the study by Fu *et al.* (2008), both MPA and Prog, alone or in combination with 17β-estradiol (E_2), increased breast cancer cell migration and invasion by a mechanism involving the activation of the actin-binding protein moesin, and the initiation of actin remodelling. The authors suggest that MPA may play a role in the development of PR^+ breast cancer by changing the ability of cancer cells to

interact with the extracellular environment, and ultimately their ability to move or invade the surrounding environment.

On the other hand, results from some *in vitro* studies investigating the effects of MPA and NET on breast cancer, do not correlate with the recently available clinical evidence suggesting an increased risk of breast cancer for HRT users. MPA has been shown to significantly decrease parathyroid hormone related peptide (PTHrP) expression in breast tissue (Sugimoto *et al.,* 1999). The level of PTHrP expressed in breast cancer tissue is closely associated with the incidence of bone metastasis. This result is consistent with the therapeutic effect of MPA when used as second line hormonal therapy for the treatment of metastatic breast cancer. Similarly, MPA has been shown to induce the expression of the metastasis suppressor gene, Nm23-H1, in a metastatic breast cancer cell line, thereby decreasing metastatic colonization (Palmieri *et al.,* 2005).

MPA also inhibits the E_2-stimulated growth of the MCF-7 cell line (Lippert *et al.,* 2001; Schoonen *et al.,* 1995), while another study MCF-7 cells showed that both MPA and NET displayed significant inhibition of cell proliferation (Seeger *et al.,* 2003). The above *in vitro* studies, in contrast to recent clinical evidence for MPA and NET, seem to indicate that both these progestins may reduce the risk of breast cancer. Cyclin D1, which is required for cell cycle progression in G1, has been implicated in the pathogenesis of breast cancer. When the T47-D breast cancer cell line was treated with MPA, increased expression of cyclin D1 mRNA and protein was observed after 24 hours, while a decrease was observed after 72 hours (Thuneke *et al.,* 2000). In contrast to the recent clinical evidence, the authors suggest that long-term MPA

administration may therefore have an inhibitory influence on the proliferative activity of breast cancer cells.

Taken together, the evidence in the literature indicates that the role of MPA in breast cancer development is not straightforward. At high dosages such as those in cancer therapy, MPA has anti-proliferative effects on breast cancer cells, elucidating its therapeutic effects in the treatment of metastatic cancer. In contrast, the role of MPA in breast cancer development becomes blurred when used for contraception and HRT. Some studies indicate that MPA enhances the development of breast cancer (Ganzina *et al.,* 1979, Etienne *et al.,* 1992; Bentel *et al.,* 1999; Hofseth *et al.,* 1999; Thuneke *et al.,* 2000; Cavalli *et al.,* 1984), while others show that does not increase breast cancer risk (reviewed by Kaunitz, 1996; Dillis and Schreiman, 2003; Palmieri *et al.,* 2005; Lippert *et al.,* 2001; Schoonen *et al.,* 1995; Thuneke *et al.,* 2000). Similarly, NET has also been shown to significantly inhibit cell proliferation at a concentration range of 0.01 nM to 10 μM (Seeger *et al.,* 2003), but also to increase breast cancer proliferation at HRT doses (Millions women's study collaborators). Further investigation is therefore required to determine the conditions under which MPA or NET (in contraceptive or HRT use) facilitates the development of breast cancer in women, in order to minimize the risk to users.

1.3.5.2 Endometrial, ovarian and cervical cancers

The use of MPA as an injectable contraceptive is associated with an 80% reduction of endometrial adenocarcinoma (Kaunitz, 1996). Further *in vitro* evidence for the protective effect of MPA on the endometrium involves a study on regularly cycling women treated with MPA (10 mg/day), which showed suppression of c-fos

expression in the human endometrial samples (Reis *et al.,* 1999). The proto-oncogene, c-fos is one of the putative mediators of estrogen-induced endometrial proliferation.

A number of studies have been undertaken in cell lines and tissue samples to further determine the effects and mechanisms of action of MPA and NET on uterine (endometrial) cancer. Consistent with the clinical evidence suggesting no deleterious effect of MPA on the development of uterine cancers, MPA, unlike estradiol, did not increase the expression of PD-ECGF, an angiogenic factor, in the Ishikawa cell line (Aoki *et al.,* 2003). Similarly, NET combined with estrogen in HRT has been reported to protect against the hyperplastic effects of estrogen treatment on the endometrium (Riis *et al.,* 2002).

Although NET in HRT use has been shown to have protective effects on the endometrium, its A-ring (figure 1) reduced derivatives were able to significantly increase mRNA levels of c-fos in ovariectomized rats. These results indicate that NET administration might indirectly induce estrogenic effects through the action of its 5α-dihydro and 3β, 5α-tetrahydro derivatives, an activity that may facilitate the development of uterine cancer (Mendoza-Rodriguez *et al.,* 1999). In contrast to the above study, and consistent with its protective effects on the endometrium, *in vitro* studies showed upregulation of Wnt-7a gene expression by NET-A in normal estrogen treated endometrial epithelial cells. This upregulation of Wnt-7a is thought to be associated with the antineoplastic effects of progestins on the endometrium (Oehler *et al.,* 2002). Taken together, the *in vitro* studies are consistent with the clinical evidence that neither MPA nor NET increase the risk of endometrial cancer.

However, the study by Mendoza-Rodriguez *et al*. (1999) suggests that NET metabolites may increase the risk of endometrial cancer under certain conditions.

The use of MPA as contraceptive has been reported to have no effect the risk of epithelial ovarian cancer or cervical neoplasia (Kaunitz, 1996, Shapiro *et al.,* 2003). In contrast, recent evidence suggests that MPA in HRT use increases the risk of ovarian cancers (Anderson *et al.,* 2003). To our knowledge, nothing is known on the effects of contraceptive or HRT doses of NET on the development of ovarian and cervical cancers. Further investigation is therefore required to determine whether NET (in contraceptive or HRT use) facilitates the development of these reproductive cancers in women.

1.3.6 Cardiovascular function

Cardiovascular disease (CVD) is the main cause of death in both men and women of the developed world (ESHRE Capri Workshop Group, 2006). Pre-menopausal women are considered more protected from CVD than men due to the presence of endogenous estrogen. When estrogen levels start declining after the onset of menopause, the relative cardioprotective effect by estrogen also wanes. In a study by Bairey Merz *et al.* (2003), it was shown that disruption of the ovulatory cycle in premenopausal women, indicated by estrogen deficiency and hypothalamic dysfunction, is associated with an increased risk of coronary atherosclerosis and adverse cardiovascular events (Bairey Merz *et al.,* 2003). Furthermore, lipid and lipoprotein profiles have been shown to deteriorate following menopause, resulting in increased risk of coronary heart disease (CHD), which is known to be reversed by estrogen therapy (Dubey *et al.,* 2005).

Synthetic progestins, including MPA and NET, have been shown to negate the cardioprotective effects of estrogen, in addition to producing negative cardiovascular effects when used alone (Farish *et al.,* 1986; LaRosa, 1994; Kim *et al.,* 1994; Levine *et al.,* 1996; Adams *et al.,* 1997; Register *et al.,* 1998; Chen *et al.,* 1998; Spencer *et al.,* 2000; Mishra *et al.,* 2005; Jeanes *et al.,* 2006, Fernandes *et al.,* 2008). Consistent with this, recent randomised clinical trials have suggested that estrogen plus MPA in HRT regimes does not confer cardiac protection, and may increase the risk of coronary heart disease in postmenopausal women (Rossouw *et al.,* 2002; Manson *et al.,* 2003). In contrast, a trial on estrogen alone did not show increased risk of CVD (Women's health initiative steering committee, 2004).

The PEPI study, which compared a variety of cardiovascular effects, including lipid effects, of usage of both MPA and Prog in combination with CEE, indicated that all regimens were associated with clinically significant improvements in lipoprotein levels, but that many of estrogen's beneficial effects on HDL-cholesterol (HDL-C) were reversed with the addition of MPA, but not Prog (Writing group for the PEPI trial, 1995). These results are consistent with other studies, showing that MPA, and also NET, reduces HDL levels (Ottosson *et al.,* 1985; Hirvonen *et al.,* 1981; Saarikoski *et al.,* 1990; Miller *et al.,* 1991), while progesterone either maintains or increases estrogen's positive lipid and HDL effects (Ottosson *et al.,* 1985; Saarikoski *et al.,* 1990). Ottosson *et al.* (1985) compared the lipid effects in menopausal women receiving estrogen combined with MPA or Prog, and demonstrated that those receiving estrogen combined with MPA had a significant reduction in HDL and HDL subfraction 2, while no significant changes were seen in those women receiving estrogen combined with Prog. Furthermore, a randomized trial which compared the

lipid effects in women using Prog and NET, indicated that women using NET had a significantly decreased HDL, whereas those using Prog showed no decrease in HDL.

In addition to the effects of Prog, MPA and NET on lipids, their effects on other risk factors of CVD such as atherosclerosis, vasoconstriction/vasodilation, and carbohydrate metabolism, have also been examined. The lack of correct endothelial function (endothelial dysfunction) is the initial event in the development of atherosclerosis and an early marker of cardiovascular disease (CVD) (Abdu *et al.,* 2001). It starts with injury of the vascular endothelium and results in an increase of monocytes and macrophages in the subendothelium by increasing the expression of cellular adhesion molecules such as intercellular adhesion molecule (ICAM-1), vascular and cell adhesion molecule-1 (VCAM-1) and E-selectin, as well as increased production of chemokines like monocyte attracting protein (MCP-1) (Seeger *et al.,* 2007). Consequently, smooth muscle cells migrate and proliferate resulting in remodeling of the vascular wall. When a further buildup of inflammatory cells and lipids occurs, atherosclerotic plaques form. When these plaques rupture, it may cause myocardial infarction or stroke. A number of studies have shown that unlike Prog, which inhibits atherosclerotic plaque formation, MPA promotes plaque formation and also prevents the plaque-inhibiting and lipid-lowering actions of estrogen (Levine *et al.,* 1996; Adams *et al.,* 1997; Register *et al.,* 1998).

Both MPA and NET-A have been shown to have deleterious effects on the vasculature by dose dependently increasing the expression of the adhesion molecules, ICAM-1 and VCAM-1, in human umbilical venous endothelial cells (HUVECs) (Tatsumi *et al.,* 2002). Similarly, a study in human vascular endothelial

cells, showed that unlike Prog, MPA did not inhibit the expression of VCAM-1 (Otsuki *et al.,* 2001). As already mentioned, monocyte adhesion to endothelial cells is an important initial event at the onset of atherosclerosis, thus the above data suggests that MPA and NET may contribute unfavourably to this disease (Tatsumi *et al.,* 2002). Furthermore, treatment of vascular smooth muscle cells with MPA, in contrast to NET, upregulated PAR-1 expression, which markedly potentiated the vascular procoagulent effects of thrombin, an effect thought to be due to the glucocorticoid-like activity of MPA (Herkert *et al.,* 2001). In addition, long-term administration of MPA to ovariectomised rats increased PAR-1 protein levels in the arterial wall, which resulted in increased responsiveness of isolated aortic rings to thrombin (Herkert *et al.,* 2001). Interestingly however, MPA unlike NET antagonised the estradiol-induced significant reduction of MCP-1 in human female coronary endothelial cell cultures (Mueck *et al.,* 2002). Taken together, a large number of studies indicate that unlike Prog, MPA and NET-A have adverse effects on early stages of atherosclerosis, while the study by Mueck *et al.* (2002) infers that MPA and NET-A may differ in their effects, and that NET-A may be protective under some conditions.

Two recent studies have also indicated that MPA promotes endothelial dysfunction (Meendering *et al.,* 2008; Lizarelli *et al.,* 2009), which could subsequently lead to increased stroke risk. Meendering *et al.* (2008) demonstrated that MPA counteracts the beneficial effects of estradiol on endothelium-dependent vasodilation in young women. In particular, their data suggests that the decrease of the vasoconstrictor, endothelin-1, by estradiol is reversed by MPA. A study in isolated human endothelial cells as well as in ovariectomized rat aortas, demonstrated that MPA has no effect on endothelial nitric oxide (NO) production, while Prog increases its production

(Simoncini *et al.,* 2004). Production of NO, a vasodilatory agent, is essential for normal vascular endothelial functioning, and impaired synthesis of NO would result in atherosclerosis (Yasa and Türkseven, 2005). Thus, the study by Simoncini *et al.,* 2004, implies that Prog would have beneficial effects on the vasculature, while MPA would not. In addition, that study showed that MPA inhibits the expression of leukocyte adhesion molecules and moreover, when MPA was used in combination with estradiol, it hinders the favorable estradiol signaling, while Prog enhances the effects of estradiol. Overall, these results highlight the fact that Prog and MPA have distinct biological effects, with MPA generally having a more negative effect on the vascular system.

A number of studies have shown that coronary artery spasm, which increases the risk for heart attack and stroke, is reduced with the use of estrogen and/or Prog, while the addition of MPA to estrogen has the opposite effect, resulting in vasoconstriction, thus increasing the risk for ischemic heart disease (Miyagawa *et al.,* 1997; Minshall *et al.,* 1998; Mishra *et al.,* 2005). For example, Miyagawa and co-workers (1997) showed that primates pretreated with estradiol combined with Prog, did not experience vasospasm, while all of those treated with MPA showed significant vasospasm. Similarly, Mishra *et al.* (2005) recently also showed that Prog protected against coronary hyperreactivity, while MPA had the opposite effect and induced coronary constriction. A study investigating the effects of MPA and NET-A in an animal model of the human menopause (ovariectomised *New Zealand white rabbits*), indicated that potassium and endothelin-1 induced higher contractile responses in the distal coronary artery from animals treated with MPA compared to animals treated with NET-A (Pedersen *et al.,* 2004). Furthermore, another study by

this group, investigating vascular function in coronary arteries of hyperlipidemic rabbits, showed that when estradiol is combined with MPA or NET-A, the beneficial effects of estradiol on NO and Ca^{2+}-mediated vascular reactivity are negated by MPA, but not by NET-A (Pedersen *et al.,* 2006). Taken together, the results of the two latter studies show adverse effects with MPA but not NET-A, clearly indicating that MPA and NET-A cause different vascular effects, emphasizing that the choice of progestin is crucial in HRT.

An increase in insulin resistance and subsequent deterioration in glucose tolerance (associated with menopause) could result in type 2 diabetes as well as CVD. MPA and NET-A have been shown to significantly increase insulin resistance (Wagner *et al.,* 1996; Godsland *et al.,* 1992*;* Christodoulakos *et al.,* 2005; 1993; Spencer *et al.,* 2000; Fernandes *et al.,* 2008) when compared with estrogen and progesterone (Godsland *et al.,* 1993; Spencer *et al.,* 2000). Christodoulakos *et al.* (2005), comparing insulin resistance in postmenopausal women receiving different regimens of HRT, demonstrated that estrogen combined with NET-A showed no association with insulin resistance, while when estrogen was combined with MPA, increased insulin resistance was found. Similarly, a placebo-controlled trial showed that a 6-month treatment with estrogen and NET-A, did not affect insulin sensitivity (Walker *et al.,* 2001). Conversely, Fernandes *et al.* (2008) showed that in postmenopausal women receiving HRT, estrogen alone caused an improvement insulin resistance, while when combined with NET-A, this positive effect was no longer seen. Taken together, these data indicate that MPA may worsen glucose tolerance causing insulin resistance, thereby increasing risk of CVD. However, the effects of NET are

controversial, and more research is needed to establish the effect of NET on glucose metabolism.

Hypertension is one of the greatest risk factors of CVD (Mueck and Seeger, 2004). When used in HRT, synthetic progestins, such as MPA and NET-A, that are reported to lack the anti-mineralocorticoid activity of Prog (Winneker *et al.,* 2003; Palacios *et al.,* 2006), could lead to cardiovascular complications, as they are not able to antagonize the salt-retaining and blood pressure raising effects of estrogens. Estrogen acts on the renin-angiotensin-aldosterone system (RAAS), leading to increased synthesis of angiotensinogen, which subsequently increases aldosterone levels thereby promoting sodium and water retention (Oelkers, 1996). Thus estrogen indirectly leads to increases in weight and blood pressure. On the other hand, Prog is a potent antagonist of aldosterone (Wambach *et al.,* 1979; Rafestin-Oblin *et al.,* 1992; Quinkler *et al.,* 2002) via binding to the mineralocorticoid receptor (MR). Thus Prog, acting via the MR, will cause increased sodium excretion and prevent sodium retention, conferring potential blood pressure benefits. As MPA and NET-A are unable to antagonize the salt-retaining and blood pressure raising effects of estrogens, it would lead to weight gain and increased blood pressure, which may subsequently lead to cardiovascular complications.

Consistent with the above effects of Prog on the RAAS, Rylance *et al.* 1985 observed no changes in blood pressure with progesterone in normotensive postmenopausal women, but a slight reduction in blood pressure was observed in hypertensive women. In contrast, NET-A in combination with estradiol in HRT has been reported to result in increases in blood pressure (Activelle package insert reg. no.

33/21.8.2/0532, Novo Nordisk). Similarly, Rosano *et al.* (2001) showed that estradiol associated with a high dose of NET-A (10 mg) increased blood pressure in hypertensive postmenopausal women. In another study, NET-A in HRT was associated with an increased risk of stroke among hypertensive but not normotensive women. The authors thus suggested that hypertensive postmenopausal women should not use HRT regimes containing NET-A (Lokkegaard *et al.,* 2003). A study in ovariectomized female Wistar rats treated with Ald and salt to induce renal injury, showed that estradiol in combination with MPA, increased the development of kidney injury, sodium retention and increased blood pressure (Arias-Loza *et al.,* 2009). In contrast to the risk for HRT users, contraceptive usage of MPA or NET-EN is reported to have a relatively minor effect on blood pressure. The Cochrane comparative review on these two progestins reported similar relatively small changes in blood pressure, which are not clinically relevant, for both MPA and NET-EN (Draper *et al.,* 2006).

In contrast to the studies showing negative effects of MPA on CVD, and variable effects for NET (negative and positive), a study in human vascular coronary cells demonstrated that estrogen in combination with MPA and NET significantly reduced the inflammatory markers, monocyte chemoattractant protein-1 (MCP-1), E-Selectin and ICAM-1, as well as the plaque marker PAI-1 (Seeger *et al.,* 2007), suggesting that MPA and NET can also positively influence the vasculature. PAI-1, synthesized by the endothelium, is an independent risk factor for the development of CVD and a promoter of thrombosis after plaque rupture. Similarly, a prospective randomized study investigating the pro-coagulant and fibrinolytic activity of HRT showed that estrogen combined with MPA or NET, reduced PAI-1 levels (Taner *et al.,* 2006). MPA

and NET also enhanced the positive effect of estradiol on the precursor of matrix metalloproteinase-1 human female coronary endothelial cell cultures (Mueck *et al.,* 2002). Consistent with positive effects on the vasculature with MPA usage, but in contrast to previous studies, a recent study showed that MPA combined with estrogen does not impair the improvement in flow-mediated dilatation in resistance arteries of postmenopausal women, and that MPA alone has no effect (Kublickiene *et al.,* 2008). These data suggest that use of MPA or NET, either alone or in combination with estrogen, has a positive effect on the function of resistance arteries, and may prevent endothelial dysfunction.

Understanding the effects of MPA and NET on cardiovascular mechanisms and how they translate into clinical risk, together with the current epidemiological knowledge, should thus be considered when prescribing the optimal progestin for HRT, especially in women susceptible to cardiovascular disease.

1.3.7 Immune function

The possibility that MPA, and to a lesser extent NET, can exert immunosuppressive effects on a physiological level was raised many years ago by Hulka *et al.* (1965). In their study in rabbits, they observed that MPA treatment (5 mg daily) suppressed circulating antibody production to the same extent as observed with cortisol treatment (50 mg twice a week). Similarly, MPA was shown to suppress the primary humoral antibody response in rabbits and also to extend survival of dogs with renal allografts as well as survival of rabbits with skin allografts (Turcotte *et al.,* 1968). In the guinea pig, MPA decreased expression of splenic macrophage Fcgamma receptors, which play a critical role in host defence against infection (Gomez *et al.,* 1998). In contrast,

MPA was shown to enhance antibody production in mice *in vivo* and *in vitro* in cultures of splenocytes, bone marrow cells or lymph node cells (Vermeulen *et al.,* 2001).

Surprisingly, little research appears to have been carried out in humans on the effects of contraceptive doses of MPA on immune function. Two early findings show that MPA increases cell-mediated immune reactivity in the skin (Gerretsen *et al.,* 1979) and has a profound effect on the efferent phase of the immune response (Gerretsen *et al.,* 1980). However, MPA was found to compromise cell-mediated immune status in Bangladeshi women receiving these compounds as injectable contraceptives (Majumder *et al.,* 1987). Doses of MPA used in HRT also modulate human immune function, selectively affecting various immune cell subsets (Brunelli *et al.,* 1996, Malarkey *et al.,* 1997). Furthermore, MPA has been shown to have anti-inflammatory activity, by decreasing the levels of the plasma proinflammatory markers, C-reactive protein and serum amyloid A protein, that are increased by estrogen, in postmenopausal women using the CEE/MPA HRT treatment (Wakatsuki *et al.,* 2002). In addition, MPA used together with estrogen, has been shown to downregulate release of the pro-inflammatiry cytokines interleukin-2 (IL-2) and interferon gamma (IFN-γ) by phytohemaglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) isolated from postmenopausal women using HRT therapy (Stopińska-Głuszak *et al.,* 2006). The authors propose that the use of estrogen combined with MPA in HRT may affect cell-mediated immunity which could lead to the development of neoplastic disease.

At higher doses (up to 1500 mg orally per day), MPA has been shown to cause significant immunosuppression in patients (Brunelli *et al.,* 1996; Malarkey *et al.,* 1997; Yamashita *et al.,* 1996; Mallmann *et al.,* 1990; Scambia *et al.,* 1988; Kurebayashi *et al.,* 2003; Naglieri *et al.,* 2002). Mallman *et al.* (1990) observed decreased T cell numbers in breast cancer patients receiving high doses of MPA, while another group showed that MPA could inhibit lymphocyte proliferation *in vitro* (Kontula *et al.,* 1983). Furthermore, MPA treatment of PBMCs from cancer patients was shown to significantly reduce the proliferative response to PHA (Mantovani *et al.,* 1997). Similarly, MPA at 100 ng/ml was shown to exert a significant inhibitory effect on lymphocyte response to mitogens in healthy volunteers (Corsini and Puppo, 1982-1983). In contrast, Grönroos and Eskola (1984) showed no effect of MPA on lymphocyte proliferation, or in the proportion of T and B cells in endometrial cancer patients. Furthermore, MPA at 0.2 μg/ml has been shown to decrease the levels of IL-1β, IL-6, tumor necrosis factor-alpha (TNFα) and serotonin (5-HT) produced in culture by PHA-stimulated PBMCs from cancer patients (Mantovani *et al.,* 1997). Moreover, treatment of serotonin-induced myometrial smooth muscle cells with MPA resulted in a marked decrease in the expression of IL-1α mRNA and protein (Lan *et al.,* 1999). Similarly, IL-1 and IL-6 were strongly suppressed by MPA (≥ 100 nM), but not Prog, in normal human lymphocytes (Bamberger *et al.,* 1999). Koubovec *et al.* (2005) showed, using promoter-reporter assays, that MPA elicited more potent transrepression of the IL-8 promoter than Prog, while NET-A only repressed the reporter by 22% at a concentration of 10 μM. Furthermore, studies have shown that 1 μM MPA could inhibit IL-8 production in endometrial explants and chorion cells (Kelly *et al.,* 1994), as well as β-chemokine production in choriodecidual cells (Kelly *et al.,*

1997). In contrast Arici *et al.* (1996a) found that 100 nM MPA increased the expression of IL-8 mRNA in HESCs.

Interestingly, MPA is widely used to facilitate infection of sexually transmitted diseases in animal models. Kaushic *et al.* (2003) showed that MPA treatment (up to 2 mg/mouse) changed susceptibility and local immune responses to genital herpes simplex virus type 2 (HSV-2) infections. Similarly, longer exposure to MPA lead to poor innate and adaptive immune responses to HSV-2 that failed to protect mice from subsequent genital challenges (Gillgrass *et al.,* 2003). Progesterone implants, which could mimic hormonally based contraceptives like MPA, were shown to thin the vaginal epithelium and enhanced SIV vaginal infection in monkeys (Cohen, 1996; Marx *et al.,* 1996). Interestingly, a recent animal study showed increased susceptibility to vaginal simian-human immunodeficiency virus (SHIV) transmission and suppression of the antiviral cellular immune response in SHIV-infected rhesus macaques administered the injectable MPA contraceptive (Trunova *et al.,* 2006), indicating an immune- rather than transmission-based mechanism. In addition, the use of MPA at contraceptive doses has been associated with increased acquisition of cervical chlamydial and gonococcal infections (Morrison *et al.,* 2004). This increase could be related to significant changes observed in the vaginal intraepithelial leukocyte population of MPA contraceptive users, which was thought to reflect altered local immune capacity (Ildgruben *et al.,* 2003). In contrast, in a retrospective cohort study among HIV-infected women, MPA contraceptive use did not appear to enhance the risk of sexually transmitted infection (STI) acquisition (Overton *et al.,* 2008). With the exception of the latter study, the above-mentioned studies suggest that MPA may exert a negative influence on local defence mechanisms in the human

female genital tract, which is the primary route for heterosexual transmission of HIV. During male-to-female transmission, it is thought that virus in the semen enters the stratified squamous epithelium of the vagina or the columnar epithelium of the endocervix to infect cells within or below the epithelium (reviewed in Pope and Haase, 2003). It is not clear which cells are the first to interact with, and to be infected with the virus, whether environmental factors in the reproductive tract tissues may influence the infection, or whether there are local immune-system responses directed at the virus. Understanding these processes is critical, particularly for developing effective prophylactic microbiocides and vaccines (Boggiano and Littman, 2007).

When used as a contraceptive, MPA was found to increase HIV viral shedding (Mostad *et al.,* 1997) and HSV cervical shedding in HIV-infected women (Mostad *et al.,* 2000). Although Mostad *et al.* (1997) did not investigate the molecular mechanism of these effects, they postulate that the effects may be mediated by factors such as direct effects on the virus, effects on local genital tract physiology or effects on immune modulation of viral replication, or a combination of these effects. Similarly, MPA used as a contraceptive causes a modest but significant increase in shedding of HIV-1 DNA in humans (Wang *et al.,* 2004). Moreover, MPA users infected with HIV have more viral variants and higher viral loads than women who are not on hormonal contraception (Lavreys *et al.,* 2004). Female sex workers in Kenya who used MPA were found to have an increased incidence of HIV-1 infection (Martin *et al.,* 1998). Taken together, the above studies raise concern, as they suggest that MPA exerts not only systemic but also local immunosuppressive side-effects *in vivo*,

and when used as injectable contraception has deleterious effects on HIV viral shedding.

In contrast to the above studies, recent data suggest that MPA as a contraceptive does not increase the risk of HIV infectability in women (Myer *et al.,* 2007; Kleinschmidt *et al.,* 2007; Morrison *et al.,* 2007). Although the results from the study by Morrison *et al.* (2007), called the Hormonal Contraception and Risk of HIV Acquisition (HC-HIV) study, found no overall increased risk of HIV acquisition among MPA users in Uganda and Zimbabwe, they observed a link between HIV, MPA and herpes simplex virus-2 (HSV-2). They indicated that whether or not women were infected with HSV-2, was a determining factor for whether women using MPA were at risk of HIV infection. They reported that women using MPA only had increased risk of HIV infection if they were not already infected with HSV-2 (Morrison *et al.,* 2007), indicating that HSV-2 infection appears to protect against HIV infection in MPA users. This result is surprising, as there is some evidence that genital tract shedding of HSV increases rather than decreases HIV acquisition and also other STIs (Ramaswamy *et al.,* 2007; Corey *et al.,* 2007). However, whether MPA influences susceptibility to HIV remains a controversial issue.

In contrast to what is known for MPA, very little is known about the effects of NET on immune function. Early studies in animal models on the effects NET (5 mg daily) on the immune system reported significantly prolonged survival of skin allografts and a moderate suppression of antibody production (Hulka *et al.,* 1965). Similary to MPA, NET also decreases splenic macrophage Fcgamma receptor expression in the guinea pig, thereby impairing the clearance of IgG-coated erythrocytes (Gomez *et al.,*

1998). Furthermore, in humans, NET like MPA compromised cell-mediated immune status in Bangladeshi women receiving these compounds as injectable contraceptives (Majumder *et al.,* 1987). In a study in men, contraceptive doses of NET-EN upregulated IL-6 (proinflammatory cytokine), while downregulating IL-10 (anti-inflammatory cytokine), implying an inflammatory profile in healthy men (Zitzmann *et al.,* 2005). In a study in healthy postmenopausal women however, NET-A in combination with estrogen, similarly to estrogen only therapy, had no effect on IL-6 levels (Žegura *et al.,* 2006). Whether or not NET is associated with an increased risk of HIV/HSV acquisition and shedding and viral load and viral diversity remains to be determined. In this regard, two recent studies in a cohort of South African women, showed no association between risk of HIV infection and NET-EN at contraceptive doses (Myer *et al.,* 2007; Kleinschmidt *et al.,* 2007).

To date, very little is known about the molecular mechanisms of action of MPA and NET on immune function, in particular the target cells, target genes and dose responses. In this regard, MPA has been reported to modulate transcription of a number of genes via the glucocorticoid receptor (GR), such as IL-2 in normal human lymphocytes (Bamberger *et al.,* 1999), IL-6 and IL-8 in mouse fibroblast cells (Koubovec *et al.,* 2004) and the *nm-23* tumor suppressor gene in breast cancer cells (Ouatas *et al.,* 2003). In contrast, MPA's suppression of the RANTES gene in endometrial cells was progesterone receptor (PR)-mediated (Zhao *et al.,* 2002). Thus, the above results suggest that the immune suppression manifested by MPA is mostly mediated by its glucocorticoid rather than progestogenic actions (Kurebayashi *et al.,* 2003; Koubovec *et al.,* 2004). Consistent with this idea is the finding that MPA, but not Prog, possesses potent anti-inflammatory properties that contribute to its

ability to prevent inflammation-induced pre-term birth in mice (Elovitz and Wang, 2004), indicating that MPA has more pronounced immunosuppressive effects than Prog.

Considering the above data, it is clear that more research on the effects of MPA and NET on target genes involved in immune function in specific target cells, is needed at the molecular level.

1.4 Mechanism of action

There are many unanswered questions about the relative actions of MPA and NET at the cellular level. It is known, however, that both these progestins bind to, as well as regulate, serum levels of steroid-binding proteins such as corticosteroid-binding globulin (CBG) (Pugeat *et al.,* 1981; Misao *et al.,* 1998a; van der Vange *et al.,* 1990) and sex hormone-binding globulin (SHBG) (Pugeat *et al.,* 1981; Misao *et al.,* 1998b; Rodriguez-Aleman *et al.,* 2000; Onobrakpeya *et al.,* 2001; Fotherby, 1988; Biglia *et al.,* 2003; Ylikorkala *et al.,* 2003). This means that they could possibly alter serum concentrations, and thus the bioavailability, of endogenous steroids to various target tissues. Moreover, it is known that the intracellular effects of MPA and NET, are mediated via binding to not only the progesterone receptor (PR), but also other steroid receptors such as the GR, MR, androgen (AR), and possibly the estrogen (ER) receptor, thereby regulating an array of genes. In the sections below, we briefly review what is known at the molecular level about the effects of MPA and NET on serum-binding proteins, followed by a detailed review on the known effects of MPA and NET via the different members of the steroid receptor family.

1.4.1 Serum binding globulins

When steroids enter the bloodstream, they interact with several serum-binding proteins, and their biological activity depends on the ratio of bound to free steroid. Steroids that are bound to serum-binding proteins are unavailable to tissues, whereas steroids that are unbound are free to diffuse from the blood into the cells of target tissues where they either get metabolised or interact with specific steroid receptors. CBG and SHBG are low abundance serum-binding proteins with high steroid-binding affinity and specificity (Hammond, 2002). They are the main transporter proteins that carry steroid hormones in the plasma. SHBG preferentially binds to testosterone, while CBG preferentially binds to cortisol (Pugeat *et al.,* 1981). Once MPA and NET-EN reach the bloodstream, they demonstrate very different binding properties towards these serum proteins, resulting in different effects on their free (bioavailable) concentrations and those of endogenous steroids which compete for binding to serum proteins (Schindler, 2003; Pugeat *et al.,* 1981).

CBG is a glycoprotein synthesized mainly in the liver (Scrocchi *et al.,* 1993), with high affinity for glucocorticoids and progestins, and low affinity for androgens, estrogens and mineralocorticoids (Breuner and Orchinik, 2002). It is the major transport protein for natural glucocorticoids (GCs) in all mammalian species including humans (Hammond, 1990). The main function of CBG is to transport and regulate the bioavailability of GCs to a range of target tissues (Hammond, 1990). In this way, CBG may be protecting tissues against potentially harmful effects of elevated GCs (Breuner and Orchinik, 2002). CBG has also been shown to influence metabolic clearance rates of GCs as the greater the fraction of bound hormone, the slower the metabolic clearance (Siiteri *et al.,* 1982). Although CBG is reported to have high

binding affinity for progestins, Schindler *et al.* (2003) have previously reported that MPA has no binding affinity for CBG. Similarly, a recent study observed very low relative binding affinities (RBAs) of Prog, MPA, as well as NET, for human CBG (less than 1%, compared to 100% for cortisol) (Sasagawa *et al.,* 2008). This result for MPA and NET-A is in agreement with an earlier study showing that these progestins could not compete with cortisol for binding to CBG (Ottosson *et al.,* 1985). In contrast to the study by Sasagawa *et al.* (2008), Ottosson *et al.* (1985) showed that Prog could compete with cortisol for binding to CBG. Thus there are a number of inconsistencies in the literature, which should be addressed by further research.

Little is known on the effects of MPA or NET on CBG gene regulation. One study showed that MPA at 1 μM suppressed CBG mRNA expression in an endometrial cancer cell line (Misao *et al.,* 1998a), which could influence bioavailability of GCs to target tissues. In contrast, oral contraceptive doses of NET resulted in an increase in serum CBG levels in healthy women (van der Vange *et al.,* 1990).

SHBG is a homodimeric glycoprotein produced by hepatocytes (Jänne *et al.,* 1998) that binds testosterone and estradiol with high affinity (Hammond, 2002). Its primary function is to transport these two biologically active steroids in the blood, thus playing an important role in regulating the levels of free fraction of testosterone and estradiol available to target cells (Siiteri *et al.,* 1982). Furthermore, several reports have shown that SHBG can exit the blood circulation and enter the extravascular compartments of various tissues (Bordin and Petra, 1980; Sinnecker *et al.,* 1988). This may regulate the local access of testosterone and estradiol to target cells that are not in the immediate vicinity of the blood supply.

MPA, NET and NET-A have been shown to influence SHBG levels in a number of studies. In the Ishikawa cell line (human endometrial cancer cells), low MPA concentrations (0.1 nM) added together with estradiol (10 nM) were found to increase SHBG mRNA expression, whereas the addition of high concentrations (1-10 μM) of MPA with or without estradiol (10 nM) suppressed it (Misao *et al.,* 1998b). Similarly, serum SHBG concentrations investigated in postmenopausal women decreased 58% with a daily treatment of 60 mg MPA for two weeks (Saaresranta *et al.,* 2000). This decrease in SHBG concentrations in response to MPA is significant, as low levels of circulating SHBG may predict diabetes and cardiovascular diseases (Pugeat *et al.,* 1995; Hajamor *et al.,* 2003). In contrast, serum SHBG levels were significantly increased in postmenopausal women treated with MPA at 2.5- to 10 mg daily in combination with 2 mg per day estradiol valerate (Rodriguez-Aleman *et al.,* 2000). Furthermore, postmenopausal women using the CEE/MPA regimen for HRT exhibited 2.5-fold higher SHBG levels, with a consequent corresponding 3.5-fold lower free androgen level, compared to non-users (Christodoulakos *et al.,* 2005).

Postmenopausal women treated with NET-A (5 mg/day) for 9 weeks showed a significant decrease in SHBG levels (Onobrakpeya *et al.,* 2001). Similarly, increasing concentrations of NET antagonised the stimulatory effect of estrogen and resulted in a reduction of serum SHBG concentrations (Fotherby, 1988). However, in another study of postmenopausal women receiving E_2/NET-A this regimen increased SHBG levels and decreased free testosterone (Dören *et al.,* 2001). Consistent with this study, SHBG levels increased in osteoporotic elderly women receiving 1 mg NET-A in combination with 2 mg estradiol over a period of one year (Ylikorkala *et al.,* 2003).

In contrast to these studies showing either decreased or increased SHBG levels, some studies have shown no variations in serum levels of SHBG in postmenopausal women receiving NET-A in combination with estradiol (Biglia *et al.,* 2003; Christodoulakos *et al.,* 2005).

In addition to regulating SHBG levels, NET, to a greater extent than MPA, has been shown to bind to and displace [^{3}H]-testosterone from SHBG (Pugeat *et al.,* 1981; Fotherby, 1988; Darney, 1995; Activelle package insert reg. no. 33/21.8.2/0532, Novo Nordisk). The results from a recent study showing RBAs of NET for human SHBG as 19.5%, and less than 1% for both MPA and Prog, relative to testosterone = 100%, are consistent with this (Sasagawa *et al.,* 2008). This may result in the displacement of endogenous steroids from SHBG such as testosterone and estrogen, which may increase their bioavailability to target tissues.

1.4.2 Steroid receptors

The effects of steroid hormones are mediated through binding to intracellular steroid receptors, a subfamily of the nuclear receptor superfamily. The steroid receptor family comprises the PR, GR, MR, AR, and ER. These receptors are hormone-activated transcription factors that share a high level of similarity with regards to their structure (Figure 2), as well as their mechanism of action. The receptors all contain a variable amino-terminal transactivation domain (NTD), followed by the well conserved DNA-binding domain (DBD) and the moderately conserved carboxy-terminal domain, containing the ligand-binding domain (LBD) (Mangelsdorf *et al.,* 1995). Furthermore, they have two major transcriptional activation domains i.e. activation function 1 (AF-1) and activation function 2 (AF-2) located in the NTD and LBD, respectively. Co-

activators bind to the AF-2 site, and are thus important for the induction of transcriptional activity of the receptor, while the AF-1 site is required for optimal transcriptional activity. In the absence of hormone, steroid receptors are mostly associated with heat-shock proteins.

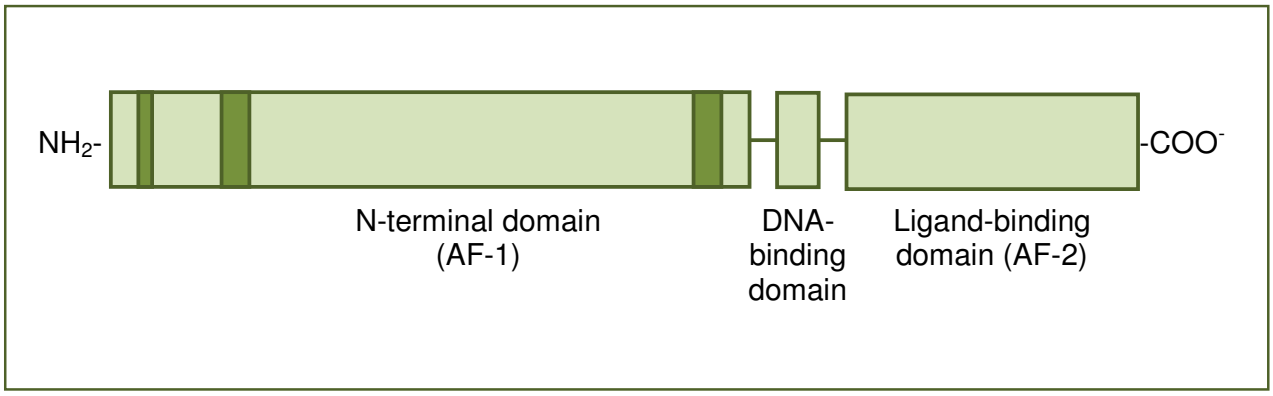

Figure 2. A schematic representation of the general structural and functional domains of steroid hormone receptors.

Although all steroid receptors function by essentially identical mechanisms, their target genes and also target tissues, may differ (McKenna and O'Malley, 2002). The inactive receptor is activated by hormone binding after which the hormone receptor-complex translocates to the cell nucleus where it binds to specific DNA sequences, called hormone response elements (HREs), in the promoter regions of target genes, and subsequently regulates gene expression (reviewed in Evans, 1988 and Beato, 1989). For example, the GR binds to HREs called glucocorticoid response elements (GREs). When receptors bind directly to the DNA of target genes, expression of specific target genes is modulated by activating (transactivation) or repressing (transrepression) transcription. Transrepression can take place without direct DNA binding by the receptor but rather via protein-protein interactions with other transcription factors such as nuclear factor-kappa B (NFκB) or activator protein-1

(AP-1) (McEwan *et al.,* 1997; Webster *et al.,* 1999). By far the best-studied in terms of transrepression is the GR (De Bosscher *et al.,* 2003; Koubovec *et al.,* 2005). GCs may inhibit other genes by transcriptional cross-talk where the GR mutually interferes with other signalling pathways such as those involving AP-1, NFκB, C/EBP, CREB, p53 and Smad (De Bosscher *et al.,* 2003). This may require direct or indirect binding of the GR monomer to these transcription factors, and as this effect does not require direct binding of the GR to DNA, the term "tethering GRE" is often used to describe these elements (Newton, 2000). Many genes involved in the inflammatory response such as cytokines and chemokines have been reported to be repressed in this way (De Bosscher *et al.,* 2003).

Many genes contain GREs in their promoters, including lipocortin-1 (also known as annexin I), p11/calpactin binding protein (Flower and Rothwell, 1994; Pitzalis *et al.,* 2002; Yao *et al.,* 1999), tyrosine aminotransferase (TAT) (Sassi *et al.,* 1998), and the mouse mammary tumour virus (MMTV) gene (Scheidereit *et al.,* 1983). The consensus sequence for GRE binding is the palindromic 15bp sequence GGTACAnnnTGTTCT, where *n* is any nucleotide (Adcock, 2000). This element may also mediate induction by progesterone, androgens and mineralocorticoids, via the PR, AR and MR, respectively (Beato, 1989).

Much less is known about the mechanisms by which the AR, PR and MR repress genes, but given the similar mechanism of action established thus far for transactivation, it is likely that these receptors repress genes by mechanisms similar to those of the well-studied GR. Indeed, the AR and PR have also been shown to mediate transrepression of genes via the same mechanism as the GR by tethering to

other transcription factors. For example, NFκB can be antagonised by the PR (Kalkhoven *et al.,* 1996) and the AR (Palvimo *et al.,* 1996; Bellido *et al.,* 1995), resulting in repression of genes. This antagonism involves direct protein-protein interactions between the steroid receptors and NFκB (Palvimo *et al.,* 1996; Kalkhoven *et al.,* 1996). Not much is known about the transrepression of genes via the MR. Early work by Pearce and Yamamoto (1993) showed that the MR could not repress AP-1 activity in response to aldosterone (Ald), while a later report suggested that the MR could exert repressive effects on NFκB (Liden *et al.,* 1997).

The prediction of the physiological effects of MPA and NET is not straightforward. These effects may be influenced by the fact that different cells have different levels, as well as different isoforms, of steroid receptors. A cell's response to a particular progestin may thus differ depending on which receptor or receptor isoform it binds. In addition, receptor density in a specific cell has also been reported to determine the biological character (agonist or antagonist) of steroids, as well as the transcriptional activity (transactivation versus transrepression) of steroids (Zhao *et al.,* 2003). Another level of complexity is the presence of plasma membrane steroid receptors that signal by rapid non-genomic mechanisms and crosstalk between various signalling pathways (Turgeon *et al.,* 2004). For these reasons, recent research has begun to address the cell-specific effects of MPA and NET. In the following section, we will thus review what is known of the mechanism of action of MPA and NET via steroid receptors, and their subsequent effects on target genes.

1.4.3 Effects of MPA and NET on target genes via the:

1.4.3.1 Progesterone receptor (PR)

The physiological effects of progestins in target tissues are assumed to be mediated by binding to the classic intracellular PR. Indeed, both MPA and NET have been shown to bind to the PR in a number of studies (MacLaughlin and Richardson, 1979; Bergink *et al.,* 1983; Feil and Bardin, 1979; Zhang *et al.,* 2000; Selman *et al.,* 1996; Bergink *et al.,* 1983; Deckers *et al.,* 2000; Chavez *et al.,* 1985; Kontula *et al.,* 1975), with almost equal affinity (Kontula *et al.,* 1975; Bergink *et al.,* 1983). It is noteworthy that although Bergink and co-workers (1983) observed similar relative binding affinities for both MPA and NET for the PR (44- and 38% respectively, relative to 100% binding by the synthetic progestin, Org 2058) in cytosolic fractions of the MCF-7 breast cancer cell line, MPA had a higher binding affinity (65%) for the PR than NET (41%) relative to 100% binding by Org 2058, in intact MCF-7 cells. It is unclear from the above data whether the relative binding activity was determined from K_d values or from EC_{50} curves.

There are two functional isoforms of the PR, PR-A and PR-B, which are transcribed from two promoters of a single gene (Kastner *et al.,* 1990). The ratios of the individual isoforms vary in reproductive tissues (Shyamala *et al.,* 1990), and they have different physiological functions. Considering that MPA is a progesterone derivative and NET-EN, a testosterone derivative, it is likely that they may have different binding affinities for the PR-A and PR-B isoforms, as well as different biological activities depending on the target cell. In normal human breast tissue, the ratio of the PR isoforms, PR-A:PR-B is approximately 1:1, while in a large percentage of breast cancer cells, this ratio is changed, and constitutes a risk for breast cancer

(Mote *et al.,* 2002). In a study evaluating the effects of CEE and MPA on the expression of PR-A and PR-B in breast tissue of castrated female monkeys, it was found that CEE combined with MPA decreased PR-A and increased PR-B levels, while treatment with conjugated estrogen alone increased the expression of both PR isoforms (Isaksson *et al.,* 2003). Interestingly, MPA alone did not affect the levels of either isoform. These findings suggest that MPA, when used together with estrogen in HRT, upregulates PR-B, which is responsible for both proliferation and differentiation of the mammary tissue (Sitruk-Ware and Plu-Bureau, 2004). This alteration of the normal PR-A:PR-B ratio has also been shown in another study (Vereide *et al.,* 2006), and may be a putative mechanism by which MPA, and possibly NET, when used together with estrogen in HRT, increase the risk for breast cancer.

MPA has also been shown to be a PR agonist for transactivation via synthetic HRE sequences as well as via natural promoter-reporter constructs for several target genes involved in different physiological processes such as tumour development (Bamberger *et al.,* 1999). In a study in Ishikawa endometrial adenocarcinoma cells co-transfected with either hPRA or hPRB, MPA has been shown to increase vascular endothelial growth factor (VEGF) promoter construct activity via three functional progesterone response elements (PREs) identified in the VEGF promoter (Mueller *et al.,* 2003). These results raise the possibility that increased angiogenesis in response to progestins may play a role in cell growth or metastasis in some human tumours (Hyder *et al.,* 1998). However, the role of MPA in cancer development is controversial. MPA effectively inhibited estrogen-induced growth of the T47D cell line, and also inhibited growth of the Ishikawa endometrial cancer cell line stably

transfected with PR-B in a dose-dependent manner, irrespective of the presence or absence of estrogen in the culture medium. Neither the parental Ishikawa cell line, which is PR negative, nor the cell line stably transfected with PR-A, showed growth inhibition during culture in the presence of MPA. When the PR-B cell line was cultured with MPA in the presence of RU486 (antiprogestin), the MPA-induced growth inhibition was impaired (Smid-Koopman *et al.,* 2003). In contrast, MPA induces human breast cancer cell proliferation by increasing cyclin D1 promoter activity via PR-B, but not PR-A (Saitoh *et al.,* 2005). As the cyclin D1 promoter does not have progesterone-response element-related sequences, this appears to be a nongenomic mechanism which entails activation of the PI3K/Akt/NFκB signalling cascade (Saitoh *et al.,* 2005).

A study comparing the transcriptional effects of MPA and Prog in human breast cancer cell lines expressing either PR-A or PR-B, transiently transfected with a PRE_2-luciferase construct, indicated that MPA and Prog were equally effective through both PR-isoforms (Ghatge *et al., 2*005). Interestingly, when co-transfecting exogenous PR-isoforms individually with the PRE_2-luciferase construct, MPA had lower transcriptional activity than Prog, at all doses, via both PR-A and PR-B. In contrast, expression profiling of endogenous PR-regulated genes comparing Prog and MPA, in the wild type T47Dco breast cancer cell line stably expressing equimolar levels of PR-A and PR-B, indicated that MPA and Prog have similar transcriptional activity (Ghatge *et al., 2*005).

MPA has been shown in many studies to play a significant role in various endometrial functions via the PR. MPA was shown to increase the promoter activity of insulin-like

growth factor binding proteins-1 (IGFBP-1) in HESCs co-transfected with either hPR-A or hPR-B. IGFBP's regulate insulin-like growth factor action, which is believed to play a role in endometrial differentiation. In contrast, IGFBP3 (the major binding protein of IGF-1) is downregulated by MPA in the Ishikawa endometrial cell line stably transfected with the PR-B, but not in the parental Ishikawa cells or those stably transfected with PR-A (Smid-Koopman *et al.,* 2003). Relatively high plasma IGF-1 and low IGFBP3 levels have been independently associated with an increased risk of prostate-, breast- (in premenopausal women), colorectal epithelium-, and possibly lung cancer development (Renehan *et al.,* 2005). It thus stands to reason that as the levels of IGFBP3 decrease, the amount of freely available IGF-1 consequently increases, resulting in an increased risk of cancer development.

MPA has also been shown to increase the promoter activity of a deleted glycodelin-A (GdA) promoter-reporter construct in the HEC-1B cell line (a human endometrial adenocarcinoma cell line originally derived from the glandular component of the endometrium) via the PR. The timing of expression of GdA in the glandular cells of the human endometrium is critical for the regulation of fertilisation, as it potentially inhibits fertilisation but enhances implantation (Gao *et al.,* 2001). Thus, this increase in GdA expression by MPA may possibly participate in its contraceptive mechanism of action. Furthermore, MPA at 10 mg/day also significantly enhanced decidual prolactin (PRL) gene expression (mRNA and protein) in the human endometrium *in vivo* (Reis *et al.,* 1999). Similar results were obtained in HESCs, where MPA was shown to enhance PRL gene activation in the presence of elevated intracellular cAMP levels (Brosens *et al.,* 1999). PRL is a marker of decidualisation, which is a critical process in the preparation of the uterus for blastocyst implantation and which

involves stromal cell differentiation. This synergy between MPA and cAMP on PRL expression was postulated to be dependent upon cellular levels of PR, and facilitated by transient expression of steroid receptor co-activator 1e (SRC-1e) but not SRC-1a. An additional study revealed an increase in STAT5 in response to cAMP/MPA treatment, which enhanced PRL expression in human endometrial stromal (ES) cells (Mak *et al.,* 2002). Furthermore, MPA acts via the PR to suppress functional long form leptin receptor (OB-R (L)) mRNA expression by 50% in human organ-cultured proliferative endometrial samples, as indicated by the inhibition of MPA-induced suppression by the addition of the PR antagonist, RU486 (Koshiba *et al.,* 2001). MPA may therefore alter the sensitivity of the endometrium to leptin, which is involved in the stimulation of reproductive functions and possibly in early development.

NET has also been shown to have agonist activity for transactivation via the PR on synthetic and natural promoter constructs of genes involved in various physiological functions. NET was shown to induce a PRE2-TATA-CAT reporter vector transiently transfected in the CV-1 cell line stably transfected with the rabbit PR, and this activity was inhibited by RU486 (Pasapera *et al.,* 2001). In the CHO cell line transfected with the MMTV-luciferase reporter, NET showed about 12% transactivation activity relative to 100% agonist activity of Org 2058 (Schoonen *et al.,* 2000; Deckers *et al.,* 2000). As was observed with MPA, NET also increased VEGF release into the media of cultured T47-D breast cancer cells (Hyder *et al.,*1998), an effect likely mediated via the three functional PRE elements located in the promoter (Mueller *et al.,*2003). Furthermore, NET (like MPA) was shown to increase the promoter activity of IGFBP-1 in endometrial cells co-transfected with hPRA via PRE sites present in the promoter (Gao *et al.,* 2000). A study using Affymetrix GeneChip U133A expression arrays,

demonstrated that the effects of MPA, NET-A and Prog on PR-regulated gene expression in the PR-positive T47Dco breast cancer cell line, were similar to each other (Bray *et al.,* 2005). Similarly, a recent study reported EC_{50} values for MPA, NET and Prog for the PR in the COS-1 cell line transiently co-transfected with PRE2-tk-luc, as 0.1, 0.2 and 0.4-0.8 nM, respectively (Sasagawa *et al.,* 2008). Furthermore, in the CHO cell line stably transfected with either human PR-A or PR-B, and a MMTV-luciferase reporter, Dijkema and co-workers (1998) reported similar rank order of MPA, NET-A and Prog for both the PR-A and PR-B. Upon closer inspection of their data however, it appears that the rank order for potency for PR-A is MPA > NET-A > Prog, while for PR-B, MPA = NET-A > Prog.

Both MPA and NET-A were shown to dose-dependently (0.1 to 10 nM) increase expression of two markers of vascular inflammation, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Tatsumi *et al.,* 2002). This increase was moderately blocked by the addition of RU486, but not by hydroxyflutamide, an AR antagonist, and the authors conclude that this stimulation was mediated to some extent via the PR. Considering that RU486 is also an antagonist of the GR it may be that the GR also plays a role. However, the authors do not comment on such a possibility. In human ES cells, prolonged MPA exposure (100 nM for 8 days) was shown to repress the transcription and protein levels of RANTES (Regulated-upon-Activation, Normal T cell Expressed and Secreted) via the PR (Zhao *et al.,* 2002). RANTES is a critical chemokine in the pathogenesis of endometriosis and has been suggested to play an early role in the inflammatory response (Khorram *et al.,* 1993; Hornung *et al.,* 1997)

Although the above studies shed some light on the mechanism of action of MPA and NET via the PR on various physiological functions, further pharmacological investigation into the relative binding affinity, relative agonist potency and mechanism of action of MPA and NET for both transactivation and transrepression via PR-A and PR-B are required.

1.4.3.2 Glucocorticoid receptor (GR)

A large number of studies have indicated that MPA interacts with the GR (Bojar *et al.,* 1979; Winneker and Parsons, 1981; Teulings *et al.,* 1980; Kontula *et al.,* 1983; Selman *et al.,* 1996; Bamberger *et al.,* 1999; Simoncini *et al.,* 2004; Koubovec *et al.,* 2004; Koubovec *et al.,* 2005; Ronacher *et al.,* 2009). One study, in human mononuclear leukocytes, found that MPA displays considerable binding affinity towards the GR (42%, relative to 100% binding by the synthetic glucocorticoid (GC) Dexamethasone (Dex), calculated from EC_{50} curves at the 50% competition level) (Kontula *et al.,* 1983). Interestingly, the relative binding affinity of the naturally occurring ligand, cortisol, was significantly lower (25%) than that of MPA. Similar results were obtained in human renal carcinoma cells (Bojar *et al.,* 1979) and human breast cancer cells (Teulings *et al.,* 1980). In addition, our recent study in the COS-1 monkey kidney cell line transfected with the hGR indicated a relative binding affinity of 19 nM for MPA, compared to 14 nM for Dex (Ronacher *et al.,* 2009). Kontula and co-workers also obtained a K_d value (31 nM), which is a more accurate measure of binding affinity, for MPA binding to the GR in human mononuclear leukocytes, which was shown to be only 3 times higher than that of Dex (K_d = 10 nM) (Kontula *et al.,*1983). An additional study by our group revealed a K_i value of 10.8 nM for MPA, only about 2.5 times higher than the K_d of 4.2 nM for Dex , for the hGR in a human

lung carcinoma cell line (A549) expressing endogenous GR (Koubovec *et al.,* 2005). Furthermore, MPA's high affinity for the GR was also demonstrated in the beagle dog, with a calculated K_i value of 3.7 nM for MPA, which was shown to be 4-5 times higher than that of Dex (Selman *et al.,* 1996). In another study, the affinity of the rat GR for MPA (K_d = 5.0 nM) was observed to be similar to that of Dex (K_d = 4.1 nM) (Winneker and Parsons, 1981).

In contrast to MPA, NET has been shown to bind with very low affinity to the hGR (0.1% relative to 100% binding by Dex, calculated from EC_{50} curves) and was also unable to displace [^{3}H]-MPA from the human GR in human mononuclear leukocytes (Kontula *et al.,* 1983). These results are consistent with a later study in a human leukemic IM-9 cell line, indicating binding activity of NET towards the human GR to be below 1% relative to Dex = 100% (Schoonen *et al.,* 2000). Our results obtained from a study in the COS-1 cell line transfected with a human GR expression vector also indicate that NET has a low affinity for the GR (RBA for NET=1688 nM, Dex=14 nM) (Ronacher *et al.,* 2009). In addition, we have directly compared the relative binding affinities of MPA and NET-A to each other, and to Prog, for the GR in the A549 cell line, and subsequently determined precise K_i values for MPA (10.8 nM), NET (270 nM) and Prog (215 nM) (Koubovec *et al.,* 2005).

Since many of the side-effects of conventional GCs used to treat inflammatory or autoimmune diseases may be attributed to transactivation of GRE-driven promoters (Imai *et al.,* 1993; Brasier and Li, 1996), an important goal of pharmacological and clinical research has been to identify GCs that discriminate between transactivation and transrepression. The transrepressive effect of MPA, and to a much lesser extent NET, on *in vitro* functions of human lymphocytes via the GR has previously been

investigated. Results indicate that MPA (at $2.5x10^{-7}$ M) acts as a dissociated GC, since it efficiently (same extent as Dex) transrepresses phorbol-ester-stimulated IL-2 and IL-6 transcription, but only minimally activates transcription via GREs (Bamberger *et al.,* 1999). These observed effects of MPA are likely to be mediated by the GR, as Dex, the synthetic GR ligand, showed similar effects, and moreover human lymphocytes express GR, very low levels of AR, and no PR. In addition, MPA was also shown to repress transcription of the IL-2 promoter construct stimulated with tetradecanoyl phorbol acetate (TPA) and ionomycin, via co-transfected GR in the Jurkat T-lymphocyte cell line (Bamberger *et al.,* 1999). Furthermore, the dose-dependent (10 nM to 10 μM) decrease in the secretion and mRNA expression of IL-6 by MPA in a human thyroid cancer cell line (KTC-2 cells), was also suggested to be GR-mediated (Kurebayashi *et al.,* 2003).

Although NET has previously been shown to display no transactivation activity towards the GR in the leukemic IM-9 cell line (Schoonen *et al.,* 2000), it had not been established whether it displays dissociative GC properties until the work by our group. We pharmacologically characterised the effects of MPA compared to NET and Prog on GR-mediated gene regulation for both transrepression and transactivation in the human embryonic kidney (HEK293) cell line stably transfected with a rat GR expression vector, and transiently transfected with a glucocorticoid response element (GRE)-driven reporter construct or with an IL-8 promoter-reporter construct (Koubovec *et al.,* 2005). In contrast to the minimal transcriptional activity observed via GREs in human lymphocytes, our study showed that MPA displayed transcriptional activity via GREs. In fact, MPA displayed much greater glucocorticoid agonist potency than Prog for both transactivation (MPA: EC_{50} = 7.2 nM; Prog: EC_{50}

= 280 nM) and transrepression (MPA: EC_{50} = 2.7 nM; Prog: EC_{50} = 26 nM). In contrast to MPA, NET-A did not transactivate even at 10 µM, and only marginally (22%) transrepressed the IL-8 reporter at this concentration. We observed similar results in a COS-1 cell system, transiently transfected with hGR and a GRE-driven reporter construct (pTAT-GRE-E1b) or an AP-1 or NFκB promoter–reporter construct, respectively (Ronacher *et al.,* 2009). In this system, NET-A was shown to antagonize GR-mediated transactivation (Appendix C6). Interestingly, results from another recent study in the COS-1 cell line co-transfected with the GR and the PRE_2-tk-luc promoter-reporter, corroborates the results indicating that MPA is an agonist for transactivation via the GR (EC_{50} = 23.3 nM is about 11.5 times higher than the 2 nM observed by Ronacher *et al.,* 2009), but this study did not show antagonist activity for NET up to 3 x 10^{-6}M (Sasagawa *et al.,* 2008).

Consistent with agonist activity of MPA via the GR as discussed above, there are several reports of GR-mediated MPA effects on target genes in different tissues and cells. It has been suggested that MPA, but not Prog, can exert negative effects in kidney cells via the GR. MPA increased α-subunit of epithelial Na channel (α-ENaC) mRNA levels in the kidney cortex *in vivo* (in adrenalectomized mice), while MPA, but not Prog, dose dependently increased endogenous α-ENaC, as well as serum and glucocorticoid-regulated kinase 1 (sgk1) mRNA expression via the GR in two mouse cortical collecting duct (CCD) cell lines, M-1 and MDCK-C7 (Thomas *et al.,*2006). In addition, MPA was unable to stimulate an α-ENaC promoter-reporter construct where the GRE had been mutated, indicating that the stimulation of α-ENaC is mediated via the GRE in its 5'-flanking region. Furthermore, the dose-dependent (10 nM to 1µM) elevation of Nm23-H1 (metastasis suppressor) protein expression by MPA in

metastatic human breast carcinoma cells via the GR was thought to involve GREs (Ouatas *et al.,* 2003). Recently, it was shown that MPA downregulates endothelial nitric oxide synthase (eNOS) mRNA expression and the formation of nitric oxide levels in human umbilical vein endothelial cells (HUVECs) via the GR (Zerr-Fouineau *et al.,* 2007), thereby reducing the anti-aggregatory effect of endothelial cells.

Several studies indicate that the GR may play a role in the effects of MPA on the immune system. MPA, unlike NET, has been shown to induce GC-like effects including inhibition of the proliferative responses to the T-cell mitogens concanavalin A and phytohaemagglutinin (Kontula *et al.,* 1983). We have also previously demonstrated that the GR partly mediates MPA repression of IL-6 and IL-8 promoter-reporter activity at the transcriptional level, most likely via interference with NFκB and AP-1 transcription factors, in murine fibrosarcoma L929sA (Koubovec *et al.,*2004). Since GCs impact on virtually every aspect of the immune and inflammatory response (Spangelo and Gorospe, 1995; Galon *et al.,* 2002) MPA, and possibly NET, may exert side-effects due to their GC-like repressive action on target genes involved in immune function, other than those reviewed above.

In addition to genes involved in immune function, several genes involved in reproduction are negatively regulated by GCs, and may also be targets for MPA, such as the genes for the α-subunit of glycoprotein hormones (Chatterjee *et al.,* 1991) and PRL (Sakai *et al.,* 1988). Furthermore, physiological concentrations of GCs have been proposed to be essential and optimal for osteoblast proliferation and differentiation. It has recently been proposed that bone loss associated with MPA administration, at contraceptive doses or higher, is caused by decreased osteoblast

differentiation as a likely result of MPA occupying the GR, since increasing GR occupancy beyond that reached at normal GC concentrations reduces osteoblast differentiation (Ishida and Heersche, 2002).

Taken together, MPA, unlike NET-A and Prog, has high affinity for the GR (Koubovec *et al.,* 2005; Ronacher *et al.,* 2009). MPA represses cytokine genes *in vitro* (Koubovec *et al.,* 2004, Bamberger *et al.,* 1999) and displays greater GR agonist potency and efficacy than progesterone or NET-A for both transactivation and transrepression of synthetic promoter-reporter constructs (Koubovec *et al,.* 2005). In addition, MPA protected the GR from partial trypsin digestion *in vitro* to a much greater extent than NET-A or Prog at saturating concentrations, suggesting that the differences in biological activity of the progestins are not only due to differences in their affinity for the GR, but also due to the induction of different conformational changes in the liganded-GR (Koubovec *et al.,* 2005). Furthermore, the findings that GR levels can determine whether MPA acts as a GR agonist or antagonist in some systems, and can also influence its dissociative properties (extent of transactivation versus transrepression activity), could have major implications for the physiological effects of MPA, and maybe even NET-A, via the GR (Bamberger *et al.,* 1999, Zhao *et al.,* 2003). Finally, the glucocorticoid-like actions of MPA may cause side-effects in women using MPA for contraception, as well as other reproductive therapies via transactivation and transrepression of GC-responsive target genes, and in particular may compromise immune function via transrepression of cytokine genes. The above-mentioned data all indicate that MPA and NET-A display very different GC-like properties compared to each other and to Prog, confirming that all progestins are not the same as each other, and very rarely the same as Prog.

1.4.3.3 Androgen Receptor (AR)

As for the GR, a multitude of studies show that MPA binds to and is an agonist for transactivation via the AR (MacLaughlin and Richardson, 1979; Teulings *et al.,* 1980; Perez-Palacios *et al.,* 1981a; Perez-Palacios *et al.,* 1981b; Perez-Palacios *et al.,* 1983; Bergink *et al.,* 1983; Hackenberg *et al.,* 1993; Bentel *et al.,* 1999; Bamberger *et al.,* 1999; Kemppainen *et al.,* 1999). In the study by Perez-Palacios and co-workers (1983), the K_d of MPA for the cytosolic AR from rat pituitary and hypothalamus was determined as 1.7 nM and 2.9 nM, respectively. Similarly, the K_d of MPA for the AR in MFM-223 human mammary carcinoma cells was determined as 3.6 nM, compared with a K_d of 0.18 nM for the natural androgen dihydrotestosterone (DHT) (Hackenberg *et al.,* 1993). In addition, NET has also been shown to bind to and have agonist activity for transactivation via the AR (Perez-Palacios *et al.,* 1981a; Bergink *et al.,* 1983; Chavez *et al.,* 1985; Bergink *et al.,* 1985; Schoonen *et al.,* 2000; Deckers *et al.,* 2000). However, in two different studies (Schoonen *et al.,* 2000; Deckers *et al.,* 2000), the relative binding and relative agonist activity values for transactivation of NET for the AR were shown to be low: 3.2% and 1.1% of DHT (100%), respectively. Similarly, the K_d of NET for the AR was determined as 19 nM (8.1% relative binding compared to 100% binding by the synthetic androgen, R1881) in rat prostrate cytosol (Chavez *et al.,* 1985). Furthermore, when comparing MPA and NET, both progestins showed similar relative binding affinities (8- and 7% respectively, relative to 100% binding for DHT) for the endogenously expressed AR in intact human breast cancer (MCF-7) cells, yet in cytosol fractions of the same cells MPA showed a higher binding affinity (29%) relative to NET (17%) for the AR (Bergink *et al.,* 1985). It is unclear from some of the above data whether the relative binding activity was determined from equilibrium dissociation constant (K_d or K_i)

values or from relative binding affinity (RBA) values (EC_{50} values). This is relevant as K_d or K_i values are a constant value for a particular receptor, that cannot be influenced by different experimental conditions, and thus represent a more accurate measure of relative binding affinity of a ligand for its receptor than EC_{50} values. In contrast, the EC_{50} value is a relative number, which is influenced by receptor levels and radiolabeled ligand concentration (further explanation in Appendix D1). The possibility also exists that the results reported by Bergink *et al.* (1985) may be inaccurate, as these cells express steroid receptors other than the AR, to which these progestins may bind.

Several reports in the literature show that MPA acts as an agonist for transactivation via the AR on several different synthetic reporter genes as well as on endogenous genes. For example, MPA (at 0.1 nM) was shown to induce transcriptional activation of a MMTV-luciferase reporter construct in the CV-1 cell line via the AR. However, 100-fold more MPA was required to achieve the same agonist activity as DHT, and the synthetic AR agonists, mibolerone and R1881. Similarly, the androgenic activity of MPA was also confirmed in the HeLa cervical carcinoma cell line transiently co-transfected with an AR expression vector and two different promoter-reporter constructs (PRE_2-luc and MMTV-luc) (Ghatge *et al., 2*005). In another study, MPA (at 2.5×10^{-7} M) was reported to transactivate a GRE-driven reporter construct, via co-transfected AR in the Jurkat T lymphoma cell line (Bamberger *et al.,* 1999). NET, as well as its A-ring reduced derivative, 5α-NET, have also been shown to have AR-mediated transcriptional activation in the HeLa cervical cell line transiently transfected with an AR expression vector and a PRE-E1b-CAT reporter plasmid (Garcia-Becerra *et al.,* 2004). Interestingly, 5α-NET displayed a four-fold higher

androgenic activity than NET. Recently, a study has investigated agonist activity of MPA, NET and Prog for transactivation in the COS-1 cell line transiently co-transfected with the AR and PRE_2-tk-luc, and indicate EC_{50} values for agonist activity of MPA and NET of 56.8 and 19.6 nM, respectively, while no activity was observed for Prog (at 3 μM) (Sasagawa *et al.,* 2008). These investigators also looked at the antagonist activity of MPA, NET and Prog for transactivation in the COS-1 cell line transiently co-transfected with the AR and PRE_2-tk-luc, and indicated that, unlike Prog, neither MPA nor NET antagonized DHT-mediated transcription via the AR (up to 3 μM) (Sasagawa *et al.,* 2008).

Despite the established agonist activity of MPA, the mechanism whereby MPA acts as an AR agonist appears to differ from that for DHT. Kemppainen *et al.* (1999) investigated the mechanism of AR activation by MPA, using a mammalian two-hybrid assay in the CHO cell line, and showed that MPA (up to 1 μM) failed to promote the AR NH_2-terminal and carboxy-terminal (N/C) interaction, which is usually induced by high-affinity agonists and believed to be inhibited by antagonists. Although not an AR antagonist, MPA was shown to be a potent antagonist of the DHT-induced AR N/C-interaction in the same cells. In contrast, NET was able to induce the AR N/C-interaction, and did not antagonize the DHT-induced AR N/C-interaction (Tamzin Tanner in Hapgood laboratory, MSc thesis). In addition, partial proteolytic digestion of *in vitro* translated AR, demonstrated that both NET and 5α-NET, induced a similar conformational change to each other, and to DHT, in the AR (Garcia-Becerra *et al.,* 2004). Recently, Birrell and co-workers (2007), formulated a molecular model in which MPA-bound AR forces the displacement of a critical amino acid residue, Phe874, thereby disrupting the well-defined AF-2 cleft observed for the DHT-bound

AR, taking on an atypical ligand binding domain structure distinct from that mediated by DHT. The authors thus hypothesized that this may explain divergent effects of MPA and DHT on AR-regulated gene expression. Indeed, a recent comparative gene expression profiling study in human breast cancer cell lines showed that MPA and DHT regulated distinct subsets of genes (Ghatge *et al.,* 2005). However, they also showed that, unlike Prog, MPA at physiological (10 nM) and pharmacological (1 μM) doses, is as potent as DHT in regulating AR-mediated gene expression. In agreement with its agonist activity for transactivation, MPA has been reported to act as an agonist for transrepression via the AR. For example, Bamberger *et al.* (1999) showed that MPA (at 2.5 x 10^{-7} M) transrepressed an IL-2 promoter-reporter construct stimulated with TPA and ionomycin, via co-transfected AR in the Jurkat T lymphoma cell line.

In agreement with results showing that MPA acts as an AR agonist, there is evidence that several physiological effects of MPA administered to patients are mediated via the AR. Clinical studies indicate that the response of breast tumours to high-dose MPA therapy is dependent on the expression of the AR (Birrell *et al.,* 1995). Further evidence that MPA acts via the AR in breast cancer cells has been provided by studies indicating inhibition of proliferation of AR-positive, but not AR-negative, cells by MPA, and the reversal of this inhibition with AR-specific antagonists (Hackenberg *et al.,* 1993; Bentel *et al.,* 1999). This argument for MPA effects being mediated by the AR, was strengthened by the study of Ghatge *et al.* (2005) showing that, in contrast to Prog, MPA is as potent as DHT in regulating AR-mediated gene expression in human breast cancer cell lines. In addition, the inability of some tumors in advanced breast cancer to respond to MPA therapy is related to reduced AR levels

or impaired AR function (Buchanan *et al.,* 2005). Moreover, a recent study in an epithelial ovarian cancer cell line expressing endogenous AR indicated that MPA is more effective than DHT, in increasing the invasive potential of these cells via the AR (Gogoi *et al,* 2008). Consistent with the above-mentioned studies, an earlier study suggested that the significant inhibition of serum gonadotropin levels in postmenopausal women who had received either MPA or NET-EN, in contrast to Prog, was due to their actions via the AR (Perez-Palacios *et al.,* 1981a). Additionally, it is postulated that the androgenic properties of MPA are responsible for the favourable decrease in the levels of the plasma proinflammatory markers, C-reactive protein and serum amyloid A protein, increased by estrogen, in postmenopausal women using the CEE/MPA HRT treatment (Wakatsuki *et al.,* 2002).

In contrast to the data available for MPA, much less is known about the biological activity of NET via the AR. Although the recent study by Sasagawa *et al.* (2008) has now characterized MPA and NET pharmacologically in terms of potency for transactivation, further work is still required to pharmacologically characterise effects of MPA vs. NET vs. Prog, in terms of potency and/or efficacy, on AR-mediated gene regulation, for transactivation and transrepression of various target genes in the same system. Furthermore, no direct comparison of the relative equilibrium dissociation constants of the two progestins has been reported. Further studies are therefore required to determine precise K_i values for both MPA and NET for the AR, as well as studies to characterise the mechanisms and effects of MPA vs. NET vs. Prog on AR-mediated gene regulation for both transrepression and transactivation of various target genes in the same system.

1.4.3.4 Mineralocorticoid Receptor (MR)

Prog has a high affinity for the MR, displaying weak partial agonist[2], transactivation activity, but potent antagonist[3] activity for Ald, via the MR (Wambach *et al.,* 1979; Rafestin-Oblin *et al.,* 1992; Quinkler *et al.,* 2002). In contrast, both MPA and NET-A are reported to bind weakly to the cytoplasmic MR in rat kidney (Wambach *et al.,* 1979), and their agonist activity for transactivation via the MR, as well as antagonist activity for Ald via the MR, is controversial. The RBA's for the MR were obtained from EC_{50} curves and determined as 4% for MPA and <1% for both NET and NET-A, compared with 100% binding by Prog. A number of studies report that MPA and NET do not display any agonist or antagonist properties via the MR (Winneker *et al.,* 2003 and references therein, Palacios *et al.,* 2006). In contrast, Sasagawa *et al.* (2008) showed that although MPA and NET have no agonist activity via the transiently transfected MR co-transfected with PRE_2-tk-luc in the COS-1 cell line, both MPA and NET were able to antagonize Ald-mediated transcription via the MR, albeit to a much lesser extent than Prog. The difference in the antagonistic activity of MPA and NET relative to Prog via the MR may have serious implications for post-menopausal women using estrogen/progestin treatment for HRT, as they would not be able to antagonize the transcriptional activity of Ald via the MR, thus leading to side-effects such as weight gain and increased blood pressure. Since high blood pressure is a cardiovascular disease risk factor, using synthetic progestins, such as MPA and NET-A, that lack anti-mineralocorticoid activity in HRT, could lead to cardiovascular

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[2] A drug or ligand that elicits a response less than the maximal response of the full agonist (See Appendix B1).

[3] A drug or ligand that does not elicit a response itself, but inhibits agonist-mediated responses (See Appendix B1).

complications. In addition, it has not yet been established whether these progestins can transrepress target genes via the MR. Further work is thus required to characterise the effects of MPA vs. NET on MR-mediated gene regulation for both transrepression and transactivation of various target genes in the same system. Findings of such studies may result in an improved understanding of the relative effects of MPA and NET on blood pressure and in cardiovascular disease, as has been highlighted by the drive to find new progestins, such as trimegestone (Winneker *et al.,* 2003) and drospirenone (Elger *et al.,* 2003, Palacios *et al. ,*2006), with anti-mineralocorticoid activity.

1.4.3.5 Estrogen Receptor (ER)

Evidence for activity of both MPA and NET via the estrogen receptor is contradictory. Two functional ERs, ERα and ERβ, transcribed from different genes, have been identified (Nilsson *et al.,* 2001). Both MPA and NET-A have previously been reported to bind estrogen receptors both *in vivo* (in the rat uterus) and *in vitro* (Di Carlo *et al.,* 1983). Contrary to these studies, two other studies showed that MPA does not bind to the ER (Teulings *et al.,* 1980; Bergink *et al.,* 1983), while another study indicated that it does not have any estrogenic effects (Markiewicz and Gurpide, 1994). Reports on the estrogenic activity of NET are similarly contradictory. NET has been reported to have no binding or transactivation activities via the ER (Schoonen *et al.,* 2000; Bergink *et al.,* 1983). However, in other studies NET (Markiewicz and Gurpide, 1994) and its A-ring reduced metabolites (Mendoza-Rodriguez *et al.,* 1999) have been reported to show intrinsic estrogenic activity. Furthermore, a 5α-reduced metabolite of NET (3β, 5α-NET), has been shown to selectively activate ERα at low concentrations, while ERβ agonistic activity was observed only at high concentrations

(1 μM) (Larrea *et al.,* 2001). Interestingly, a recent study in the COS-1 cell line transiently transfected with the ERα or ERβ expression vectors and a synthetic promoter-reporter construct (ERE3-tk-luc), revealed that NET, but not MPA or Prog, showed preferential agonistic activity towards ERα (EC_{50} = 39.3 nM), and also minimal agonistic activity towards ERβ (EC_{50} = 1097.2 nM) (Sasagawa *et al.,* 2008). In summary, data on the activity of MPA and NET via the ER is controversial. The differences in the results of the various studies may be due to different extents of metabolism of these progestins in different cells. Thus it may be that MPA and NET themselves do not bind to the respective ER isoforms, but that their metabolites do. However, it is hard to discriminate between the binding of the test compound vs. its metabolites. Further research is thus needed to determine the mechanisms and possible clinical implications of progestin activity via ER isoforms.

1.5 Conclusion

The synthetic progestins, MPA, NET-EN and NET-A, are widely used by millions of women in contraception and HRT. Although both progestins have been shown to exert a range of side-effects when used *in vivo*, little is known about their mechanism of action at the cellular level. The importance of investigating the mechanism of action of synthetic progestins at the molecular level, is highlighted by recent clinical evidence showing that MPA and NET increase the risk of the development of breast cancer in HRT users (Rossouw *et al.,* 2002, Millions Women's study collaborators). In contrast, Prog has been shown to have anti-proliferative, and anti-estrogenic effects on breast tissue. Whether MPA and NET, either alone or in combination with estrogen in HRT, have beneficial or deleterious cardiovascular effects, is still controversial. Certainly, the results of the WHI trial, and a number of other studies

(Rossouw *et al.,* 2002; Register *et al.,* 1998; Manson *et al.,* 2003; Meendering *et al.,* 2008), seem to suggest that MPA is not cardioprotective. In contrast, some studies suggest that MPA does not have any detrimental cardiovascular effects (Taner *et al.,* 2006; Seeger *et al.,* 2007). Similarly, a number of studies have indicated that NET has harmful cardiovascular effects (Rosano *et al.,* 2001; Lokkegard *et al.,* 2003; Fernandes *et al.,* 2008), while other studies show that it does not (Mueck *et al.,* 2002; Pedersen *et al.,* 2004; Pedersen *et al.,* 2006, Seeger *et al.,* 2007). On the other hand, Prog has not been shown to have adverse cardiovascular effects (Ottoson *et al.,* 1985; Saarikoski *et al.,* 1990; Simoncini *et al.,* 2004; Mishra *et al.,* 2005). These conflicting data highlight a need for further research to clarify the role of MPA and NET on breast cancer and CVD markers at the cellular level.

MPA used as contraception has also been shown to increase viral shedding (Mostad *et al.,* 1997), which raises concern as to its impact on the spread of viral diseases. Several clinical studies have shown no link between MPA or NET-EN usage and HIV infection (Myer *et al.,* 2007; Kleinschmidt *et al.,* 2007). However numerous reports suggest there is a link between progestins and HIV infection (Martin *et al.,* 1998, Lavreys *et al.,* 2004; Morrison *et al.,* 2007), highlighting the ongoing controversy in this area. More research is needed on the clinical effects, and mechanisms of action of these progestins, particularly with respect to HIV and other sexually transmitted infections (STIs). While MPA and NET are known to have varying effects on systemic immune function (reviewed above), their effect on local immune function in the cervicovaginal tract is likely to be most pertinent to unraveling their relative effects on early STIs. Clearly more research is required to investigate the relative effects of synthetic progestins on immune function in the reproductive tract.

As Prog and these synthetic progestins have different chemical structures, they may have different effects on target cells, depending on their dose, route of administration, bioavailability, effects on endogenous steroid metabolism in the target cell, their affinity for different steroid receptors or receptor isoforms, as well as the availability of steroid-responsive promoters and cofactors. Surprisingly, there is a paucity of research addressing the above-mentioned factors, particularly the relative binding affinity and relative agonist potency and efficacy (relative to each other and to Prog) of MPA and NET-EN (and NET-A) for transrepression and transactivation via various steroid receptors as well as their effects on intracellular signalling pathways, in the same system. Our recent work highlights the importance of such investigations as we have shown that MPA displays greater GR agonist potency and efficacy than either Prog or NET-A for both transactivation and transrepression of synthetic promoter-reporter constructs, and displays a higher affinity than Prog or NET-A for the GR (Koubovec *et al.,* 2005). Furthermore, their interactions with serum-binding proteins as well as their effects on steroidogenesis require further investigation. These studies would be important in defining differences in mechanism of action of various synthetic progestins, and hence facilitate an informed choice of progestins, by women and their physicians, for reproductive intervention and therapy.

HYPOTHESES AND AIMS

From the literature it is clear that Prog, MPA and NET-A differ in their structure, metabolites, bioavailability, affinity for different steroid receptors, and ultimately in their biological activity.

To this end, the primary hypothesis of this thesis was that MPA and NET-A differ at least in some respects in their biological activity towards the hAR and hMR as compared to each other and Prog. Furthermore, it was hypothesized that MPA and NET-A would differ in their regulation of cytokine genes in the lower female genital tract. More specifically it was hypothesized that:

1) MPA and NET-A (as compared to Prog), differentially regulate the cytokine and chemokine genes, in a cell-specific manner, in cervicovaginal epithelial cells;
2) different steroid receptors are responsible for the differential regulation of these genes;
3) unlike Prog, MPA and NET-A are potent androgens;
4) MPA and NET-A have weaker anti-MR activity than Prog;
5) the aldosterone-induced upregulation of the Orm-1 (α-glycolytic protein or orosomucoid-1) gene via the hMR, is antagonized by progesterone, but not by MPA and NET-A;

The aims of this project were three-fold:

Firstly, the regulation of TNFα-induced cytokine and chemokine genes, specifically IL-6, IL-8 and RANTES, in response to the endogenous female hormone, Prog, and the two synthetic progestins, MPA and NET-A, was investigated. These studies were performed in two immortalized cell lines from the lower female genital tract namely, Ect1/E6E7 (human ectocervical epithelial cell line) and Vk2/E6E7 (human vaginal epithelial cell line). Thereafter, the hypothesis that different steroid receptors are mediating the differential regulation of these genes, was tested by re-evaluating the expression of the genes when incubating cells with the progestins, in the presence and absence of the androgen receptor-specific antagonist hydroxyflutamide and RU486, the PR and GR antagonist.

Secondly, using the COS-1 monkey kidney cell line, the mechanism of action of MPA and NET-A were directly compared to each other and Prog, via overexpressed human androgen receptor (hAR) and human mineralocorticoid receptor (hMR), respectively. Relative dissociation constants (K_i values) were determined for the progestins for each receptor, as well as the AR- and MR-mediated transcriptional activation and repression by MPA and NET-A in the COS-1 cell line.

Thirdly, the MR-mediated transcriptional regulation of a gene that is a marker of cardiovascular disease (CVD), Orm-1, was investigated in a rat cardiomyocyte cell line, with a particular focus on the antagonistic role of MPA and NET-A, as compared to Prog.

Chapter 2

Differential regulation of endogenous pro-inflammatory cytokine genes by MPA and NET-A in cell lines of the female genital tract

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Abstract

Medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives are widely used in female reproductive therapy, but little is known about their mechanism of action at the cellular level. MPA used as a contraceptive, has been shown to increase HIV (Mostad *et al.,* 1997) and HSV cervical shedding in HIV-infected women (Mostad *et al.,* 2000). Mucosal innate immune factors as well as proinflammatory cytokines are associated with elevated levels of HIV-1 shedding in the female genital tract (Gumbi *et al.,* 2008). Although use of MPA as a contraceptive has been implicated in effects on local immune function (IIdgruben et al., 2003), not much is known about the effects of MPA and NET-A in the female genital tract. In this study, we thus investigated the regulation of cytokine genes by MPA and NET-A, as compared to the endogenous hormone, progesterone (Prog), on mucosal immunity in an *in vitro* cell culture model of the female cervicovaginal environment, by comparing their effects on the regulation of the endogenous pro-inflammatory cytokine/chemokine genes, interleukin (IL)-6, IL-8 and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted). Two epithelial cell lines generated from normal human vaginal (Vk2/E6E7) and ectocervical (Ect1/E6E7) cells, immortalized by expression of the E6 and E7 genes of human papillomavirus type 16 (Fichorova *et al.,* 1997), were used as model systems. Our study is the first to show that ligand-specific and cell-specific regulation of IL-6, IL-8 and RANTES gene expression occurs with Prog, MPA and NET-A in the cervicovaginal environment. Moreover, we show that the repression of the TNFα-induced RANTES gene by MPA in the Ect1/E6E7 cell line is mediated by the androgen receptor. Collectively, these data demonstrate that different anatomical sites of the female genital tract respond differently to Prog and the synthetic

progestins, possibly due to interaction with different steroid receptors. This may have important implications for women's risk of susceptibility to infections.

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Introduction

The mucosal surface of the lower female genital tract (cervicovaginal environment) is a complex system that provides a barrier against pathogens. Epithelial cells lining the cervicovaginal mucosa are the point of entry for many viral, bacterial, and parasitic infections (Fichorova and Anderson, 1999). Therefore, these cells have features enabling them to combat infections, such as the expression of hormone receptors, hormone-dependent immune functions, and expression of genital tract-specific defensins (Press *et al.,* 1986; Kutteh *et al.,* 1998; Quayle *et al.,* 1998). The cervicovaginal epithelial cells constitutively express a wide variety of pro- and anti-inflammatory mediators, such as the cytokines, interleukin (IL)-1, IL-6, IL7, macrophage colony-stimulating factor, transforming growth factor beta, and the chemoattractant cytokines, IL-8 and RANTES (Regulated-upon-Activation, Normal T cell Expressed and Secreted), which are upregulated in response to tumor necrosis factor (TNF)-α (Fichorova and Anderson, 1999; Fichorova *et al.,* 2001a). These cells thus execute a vital role in the innate and acquired immune systems present at the mucosal surfaces.

IL-8 and RANTES (also termed CCL5) are chemotactic cytokines, or chemokines, involved in the early inflammatory response by recruiting specific leukocytes, particularly macrophages, to sites of ongoing inflammation and injury, while IL-6 is responsible for neutrophil priming to chemotactic factors (Kunkel *et al.,* 1997). Thus, the cytokine milieu in the cervicovaginal mucosa is an important determinant of resistance and susceptibility to infections. Interestingly, increased levels of the cytokines IL-1, TNF-α, IL-6 and the chemokines RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β in cervicovaginal secretions, have been associated with

human immunodeficiency virus (HIV)-1 infection and bacterial vaginosis (Fichorova *et al.,* 2001b).

The transmission of- and susceptibility to infections in women may be better understood if factors affecting the immune response in the vagina and cervix are more clearly defined. Research in animal models and in women indicates that local and regional immune responses can influence the outcome of vaginal challenge with microbial pathogens (Rakasz and Lynch, 2002). Sex hormones are examples of factors that have been shown to influence susceptibility and disease predisposition to many genital tract infections (Brabin, 2002). Furthermore, there are indications that women using antibiotics, corticosteroids (immunosuppressive therapy), oral contraceptives and hormone replacement therapy, are more susceptible to fungal vaginal infections (Spinillo *et al.,* 1995; Geiger and Foxman 1996; Magliani *et al.,* 2002).

Medroxyprogesterone acetate (MPA) used as contraceptive, has been shown to increase HIV (Mostad *et al.,* 1997) and HSV cervical shedding in HIV-infected women (Mostad *et al.,* 2000). Although Mostad and co-workers did not investigate the molecular mechanism of these effects, they postulate that the effects may be mediated by factors such as direct effects on the virus, effects on local genital tract physiology or effects on immune modulation of viral replication, or a combination of these effects (Mostad *et al.,* 1997). Interestingly, a recent animal study showed that MPA increased susceptibility to vaginal simian-human immunodeficiency virus (SHIV) transmission and suppressed the antiviral cellular immune response in SHIV-infected rhesus macaques (Trunova *et al.,* 2006), indicating an immune- rather than

transmission-based mechanism. In another animal study, it was shown that MPA treatment at contraceptive doses rendered mice 100-fold more susceptible to genital HSV-2 infection compared to untreated mice (Kaushic *et al.,* 2003). In addition, the use of MPA has been associated with increased acquisition of cervical chlamydial and gonococcal infections (Morrison *et al.,* 2004).

At the molecular level, MPA has been reported to modulate transcription of a number of genes via the glucocorticoid receptor (GR). For example, IL-2 in normal human lymphocytes (Bamberger *et al.,* 1999), IL-6 and IL-8 in a mouse fibroblast cell line (Koubovec *et al.,* 2004) and the *nm-23* tumor suppressor gene in a breast cancer cell line (Ouatas *et al.,* 2003). In contrast, MPA's suppression of the RANTES gene in endometrial cells was progesterone receptor (PR)-mediated (Zhao *et al.,* 2002). Furthermore, MPA has been shown to regulate a number of genes via both the PR and the androgen receptor (AR) in human breast cancer cell lines (Ghatge *et al.,* 2005). It is thus apparent that even though MPA and NET were developed for the similarity of their biological actions to those of progesterone (mediating their effects by binding to the PR), they can also initiate a diverse range of biological effects by cross-reacting with other members of the steroid receptor family such as the GR, the AR and possibly the mineralocorticoid receptor (MR) (Bentel *et al.,* 1999; Philibert *et al.,* 1999; Hapgood *et al.,* 2004; Koubovec *et al.,* 2005).

An important question is thus whether, and to what extent, clinical doses of MPA, and also NET-A, regulate known pro-inflammatory mediators such as IL-6, IL-8 and RANTES, in the cervicovaginal mucosa. Understanding the mechanisms of this regulation and the receptors involved, would further our understanding of differential

gene regulation by different progestins and assist in the design of new progestins with fewer side effects. Investigating these mechanisms at a site relevant to infections, such as the cervicovaginal environment, is likely to be relevant to mucosal immunity. Factors that affect immunity in the cervicovaginal environment may be important determinants of transmission risk of pathogens such viruses, and understanding these factors may shed light on molecular events occurring during infections. In the light of the above, our strategy was to investigate the effects of MPA and NET-A relative to Prog on mucosal immunity in an *in vitro* cell culture model of the female cervicovaginal environment, by comparing their effects on the regulation of the endogenous pro-inflammatory cytokine/chemokine genes IL-6, IL-8 and RANTES. Two epithelial cell lines generated from normal human vaginal (Vk2/E6E7) and ectocervical (Ect1/E6E7) cells, immortalized by expression of the E6 and E7 genes of human papillomavirus type 16 (Fichorova *et al.,* 1997), were used as model systems.

Materials and methods

Inducing compounds

4-pregnene-3, 20-dione (progesterone; Prog), 6α-methyl-17α-hydroxy-progesterone acetate (medroxyprogesterone acetate; MPA), 17α-ethynyl-19-nortestosterone 17β-acetate (norethindrone acetate; NET-A), 5α-androstan-17β-ol-3-one (dihydrotestosterone; DHT), 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one (mifepristone; RU486), 11β,16α-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione (dexamethasone; Dex), 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al (aldosterone; Ald), and recombinant TNF-α were obtained from Sigma-Aldrich, South Africa. NET-A, the acetate, was used as it

is soluble in water compared with the insoluble ester of NET-EN. *In vivo*, both NET-EN and NET-A undergo hydrolysis and are converted to NET and its metabolites (Stanczyk and Roy, 1990). Hydroxyflutamide (OHF) was obtained from Dr C. Tendler (Schering Plough Research Institute, USA). Unlabelled 17β-hydroxy-7α,17α-dimethylestr-4-en-3-one (mibolerone; MIB), [^{3}H]-MIB (76.8 Ci/mmol), unlabelled R5020, ^{3}[H]-R5020 (84.6 Ci/mmol) and [^{3}H]-Ald (87.9 Ci/mmol) were purchased from PerkinElmer Life and Analytical Science, South Africa. [^{3}H]-Dex (89 Ci/mmol) was purchased from AEC-Amersham (South Africa). All unlabelled test compounds were dissolved in absolute ethanol and stored at -20^{o}C. These compounds were then added to serum-free culturing medium such that the final concentration of ethanol was 0.1%. Control incubations (no test compounds) were performed in the presence of 0.1% ethanol.

Cell culture

Ect1/E6E7 and Vk2/E6E7 cell lines (human ectocervical and vaginal epithelial cell lines, respectively) were purchased from American Type Culture Collection (ATCC), USA and maintained in keratinocyte serum-free medium (GibcoBRL, Paisley, UK) supplemented with 50 µg/ml bovine pituitary extract (GibcoBRL, Paisley, UK), 0.1 ng/ml epidermal growth factor (GibcoBRL, Paisley, UK), 0.4 mM $CaCl_2$, 50 IU/ml penicillin and 50 µg/ml streptomycin (GibcoBRL, Paisley, UK). Cultures were maintained in 75 cm^2 culture flasks (Greiner Bio-One International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO_2. All cultures were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney RI, 1987), and only mycoplasma-negative cell lines were used in experiments.

Plasmids

Plasmids expressing the human mineralocorticoid receptor, pRShMR, and the human glucocorticoid receptor, pRS-hGRα were a kind gift from Prof. R. Evans (Howard Hughes Medical Institute, La Jolla, USA). A plasmid expressing the human androgen receptor, pSVARo (Brinkmann *et al.,* 1989) was obtained from Frank Claessens, (University of Leuven, Leuven, Belgium). The plasmid expressing the human progesterone receptor, isoform B, pSG5hPR-B (Kastner *et al.,* 1990), was obtained from Stoney Simons Jr (NIH, Bethesda, USA). The pGL2basic empty vector was obtained from Promega, Madison, USA.

Isolation of total RNA and realtime quantitative RT-PCR (QPCR) analysis of representative genes

Ect1/E6E7 and Vk2/E6E7 cell lines were maintained as described above. Cells were induced with 0.02 µg/µl TNF-α and 1 µM of the test compounds, in the absence and presence of 10 µM receptor-specific antagonists, for 24 hours. Total RNA was isolated from cells using Tri-reagent (Sigma-Aldrich, South Africa) according to the manufacturer's instructions. The integrity of the RNA (presence of intact 18S and 28S ribosomal bands) was confirmed by denaturing agarose gel electrophoresis. Total RNA (1 µg) was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. Real-time PCR was performed by using a Light-Cycler, rapid thermal cycler system (Roche Applied Science, South Africa) according to the manufacturer's instructions. Nucleotides, TaqDNA polymerase, and buffer used in the reaction were those included in the LightCycler-FastStart DNA Master^{PLUS} SYBR Green I system (Roche Diagnostics, South Africa). Agarose gel electrophoresis and melting curve

analysis was performed to confirm the generated amplicon in each sample. The amplification efficiency for each primer was determined by generating a standard curve from a cDNA dilution series. The efficiencies were 1.99, 1.92, 1.98 and 2.0 for IL-6, IL-8, RANTES and GAPDH, respectively. Relative IL-6, IL-8 and RANTES transcript levels were calculated with the Fit Points method described by Pfaffl (2001), and were normalized to relative glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels. Primer sets are as described in Table 1.

Table 2.1: QPCR primer sets

Gene	Primers (5' - 3')	Strand	Length of amplicon (bp)	Annealing temperature (^{o}C)	Reference
RANTES	TACCATGAAGGTCTCCGC GACAAAGACGACTGCTGG	Forward Reverse	199	60	Wolf *et al.* (2002)
IL-6	TCTCCACAAGCGCCTTCG CTCAGGGCTGAGATGCCG	Forward Reverse	193	60	Wolf *et al.* (2002)
IL-8	TGCCAAGGAGTGCTAAAG CTCCACAACCCTCTGCAC	Forward Reverse	197	60	Wolf *et al.* (2002)
GAPDH	TGAACGGGAAGCTCACTGG TCCACCACCCTGTTGCTGTA	Forward Reverse	307	55	Ishibashi *et al.* (2003)

Western blotting

Ect1/E6E7 and Vk2/E6E7 cell lines were plated at 1 x 10^6 cells per well in 6-well plates and allowed to grow to confluency. Cells were washed with PBS and lysed in 100 µl SDS-sample buffer (Sambrook *et al.,* 1989). Lysates were boiled and subjected to electrophoresis on a 8% SDS polyacrylamide gel using β-actin expression as a loading control. Following electrophoresis, proteins were transferred

to nitrocellulose membranes. Blots were probed with an anti-AR (H-280, 1:1000), anti-GR (H300, 1:3000), anti-PR-B (sc-811, 1:500), anti-PR-AB (sc-810, 1:500) or anti-MR (H300, 1:1000) antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), as well as the anti-β-actin (1:1500) antibody from Cell Signalling Technology, Massachusetts, USA, for 16 hours at 4^{o}C. Blots were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10000, NA934VS, Amersham, South Africa) for 1 hour. Proteins were visualized using enhanced chemiluminescence (ECL) from Amersham, followed by exposure to Hyperfilm MP high performance autoradiography film (Amersham, South Africa).

Whole cell binding assays to determine steroid receptor content in the Ect1/E6E7 and Vk2/E6E7 cell lines

Competitive whole cell binding assays were performed essentially as described by Bamberger *et al.* (1995), with the following modifications. Ect1/E6E7 and Vk2/E6E7 cell lines were maintained as described above. Cells were seeded into 12-well tissue culture plates (Nunc, Denmark) at 2 x 10^{5} cells per well. On day 3, the cells were washed with phosphate-buffered saline (PBS) and incubated for 2 hours at 37°C with 10 nM ^{3}[H]-MIB (76.8 Ci/mmol) for AR, ^{3}[H]-Ald (87.9 Ci/mmol) for MR, ^{3}[H]-R5020 (84.6 Ci/mmol) for PR (^{3}[H]-MIB, ^{3}[H]-Ald, and ^{3}[H]-R5020 all from PerkinElmer Life and Analytical Science, South Africa) or ^{3}[H]-Dex (89 Ci/mmol) for GR (AEC-Amersham, South Africa), in the absence (total binding) and presence of 10 µM unlabelled MIB, aldosterone, R5020, or Dex (non-specific binding), respectively. Working on ice at 4^{o}C, cells were washed three times with ice-cold 1X PBS containing 0.2% (w/v) bovine serum albumin (BSA) for 15 min. Cells were then lysed

with 200 µl reporter lysis buffer (Promega, Madison, USA). Total binding was determined by scintillation counting as counts per minute (cpm), while specific binding was determined by subtracting non-specific binding from total binding. Protein concentrations were determined using the Bradford protein assay method (Bradford, 1976). Specific binding was normalized to the protein concentration. The reported values are averages of at least three independent assays, with each condition performed in triplicate. The receptor numbers in $f$mol/mg protein were determined as follows:

The specific activity of the tritium-labelled test compound (Ci/mmol) was converted to disintegrations per minute (dpm) per mmol, by multiplying by a factor of 2.22 x 10^{12} (1 Curie equals 2.22 x 10^{12} dpm). The dpm was multiplied by the counting efficiency to get cpm per mmole, and divided by 10^{12} to get cpm/$f$mol. Counting efficiency was 40% in our system. The specific activity was expressed as cpm/$f$mol. Dividing the specific binding cpm value by this value gives specific binding in $f$mol, which was divided by the protein content of the sample in mg, to yield the number of binding sites in $f$mol/mg protein.

Luciferase reporter assays

For transactivation assays, the Ect1/E6E7 and Vk2/E6E7 cells were maintained as above and seeded into 24-well tissue culture plates at 5 x 10^4 cells per well. On day 2, cells in each well were transiently transfected with 330 ng of the pTAT-GRE-E1b-luc construct and 33 ng of the expression vector for the hMR, hGR, hAR, hPR-B or the empty vector pGL2basic, using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. On day 3, the cells were washed with PBS and induced with 10 µM of

either Ald, Dex, MIB, or R5020. After 24 hours, the cells were lysed with reporter lysis buffer (Promega, Madison, USA). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega, Madison, USA) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale CA, USA). The values obtained were normalised to expression of β-gal, which was measured using the GalactoStar Assay Kit from Tropix (Bedford MA, USA). The reported values are averages of at least three independent experiments, with each condition performed in triplicate.

Data manipulation and statistical analysis

The Graph Pad Prism® software was used for data manipulations, graphical representations, and statistical analysis. One-way ANOVA analysis of variance and Bonferroni posttests (compares all pairs of columns) were used for statistical analysis. Statistical significance of differences is indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, whereas no statistical significance (ns) is indicated by $p>0.05$. The letters a,b,c etc. are also used to denote statistically significant differences, where all those values which differ significantly from others, are assigned a different letter.

Results

MPA and NET-A, unlike Prog, exhibit differential patterns of gene regulation on pro-inflammatory chemokines

Human Ect1/E6E7 and Vk2/E6E7 cell lines were treated for 24 hours with 0.02 µg/µl TNF-α and 1 µM Prog, MPA or NET-A, followed by QPCR analysis for expression of the IL-6, IL-8 and RANTES genes, respectively. In both the Ect1/E6E7 and Vk2/E6E7

cell lines, results show that unlike Prog which upregulates IL-6 gene expression, both MPA and NET-A have no effect (figure 2.1A and 2.1B). In contrast, MPA and NET-A differentially regulate both the IL-8 (figure 2.2A) and RANTES (figure 2.3A) genes in the Ect1/E6E7 cell line. The results show that MPA, like Prog upregulates IL-8, (10.3- vs. 12-fold, respectively), while NET-A upregulates the gene to a significantly lower extent (2.8-fold) (figure 2.2A). Although not statistically different, Prog and NET-A seemed to upregulate the IL-8 gene in the Vk2/E6E7 cell line to a similar extent, while MPA showed the greatest level of upregulation (figure 2.2B). Unlike Prog which upregulates RANTES gene expression in the Ect1/E6E7 cell line, MPA downregulates the RANTES gene expression, while NET-A has no effect (figure 2.3A). Conversely, both MPA and NET-A, like Prog, upregulate the expression of the RANTES gene in the Vk2/E6E7 cell line (figure 2.3B). Taken together, MPA and NET-A exhibit differential patterns of gene regulation on expression of the IL-8 and RANTES genes, as compared to Prog, in a promoter- and cell- specific manner. In addition, unlike Prog, MPA and NET-A do not upregulate IL-6 gene expression.

The PR, AR and GR are expressed in both ectocervical and vaginal cell lines

As these progestins are known to interact with the PR, GR, AR and possibly the MR (Philibert *et al.,* 1999), we hypothesized that the differential regulation of genes by MPA and NET-A, particularly on the RANTES gene, was due to their action via different steroid receptors, or differing activities via the same steroid receptor. To test this hypothesis, we firstly investigated which members of the steroid receptor family were expressed in these cell lines using a combination of Western blotting (figure 2.4A) and whole cell binding (figure 2.4B and 2.4C).

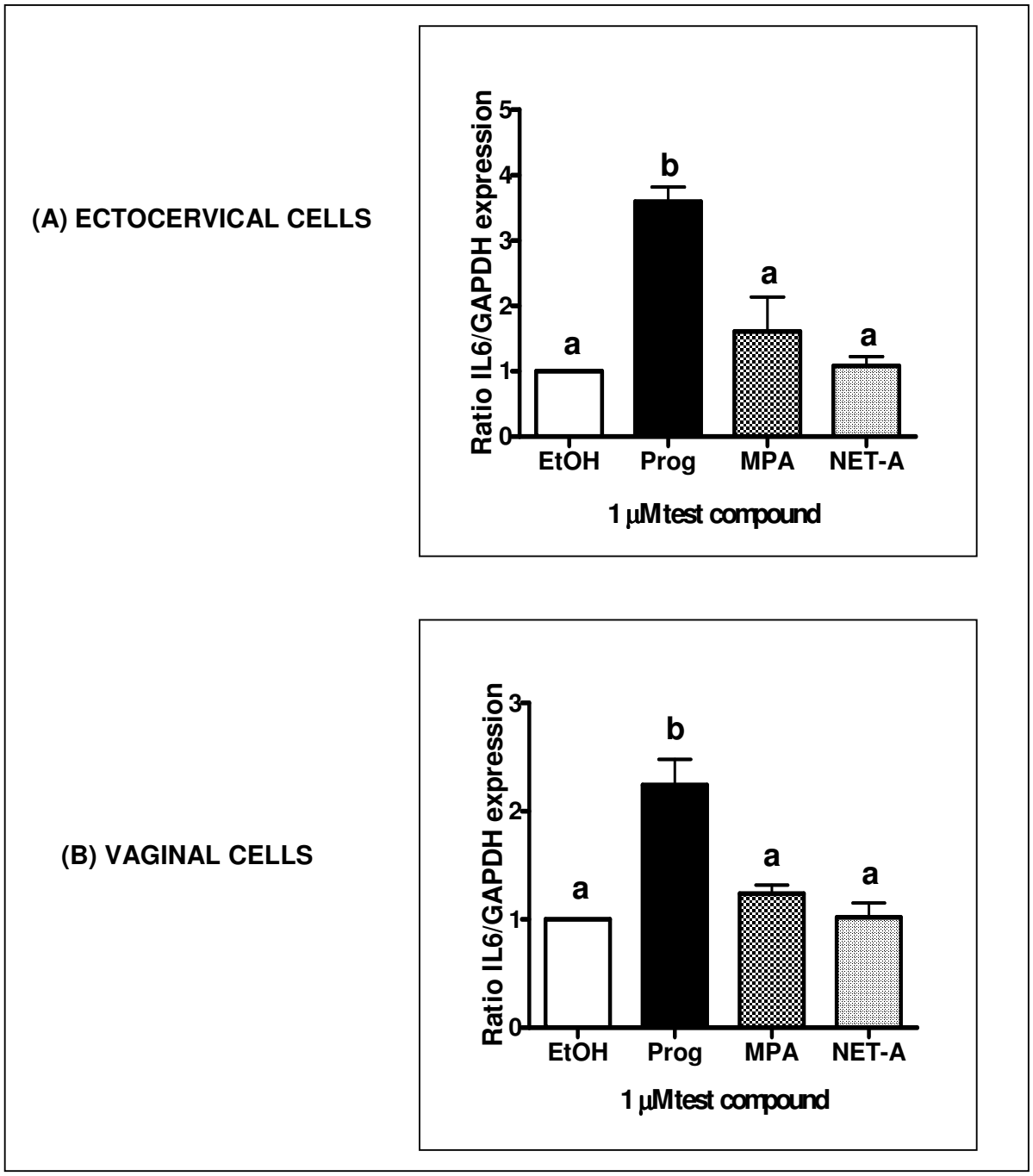

Figure 2.1: Effect of MPA and NET-A on the TNF-α-induced expression of the IL-6 gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7- and **(B)** Vk2/E6E7 cell lines were incubated with 0.02 µg/µl TNF-α in the presence of 0.1% EtOH or 1 µM Prog, MPA or NET-A for 24 hours. Total RNA was extracted, cDNA synthesized and expression levels of IL-6 and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments.

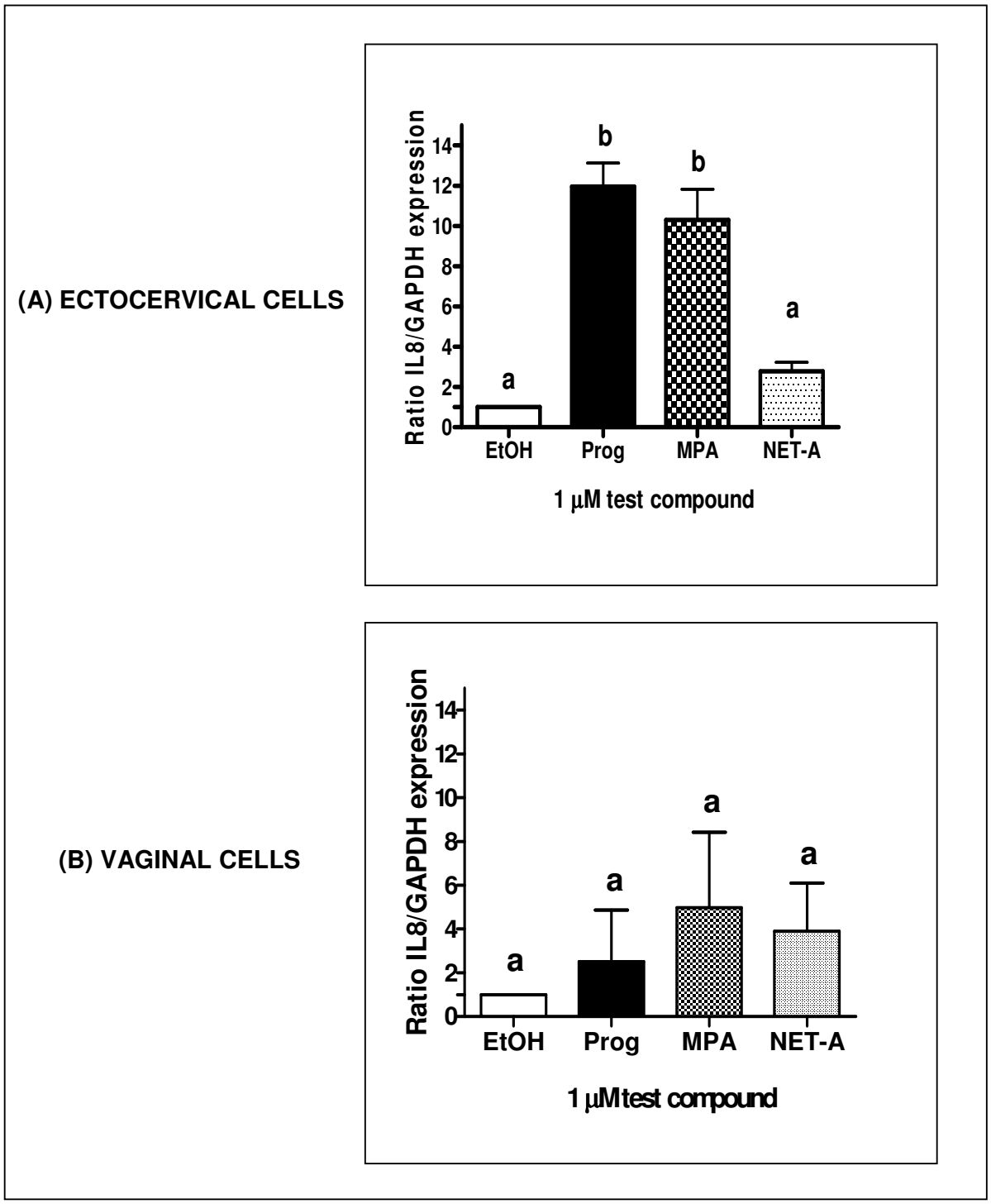

Figure 2.2: Effect of MPA and NET-A on the TNF-α-induced expression of the IL-8 gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7- and **(B)** Vk2/E6E7 cell lines were incubated with 0.02 µg/µl TNF-α in the presence of 0.1% EtOH or 1 µM Prog, MPA or NET-A for 24 hours. Total RNA was extracted, cDNA synthesized and expression levels of IL-8 and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments.

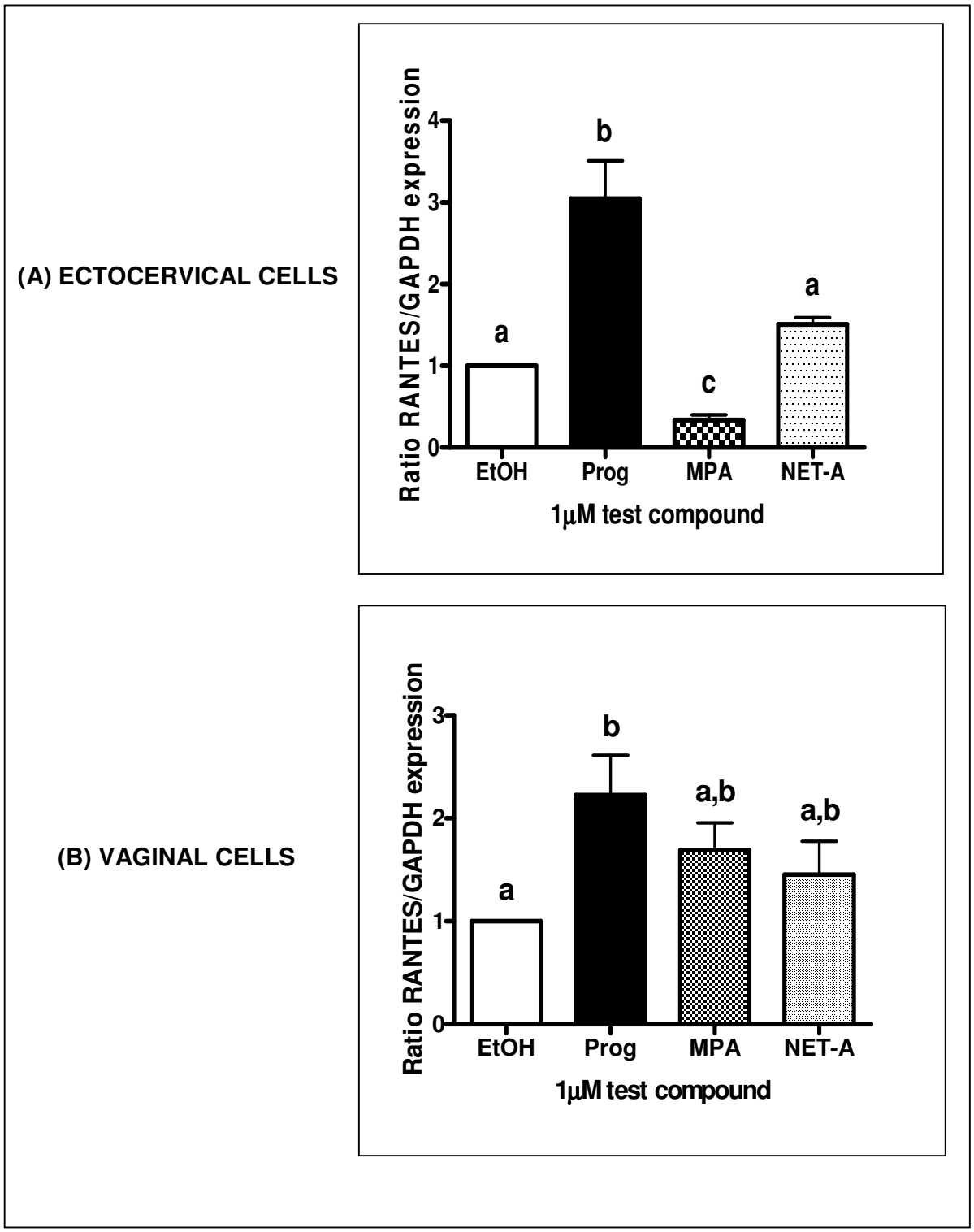

Figure 2.3: Effect of MPA and NET-A on the TNF-α-induced expression of the RANTES gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7- and **(B)** Vk2/E6E7 cell lines were incubated with 0.02 µg/µl TNF-α in the presence of 0.1% EtOH or 1 µM Prog, MPA or NET-A for 24 hours. Total RNA was extracted, cDNA synthesized and expression levels of RANTES and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments.

Whole cell binding results showed that the GR, AR, and PR, are expressed in both these cell lines, while relatively very low amounts of MR are expressed only in the Vk2/E6E7 cell line (figure 2.4C). Western blot analysis using an antibody specific for the MR (H-300), confirmed the absence of the MR in the Ect1/E6E7 cells (data not shown-Renate Louw, MSc student). However, the low levels of MR in the Vk2/E6E7 cell line observed in the whole cell binding assay, could not be detected using Western blotting (data not shown-Renate Louw, MSc student).

We subsequently determined the endogenous steroid receptor levels in both cell lines. The results show that there is significantly ($p<0.001$) more PR than AR or GR in the Ect1/E6E7 andVk2/E6E7 cell lines, respectively. Moreover, the levels of AR vs. GR are similar to each other in both cell lines ($p>0.05$), (figure 2.4B and 2.4C). The expression of the GR and AR in both cell lines was confirmed by Western blot analysis using antibodies specific for the GR and AR (figure 2.4A). As the GR is ubiquitously expressed (reviewed in Gross and Cidlowski, 2008), it is difficult to find a cell line that does not show low levels of GR. As expected, we detected low levels of endogenous GR in the untransfected COS-1 cell line (negative control). There are two functional isoforms of the PR, PR-A and PR-B (Kastner *et al.,* 1990; Kraus and Katzenellenbogen, 1993). Western blot analysis using an antibody specific for the PR-B isoform showed that both these cell types did not express PR-B. We were unable to optimize the commercial antibody detecting the PR-A isoform (anti-PR-AB (sc-810), Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)), and thus could not determine by Western blotting whether the PR-A isoform is expressed in these cell lines. Taken together, the results showing the expression of the PR, AR, and GR isoforms in both the Ect1/E6E7 and Vk2/E6E7 cell lines, support our hypothesis that

the differential effects of MPA and NET-A could be a consequence of their action via different steroid receptors.

Receptor-specific antagonists indicate a role for the AR in the downregulation of the RANTES pro-inflammatory chemokine gene by MPA in the ectocervical cell line

Having determined that these cell lines mostly express AR, GR and PR, our strategy was to determine which of these steroid receptors mediate the differential effects of MPA and NET-A on the RANTES genes in the Ect1/E6E7 cell line. Although differential effects were also observed for MPA and NET-A on the expression of the IL-8 gene, it was decided to continue the investigation only for the RANTES gene, as MPA and NET-A exhibited differential effects, not only to each, but also to Prog on this gene. For this reason, Ect1/E6E7 cells were incubated with 0.02 μg/ml TNF-α and 1 μM MPA or NET-A, in the absence and presence of 10 μM hydroxyflutamide (OHF), the classical androgen antagonist or mifepristone (RU486), the PR and GR antagonist. The addition of OHF prevented the inhibition by MPA, indicating that, in this cell line, the repression by MPA on the RANTES gene is mediated by the AR (figure 2.7A).

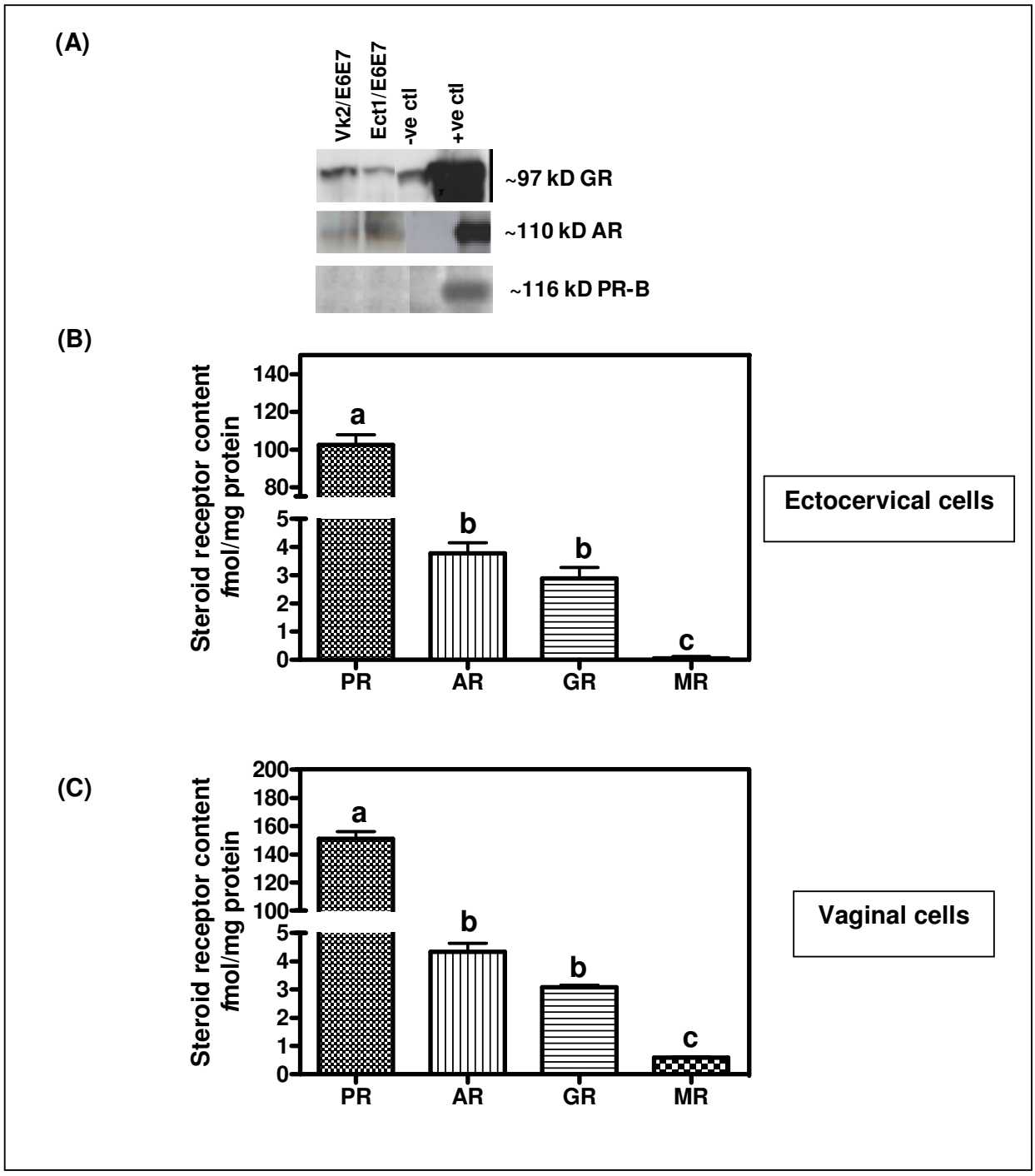

Figure 2.4 legend on next page

Figure 2.4: PR, AR and GR are expressed in human ectocervical and vaginal epithelial cell lines. (A) Whole cell extracts were prepared from human Ect1/E6E7 and Vk2/E6E7 cell lines, untransfected COS-1 cell line (negative control (-ve ctl)), COS-1 cell line transfected with pRS-hGRα, pSVARo, or pSG5hPR-B expression vectors (positive controls (+ve ctl)). Equal amounts of protein (20 µg) were analyzed by Western blotting with the GR or AR or PR-B antibodies. **(B)** Human Ect1/E6E7- and **(C)** Vk2/E6E7 cell lines were incubated with 10 nM **[^{3}H]-R5020 or [^{3}H]-Dex or [^{3}H]-MIB or [^{3}H]-Ald** in the absence (total binding) and presence of 10 µM unlabelled (non-specific binding) R5020 or Dex or MIB or Ald, respectively. Specific binding (total binding minus non-specific binding) is plotted. Results shown are the averages (±SEM) of at least three independent experiments with each condition performed in triplicate. The number of binding sites in *f*mol/mg protein was calculated from the specific activity of the radiolabelled ligands and the specific binding cpm value, as described in the materials and methods section.

Only the GR was transcriptionally active in promoter-reporter transactivation assays in both ectocervical and vaginal cell lines

Having established that the Ect1/E6E7 and Vk2 cell lines both express PR, GR, AR, and very low levels of MR in the Vk2/E6E7 cell line, we next determined whether these receptors are transcriptionally active by transiently transfecting these cells with a glucocorticoid response element (GRE)-driven reporter construct containing two copies of the rat tyrosine amino transferase (TAT) GRE. The cells were subsequently exposed to 10 µM of the receptor-specific agonist Dex for the GR, MIB for the AR, R5020 for the PR and Ald for the MR. The results show that only the GR is transcriptionally active in the Ect1/E6E7 (figure 2.5) and Vk2/E6E7 (figure 2.6) cell lines. However, this does not mean that the other endogenously expressed receptors are not biologically active in other assays. Results from transrepression experiments via a synthetic NFκB-promoter have indicated transcriptionally active GR and AR, but not PR in the ectocervical cells (data not shown-Renate Louw, MSc student). In addition, it has previously been shown that the potency of a ligand for transactication via a GRE is less than the potency for transrepression via NFκB (Ronacher *et al.,* 2009). Thus the other endogenous receptors may still be active on other synthetic

and endogenous genes, such as in transrepression assays. These results with transfected receptors do however also show that the agonist used are active in these cell lines.

Consistent with this result, dihydrotestosterone (DHT), the natural androgen, also repressed the RANTES gene in this cell line via the AR (figure 2.7A). Interestingly, the addition of OHF did not significantly inhibit the effects of Prog or NET-A, indicating that the effects of these compounds on the RANTES gene are not mediated via the AR. A similar experiment with OHF in the Vk2/E6E7 cell line indicated that the AR is not involved in any of the responses by Prog, MPA or NET-A on the RANTES gene (Appendix A1).

RU486 did not inhibit the effects mediated by the positive controls: dexamethasone (Dex), a synthetic GR agonist, or promegestone (R5020), a synthetic PR agonist (figure 2.7B), indicating that RU486 is not working as a GR or PR antagonist in the Ect1/E6E7 cell line, but rather as an agonist. It has previously been reported that RU486 may act as an agonist in some cells and as an antagonist in others (Bamberger and Chrousos, 1995). The fact that the expression of the RANTES gene is enhanced when RU486 is added together with Prog, MPA or NET-A, is difficult to interpret. Taken together, conclusions cannot be drawn as to the role of the GR or PR in mediating the repression of the TNFα-induced RANTES gene by MPA. Using a PR antagonist, like Org31710, which has little anti-glucocorticoid activity compared to RU486 (Kloosterboer *et al.,* 1994), or a GR-specific antagonist like DO6 (Miner *et al.,* 2003), was not possible since these antagonists are not commercially available.

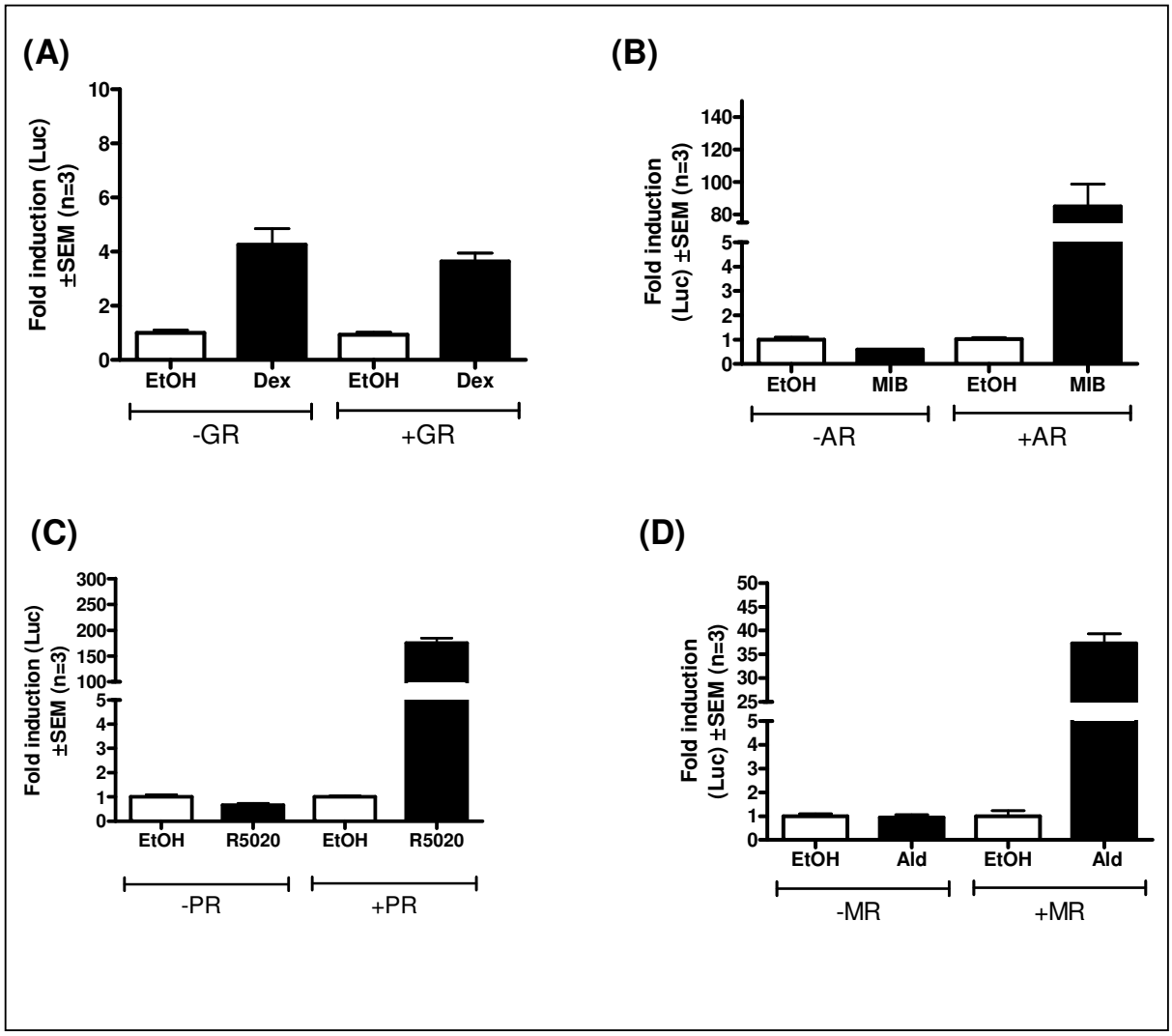

Figure 2.5: Transactivation of a transiently transfected GRE-containing promoter-reporter construct via endogenous or expressed GR, AR, PR, or MR. Ect1/E6E7 cells ($5x10^4$ cells per well in a 24-well plate) were transiently transfected with 330 ng pTAT-GRE2-Elb-luc and 33 ng pRS-hGRα, pSVARo, pSG5hPRB , pRS-hMR or pGL2basic (empty vector control) as indicated, with Fugene 6 transfection reagent according to the manufacturer's instructions. Twenty-four hrs after transfection cells were induced for 24 hrs with vehicle (ethanol (EtoH)), 10 μM Dex, MIB, R5020 or Ald (receptor-agonists).

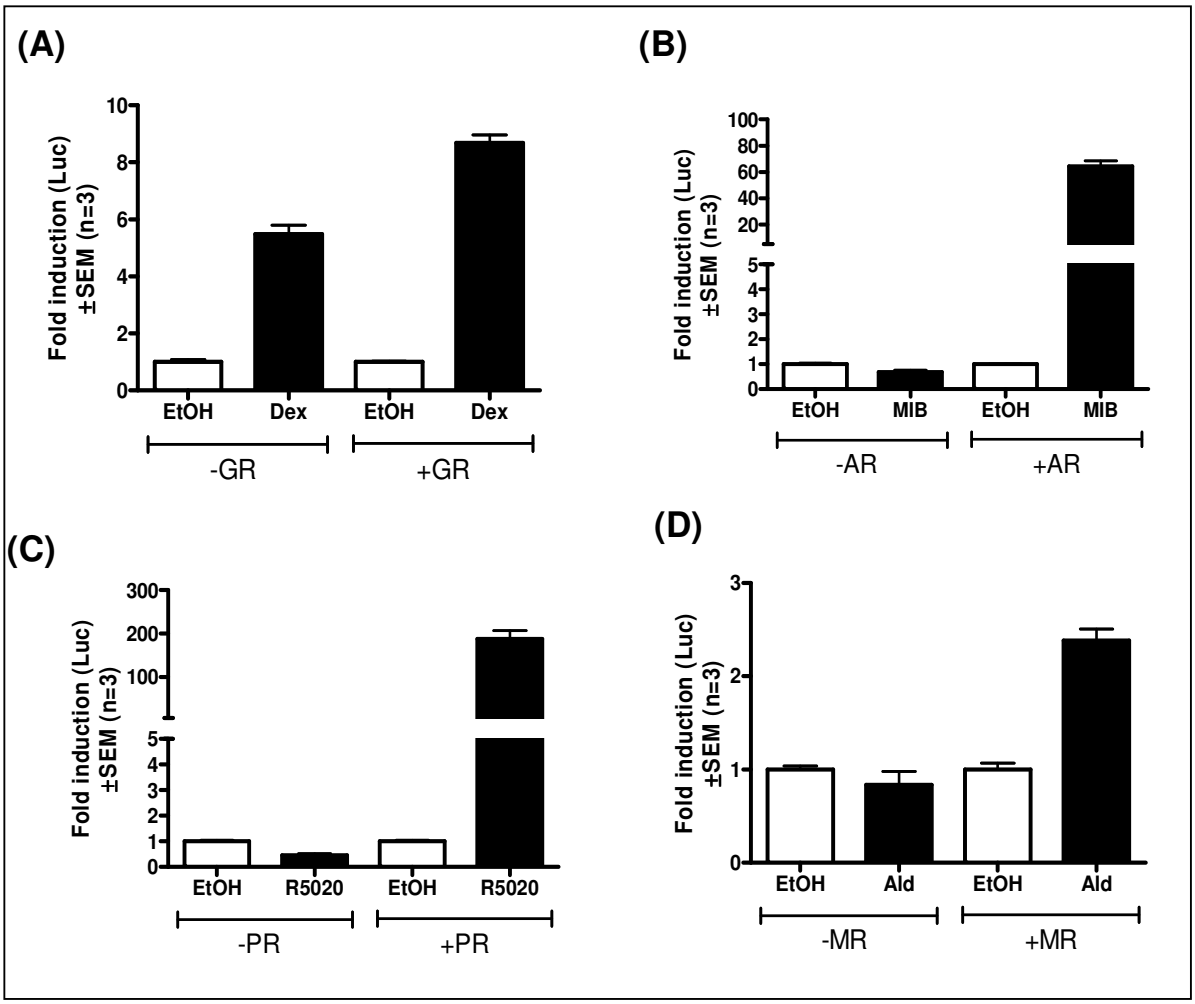

Figure 2.6: Transactivation of a transiently transfected GRE-containing promoter-reporter construct via endogenous or expressed GR, AR, PR, or MR. Vk2/E6E7 cells ($5x10^4$ cells per well in a 24-well plate) were transiently transfected with 330 ng pTAT-GRE2-Elb-luc and 33 ng pRS-hGRα, pSVARo, pSG5hPRB , pRS-hMR or pGL2basic (empty vector control) as indicated, with Fugene 6 transfection reagent according to the manufacturer's instructions. Twenty-four hrs after transfection cells were induced for 24 hrs with vehicle (ethanol (EtoH)), 10 μM Dex, MIB, R5020 or Ald (receptor-agonists).

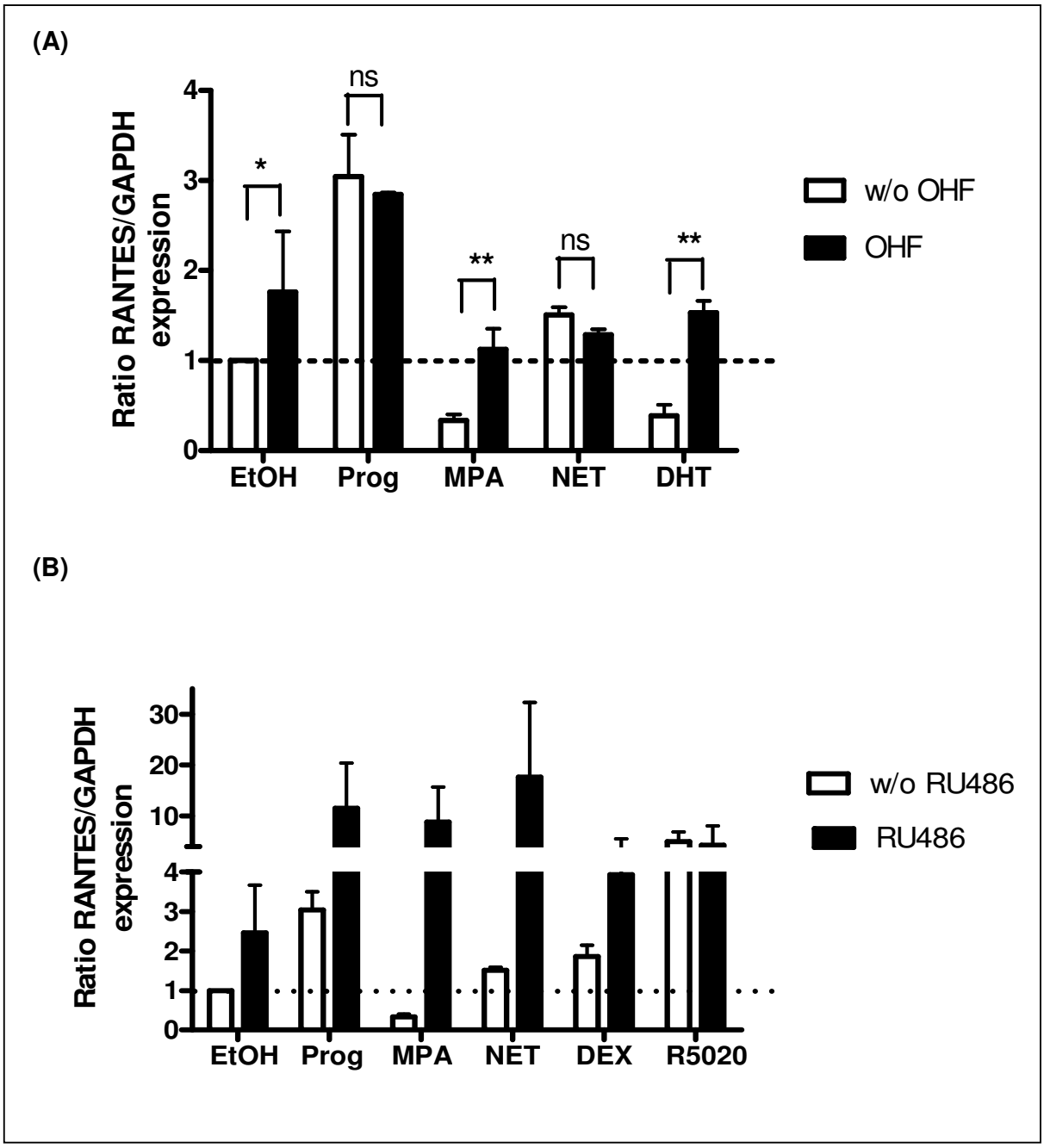

Figure 2.7: Effect of the androgen receptor antagonist (OHF) (A) or glucocorticoid/progesterone receptor antagonist (RU486) (B) on the MPA inhibition of the TNF-α-induced RANTES gene. The human Ect1/E6E7 cell line was incubated with 0.02 µg/µl TNF-α in the presence of 1 µM Prog, MPA, NET-A, DHT, DEX, or R5020, and absence or presence of 10 µM **(A)** hydroxyflutamide (OHF) or **(B)** RU486 for 24 hours. Total RNA was extracted, cDNA synthesized, and expression levels of RANTES and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least two independent experiments. Open bars are compounds and vehicle only, while the black bars are compounds plus antagonist.

DISCUSSION

Inflammation of the lower human female genital tract increases susceptibility to viral infections such as human immunodeficiency virus (HIV) (Kreiss *et al.,* 1994; Hart *et al.,* 1999) and human papilloma virus (HPV) (Castle *et al.,* 2000). In addition, excessive release of proinflammatory cytokines may alter the mucosal immune function (Alfano and Poli, 2005). Thus, understanding factors that may influence the local mucosal immune response, such as endogenous hormones or hormonal contraception, is crucial, especially since the cervicovaginal mucosa is the primary site of HIV-1 infection during male to female transmission (reviewed in Pope and Haase, 2003). Although, the synthetic progestins medroxyprogesterone acetate (MPA) and norethindrone acetate (NET-A) are widely used in both contraception and hormone replacement therapy (HRT), little is known about their effects on the cervicovaginal environment, and whether they interfere with local epithelial immune function. In this study we investigated the effects of MPA, NET-A and Prog on two immortalized, morphologically and functionally different epithelial cell lines, one from a normal human ectocervix and the other from a normal human vagina (Fichorova *et al.,* 1999). Although immortalized, the authors report that the morphological and immunocytochemical properties of these cell lines resemble not only their tissues of origin, but also those of primary cultures (Fichorova *et al.,* 1997). Notably, this is not the case for the HeLa cervical cell line, which is probably the most frequently used cell line derived from the human female lower genital tract mucosa (Fichorova *et al.,* 1997). The Ect1/E6E7 and Vk2/E6E7 cell lines express characteristics of stratified squamous nonkeratinizing epithelia, and constitutively express IL-8, while IL-6 and RANTES are produced when the cell lines are stimulated with either TNFα or interferon gamma (IFNγ) (Fichorova and Anderson, 1999).

Prog increases the expression of IL-6, IL-8 and RANTES, in both the Ect1/E6E7 and Vk2/E6E7 cell lines, while NET-A had little to no effect. We found that Prog upregulated the TNFα-induced proinflammatory cytokine interleukin (IL)-6 in both cell lines, while MPA and NET-A had no effect (figure 2.1A and 2.1B). This is inconsistent with previous studies that have shown the repression of IL-6 by MPA in other cell types (Mantovani *et al.,* 1997; Koubovec *et al.,* 2004). Similarly, NET-A has previously been shown to either upregulate (Zitzmann *et al.,* 2005) or downregulate (Wessel Kriek, MSc thesis) IL-6. In contrast, NET-A in combination with estrogen, like estrogen only, had no effect on IL-6 levels in healthy postmenopausal women (Žegura *et al.,* 2006). In the Ect1/E6E7 cell line, the IL-8 gene was upregulated by Prog, MPA and NET-A, albeit to different extents, and statistical analysis showed that while the extent of upregulation by Prog and MPA is similar ($p>0.05$), NET-A's upregulation is significantly different from both Prog ($p<0.001$) and MPA ($p<0.01$) upregulation (figure 2.2A). A similar pattern was observed for the compounds on the IL-8 gene in the Vk2/E6E7 cell line (figure 2.2B). However, due to variability in the extent of the response between experiments, when the results from different independent experiments were combined, the changes in IL-8 mRNA levels were not statistically significant. However, these results follow a similar trend between experiments, and are most likely physiologically relevant. Consistent with our results, both Prog and MPA have previously been shown to upregulate IL-8 mRNA levels in human endometrial stromal cells (Arici *et al.,* 1996). As Prog has previously been shown to have a protective role in the vagina (Beaver, 1960), our results showing that MPA and NET-A are similar to Prog in upregulating IL-8, albeit to different extents, in the Ect1/E6E7 and Vk2/E6E7 cell lines, could indicate that these

progestins may provide an enhanced local defense mechanism against infections in the vaginal mucosa.

MPA substantially and significantly downregulates the expression of the proinflammatory RANTES gene in the Ect1/E6E7 cell line, (figure 2.3A), while it is upregulated by MPA in the Vk2/E6E7 cell line (figure 2.3B). Thus, the same compound, MPA, exhibits differing effects on the same gene (RANTES), in different cell types, indicating cell-specific effects of MPA. In addition, NET-A had no statistically significant effect on the RANTES gene in both cell types, indicating differential regulation of RANTES by the two synthetic progestins thought to have similar biological activity. Trying to understand the physiological implications of this result on the RANTES gene is not easy. In epithelial cells, RANTES recruits immune cells into the reproductive tract, in this manner contributing to inflammation. Inhibition of RANTES by MPA would thus be expected to suppress inflammation, thereby blocking infiltration of immune cells (Cho *et al.,* 2006). This lack of recruitment of immune cells into the reproductive tract may have implications for women using MPA. In terms of HIV-1 infection, which has been associated with increased RANTES in cervicovaginal secretions (Behbahani *et al.,* 2007), these implications may be positive in terms of less HIV-infection due to decreased inflammation (Cummins *et al.,* 2006). However, women using MPA in contraception and HRT are also at risk of other infections, and thus the suppression of RANTES may prevent the mounting of an effective response to combat these infections in the ectocervical environment. In addition, it is important to remember that the defense function in the cervicovaginal environment is dependent on number of regulatory factors in different anatomical sites, with a constant release of pro- and anti-inflammatory mediators.

Thus the observed effects of MPA on RANTES should be considered in the light of the fact that there may be a balance between pro- and anti-inflammatory molecules produced in the cervicovaginal environment. It would be of interest to look at the expression of RANTES in cervicovaginal lavage samples from healthy-, and HIV-1 infected- women using MPA and NET as injectable contraception, as compared to women not using these contraceptives.

In summary, the general trend that was observed for the effect of Prog, MPA and NET-A on endogenous cytokine genes in the cervicovaginal environment, is that Prog upregulates the expression of IL-6, IL-8 and RANTES, while NET-A either upregulates these genes, or has no effect. In contrast, MPA either has no effect (e.g. IL-6 gene), or upregulates (e.g. IL-8 gene), or downregulates (e.g. RANTES gene) expression of these genes. Furthermore, Prog shows regulation different to both MPA and NET-A on the RANTES gene in the Ect1/E6E7 cell line (figure 2.3A), as well as on the IL-6 gene in both the Ect1/E6E7 and Vk2/E6E7 cell lines (figure 2.1A and 2.1B). Taken together, these results show that cytokine genes in the cervicovaginal environment are regulated in a ligand-specific and cell-specific manner by Prog, MPA and NET-A.

We hypothesized that the ligand-specific and cell-specific effects regulation of cytokine genes in the cervicovaginal environment are due to ligands acting via different steroid receptors, or differing activities via the same steroid receptor. Using a combination of whole cell binding and Western blotting, we showed that the PR, AR and GR are expressed in both cell lines (figure 2.4A-C). Whole cell binding experiments showed that the amount of GR and AR in the Ect1/E6E7 and Vk2/E6E7

cell lines is similar (figure 2.4B and 2.4C). Since the whole cell binding experiments showed the presence of a large amount of PR, one would expect that the progestins would predominantly act via this receptor. However, we did not observe any PR-mediated effects in our study. In fact, the potent synthetic PR agonist, R5020, displayed no activity for transactivation or transrepression via the PR. A possible explanation for this intriguing result may be explained by the expression levels of different PR isoforms. We were unable to detect the PR-B isoform in any of the cell lines, and thus we postulate that it is the transcriptionally less active PR-A isoform that is present in these cell lines. Indeed, variation in the expression of PR-A and PR-B has previously been reported in the eutopic endometrium during the menstrual cycle (Mangal *et al.,* 1997), with no PR-B detectable during the secretory and early proliferative phases. Similarly, Attia *et al.* (2000) did not detect any PR-B protein in endometriotic tissue, and they ascribe the clinically observed resistance of endometriosis to treatment with progestins, to this absence of PR-B, and presence of PR-A.

We next determined which steroid receptor(s) are involved in the response of MPA on the RANTES gene in the Ect1/E6E7 cell line, by using receptor-specific antagonists. As we had shown that this cell line expresses AR, GR and PR (figure 2.4B), we looked at the expression of the RANTES gene in the presence of OHF, the AR-specific antagonist, as well as RU486, the PR and GR antagonist. The results show that in the Ect1/E6E7 cell line, RU486 behaves as an agonist, rather than an antagonist (figure 2.7B). RU486 had previously been shown to act as both an agonist and as an antagonist, depending on receptor density (Zhao *et al.,* 2003). Furthermore, it has been reported that RU486 is a partial agonist for both PR and GR

when selective coregulators are recruited (Jackson *et al.,* 1997; Schulz *et al.,* 2002). This approach of using RU486 as a receptor antagonist in this cell line is thus limited. Interestingly, the results using OHF showed that the repression of MPA on the RANTES gene was lifted when the AR is antagonised, revealing a possible role for the AR (figure 2.7A). The fact that OHF could lift the repression of MPA on the RANTES gene to similar levels as the control (EtOH), suggests that the PR, despite being the most abundant receptor, as well as the GR, are not involved in the MPA-mediated downregulation of the RANTES gene in the Ect1/E6E7 cell line. Considering that both MPA and NET-A have previously been reported to have androgenic properties (Bentel *et al.,* 1999; Teulings *et al.,* 1980; Bamberger *et al.,* 1999; Kemppainen *et al.,* 1999; Hackenberg *et al.,* 1993; Perez-Palacios *et al.,* 1983; Perez-Palacios *et al.,* 1981a; Perez-Palacios *et al.,* 1981b; Bergink *et al.,* 1983; Schoonen *et al.,* 2000; Deckers *et al.,* 2000), it was surprising that NET-A did not act via the AR in this cell line. However, as it is known that NET-A can be metabolized (Stanczyk and Roy, 1990), the possibility exists that NET-A is metabolized in this cell line, and that the metabolite(s), are less efficacious AR agonists than NET-A itself.

In summary, results from our study are the first to indicate not only differential regulation of the cytokine genes by Prog, MPA and NET-A, but also cell-specific effects. Moreover, we showed that the effect of MPA on the RANTES gene in the Ect1/E6E7 cell line is AR-mediated.

Acknowledgements

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Chapter 3

A comparative study of the androgenic properties of progesterone and the synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A)

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Abstract

Medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) are used by millions of women as contraceptives and in hormone replacement therapy (HRT). Surprisingly, even though both progestins are widely used, very little is known about their mechanism of action at the molecular level. The importance of investigating these mechanisms, and comparing them to those of progesterone (Prog), has recently been highlighted by clinical evidence showing that MPA and NET increase the risk of the development of breast cancer in HRT users. MPA is also implicated in increased risk of coronary heart disease in HRT users. The aim of this study was to investigate and compare the androgenic activities of these two clinically important progestins. Competitive binding in whole cells revealed that Prog, MPA and NET-A have a similar binding affinity for the androgen receptor (AR) to that of the natural androgen dihydrotestosterone (DHT) (K_i's for DHT, Prog, MPA and NET-A are 29.4, 36.6, 19.4 and 21.9 nM, respectively). Moreover, in both transactivation and transrepression transcriptional assays we demonstrate that, unlike Prog, MPA and NET-A are efficacious AR agonists, with activities comparable to DHT. The finding that both MPA and NET-A have strong androgenic effects when compared to endogenous Prog, may have important implications for women using these progestins in HRT or contraception, as it may lead to side-effects such as weight gain, lipid profile modifications or increased breast cancer risk. Furthermore, we show that MPA and NET-A exert their androgenic actions by different mechanisms, as NET-A, like DHT and other well-characterised androgens, induces the ligand-dependent interaction between the NH_2- and COOH-terminal domains (N/C-interaction) of the AR independent of promoter-context, while MPA does this in a promoter-dependent manner.

Introduction

The synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) are widely used in contraception and in hormone replacement therapy (HRT). MPA itself is the major progestogenic compound, while NET-EN and norethisterone acetate (NET-A) are hydrolysed to norethisterone (NET) and its metabolites, which together have progestogenic action (Stanczyk and Roy, 1990). The Women's Health Initiative (WHI) trial showed healthy postmenopausal women using combined estrogen and MPA therapy in HRT have an increased risk of breast cancer and cardiovascular complications (Rossouw *et al.,* 2002). A similar trial on the use of estrogen alone was only terminated two years later and its analysis, although indicating an increased risk for stroke, showed no increase in breast cancer risk (Anderson *et al.,* 2004). MPA and NET have both been shown to substantially increase the risk of breast cancer in longterm HRT users in the "Million Women study" (Beral, 2003). Surprisingly, very little information is available about the molecular mechanism of action of MPA and NET, in particular their comparative properties (Hapgood *et al.,* 2004).

Notably, a few studies have recently started investigating these mechanisms, but focus mostly on MPA, and not NET-A. MPA has been reported to modulate transcription of a number of genes via the glucocorticoid receptor (GR), such as IL-2 in normal human lymphocytes (Bamberger *et al.,* 1999), IL-6 and IL-8 in a mouse fibroblast cell line (Koubovec *et al.,* 2004) and the *nm-23* tumor suppressor gene in a breast cancer cell line (Ouatas *et al.,* 2003). Interestingly, MPA has a greater affinity than NET-A for the GR, and acts as a partial agonist for transactivation (Koubovec *et al.,* 2005), while NET-A is a GR antagonist (Appendix C6). This demonstrates that

MPA and NET do not have the same activity, nor do they act the same as Prog, via the GR. It is thus apparent that even though MPA and NET were developed so that their biological actions mimicked that of progesterone (mediating their effects by binding to the PR), they can also initiate a diverse range of biological effects by cross-reacting with other members of the steroid receptor family such as the GR and possibly the mineralocorticoid receptor (MR) (Hapgood *et al.,* 2004; Koubovec *et al.,* 2005; Philibert *et al.,* 1999). In this way, MPA and NET may exert side-effects via any of these receptors, such as the observed immunosuppressive effects of MPA via the GR. This highlights the importance of understanding the interactions of these compounds with different steroid receptors.

Both MPA and NET-A have previously been reported to have androgenic properties (Bentel *et al.,* 1999; Teulings *et al.,* 1980; Bamberger *et al.,* 1999; Kemppainen *et al.,* 1999; Hackenberg *et al.,* 1993; Perez-Palacios *et al.,* 1983; Perez-Palacios *et al.,* 1981a; Perez-Palacios *et al.,* 1981b; Bergink *et al.,* 1983; Schoonen *et al.,* 2000; Deckers *et al.,* 2000; Bergink *et al.,* 1983; Bergink *et al.,* 1985). In addition, MPA has been shown to regulate a number of genes via the AR in human breast cancer cell lines (Ghatge *et al.,* 2005). Our own work in human ectocervical cells (Ect/E6E7), indicates involvement of the AR in the downregulation of the proinflammatory RANTES (Regulated-upon-Activation, normal T cell Expressed and Secreted) gene by MPA (Chapter 2). Little research has been done to directly compare the relative androgenic activities of MPA and NET-A, as compared to the activity of Prog, and the precise mechanisms involved.

Like all members of the steroid receptor family, the AR is a ligand-dependent transcription factor which mediates the response of cells to androgens, and is structurally organized in three domains (depicted in Figure 1): the ligand binding domain (LBD) at the C-terminus of the protein, the highly conserved and centrally located DNA-binding domain (DBD) which consists of two zinc finger elements and which directly interacts with specific DNA sequences called androgen response elements (AREs), and the variable N-terminus or N-terminal domain (NTD), which is involved in transactivation. These receptors possess transcriptional activation functions located in the NTD (AF1 domain) and the LBD (AF2 domain) (Simental *et al.,* 1991; Brinkmann *et al.,* 1999).

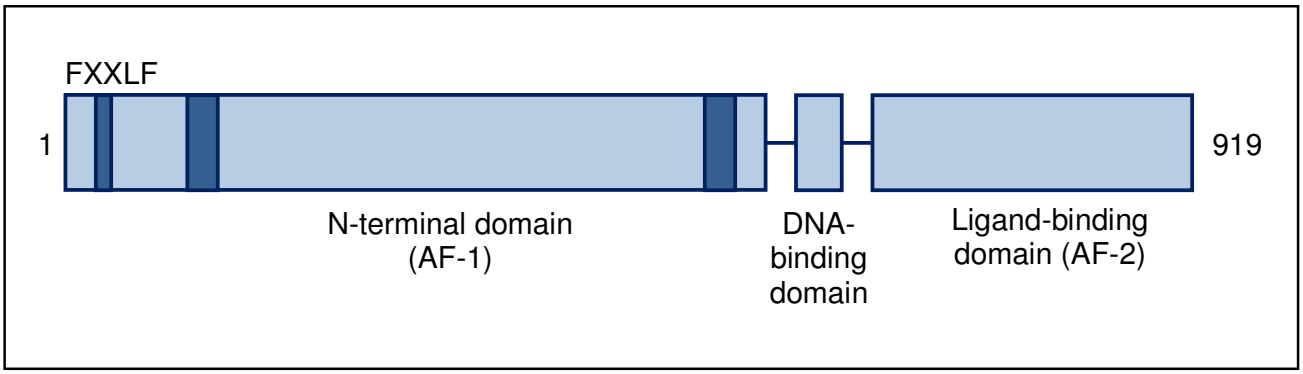


Figure 1. A schematic representation of the structural and functional domains of the 919 amino acid residue human androgen receptor. The N-terminal domain (NTD) contains the AF-1 (activation function 1), the ligand-binding domain (LBD) contains the AF-2 (activation function 2). Also indicated is the central DNA-binding domain (DBD) and the positions of the FxxLF motif. (redrawn from Trapman and Dubbink, 2007).

The first step in the process of transcriptional regulation is binding of the hormone to the AR, which promotes dissociation of the mostly cytoplasmic AR from heat-shock proteins. The liganded-AR translocates to the nucleus where it binds to AREs as a dimer, and subsequently activates (transactivation) gene expression (Evans, 1988; Beato, 1989). The liganded-AR translocates to the nucleus where it binds to AREs as

a dimer, and subsequently activates gene expression (transactivation) (Evans, 1988; Beato, 1989). The liganded-AR can also cause repression of gene expression (transrepression). This involves the ligand-activated AR binding via protein-protein interactions to promoter-bound transcription factors such as nuclear factor-kappa B (NFκB) and activator protein-1 (AP-1). Furthermore, in the presence of a classical AR agonist, the transactivation capacity of the AR is mediated primarily by an interaction between the N-terminal transactivation domain (AF-1) and the AF-2 cleft in the C-terminal LBD. Studies to further reveal the subdomains mediating this so-called "N/C interaction" implicate the F*XX*LF motif, ^{23}FQNLF^{27}, in the NTD (He *et al.,* 2000). In addition, the N-terminal WXXLF binding motif, ^{433}WHTLF^{437}, has also been reported to bind to the AF-2 domain, thereby contributing to the N/C-interaction (He *et al.,* 2000). This N/C interaction takes place both within one molecule (intramolecular), and between two AR molecules (intermolecular) (Schaufele *et al.,* 2005). Interestingly, it has previously been shown that both Prog and MPA cannot induce the AR N/C-interaction (Kemppainen *et al.,* 1999), but to the best of our knowledge, this has not been investigated for NET.

In this study we investigated the mechanism of action of MPA and NET-A via the AR and compared it to that of Prog, by evaluating and comparing binding affinities for the AR. Although a number of studies have looked at the relative binding affinities of MPA and NET-A to the AR, the data from these studies may be misleading as many performed their experiments in cell lines that express, not only endogenous AR, but also other steroid receptors to which these compounds can bind (Hackenberg *et al.,* 1993; Zhao *et al.,* 2000). Thus, we performed whole cell binding assays in the COS-1 cell line, that have negligible levels of endogenous steroid receptors, and determined

the K_d's of Prog, MPA and NET-A for the AR. In addition, we compared the transcriptional activation and repression of these compounds via the AR, as well as their ability to induce the N/C-interaction of the AR. The androgenic properties were compared with those of the synthetic androgens, mibolerone and R1881, the native ligand, 5α-dihydrotestosterone (DHT), and the synthetic progestin, mifepristone (RU486), a derivative of norethindrone, that has previously been shown to have androgenic and anti-androgenic properties (Kemppainen *et al.,* 1992; Song *et al.,* 2004) (refer to figure 2 for the chemical structures of the androgens, progesterone, and the synthetic progestins).

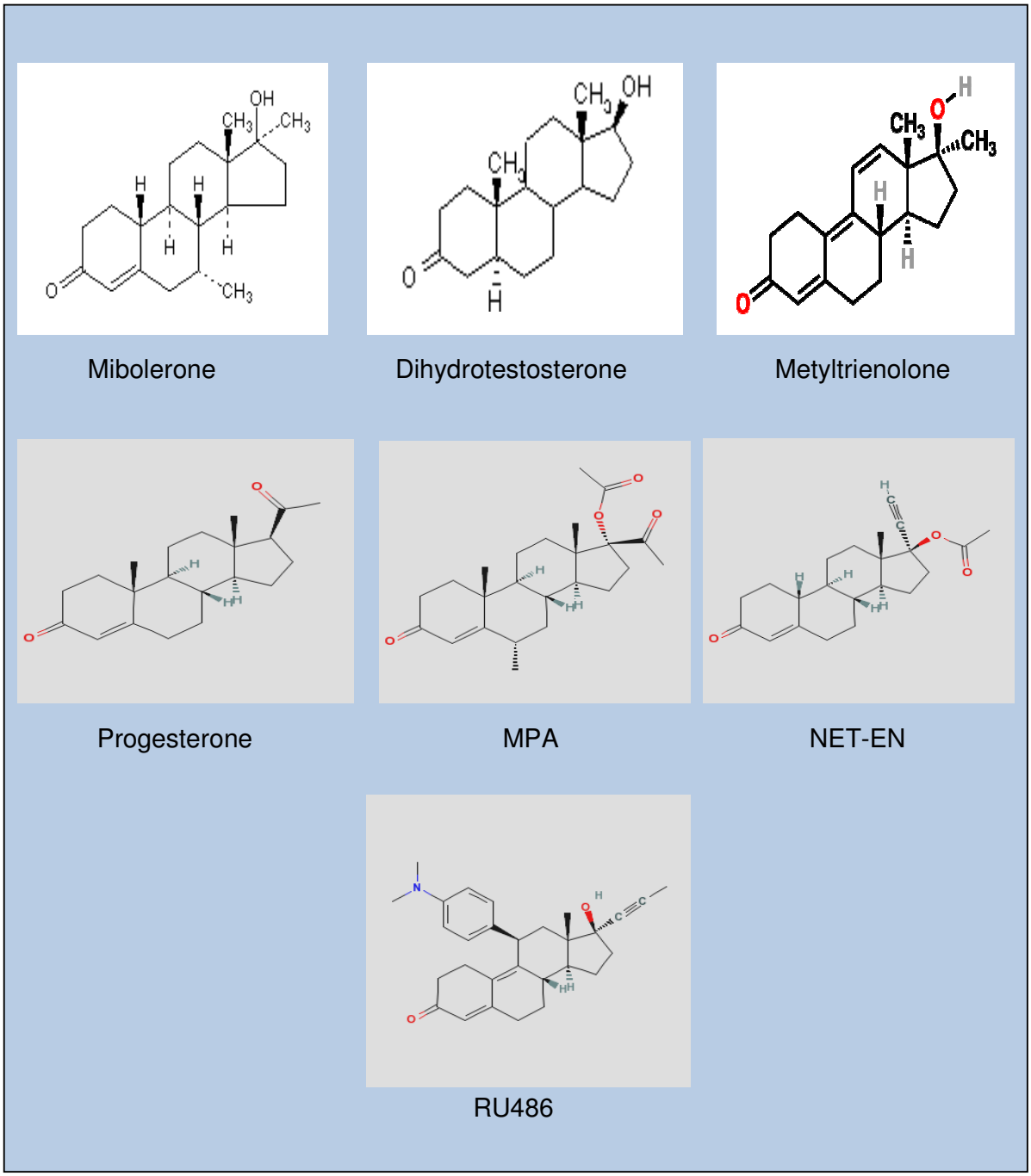


Figure 2. The chemical structures of natural (endogenous) and synthetic androgens (white boxes) and progestins (light grey boxes). The natural androgen, *dihydrotestosterone* (DHT*)*; synthetic androgens, *mibolerone* (MIB) and *methyltrienolone* (R1881); natural *progesterone* (Prog*)*, synthetic progestins *medroxyprogesterone acetate* (MPA), *norethisterone enanthate* (NET-EN) and *mifepristone* (RU486). Adapted from Ronacher *et al.,* 2009.

Materials and methods

Inducing compounds

5α-androstan-17β-ol-3-one (dihydrotestosterone; DHT), 11β-(4-dimethylamino) phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one) (mifepristone; RU486), 4-pregnene-3, 20-dione (progesterone; Prog), 6α-methyl-17α-hydroxy-progesterone acetate (medroxyprogesterone acetate; MPA), 17α-ethynyl-19-nortestosterone 17β-acetate (norethindrone acetate; NET-A), 11β,16α-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione (Dexamethasone; Dex), recombinant TNF-α and phorbol 12-myristate-13-acetate (PMA) were obtained from Sigma-Aldrich, South Africa. NET-A, the acetate, was used as it is soluble in water compared with the insoluble ester NET-EN. *In vivo*, both NET-EN and NET-A undergo hydrolysis and are converted to NET and its metabolites (Stanczyk and Roy, 1990). Hydroxyflutamide (OHF) was obtained from Dr C. Tendler (Schering Plough Research Institute, USA). Unlabelled 17β-hydroxy-7α, 17α-dimethylestra-4-en-3-one (mibolerone; MIB), 17β-17-hydroxy-17-methyl-estra-4,9,11-trien-3-one (methyltrienolone; R1881) and [^{3}H]-Mibolerone ([^{3}H]-MIB) (76.8 Ci/mmol) were purchased from (PerkinElmer Life and Analytical Science, South Africa). All unlabelled test compounds were dissolved in absolute ethanol and stored at -20^{o}C. These compounds were then added to serum-free culturing medium such that the final concentration of ethanol was 0.1%. Control incubations (no test compounds) were performed in the presence of 0.1% ethanol.

Plasmids

The plasmid pTAT-GRE-E1b-luc, driven by the E1b promoter that contains two copies of the rat TAT-GRE, has been described previously (Sui *et al.,* 1999). The

cytomegalovirus (CMV)-driven-β-galactosidase expression vector (pCMV-β-gal) was obtained from Prof. G. Haegeman (University of Gent, Belgium). The 5xNFκB-luc and 7xAP-1-luc plasmids were from Stratagene (Houston, Texas, USA). A plasmid expressing the human androgen receptor, pSVARo (Brinkmann *et al.,* 1989), the human AR (hAR) DBD-LBD expression vector, pSG5-hAR(DBD-LBD) (Alen *et al.,* 1999), the hAR NTD-VP16 fusion protein expression vector, pSNATCH-II(hAR-NTD) (Alen *et al.,* 1999), as well as the reporter constructs for selective androgen response elements (AREs), 4xSC-ARE1.2 and 4xSLP-HRE2 (Schauwaers *et al.,* 2007), were obtained from Prof. Frank Claessens (University of Leuven, Leuven, Belgium).

Cell culture

COS-1 cell lines (monkey kidney cells) were purchased from American Type Culture Collection (ATCC), USA and maintained in high glucose (4.5 g/ml) Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa), supplemented with 10% fetal calf serum (FCS) (Delta Bioproducts, Johannesburg, South Africa), 50 IU/ml penicillin and 50 µg/ml streptomycin (GibcoBRL, Paisley, UK). Cultures were maintained in 75 cm^2 culture flasks (Greiner Bio-One International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO_2. All cultures were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney RI, 1987), and only mycoplasma-negative cell lines were used in experiments.

Whole cell binding assays

Competitive whole cell binding assays were performed essentially as described by Bamberger *et al.* (1995), with the following modifications. The COS-1 cell line was maintained in DMEM as described above. Cells were seeded into 24-well tissue

culture plates (Nunc, Denmark) at 1 x 10^5 cells per well. On day 2, cells were transfected with 0.375 μg pSVARo expression vector and 0.0375 μg of the pCMV-β-gal expression vector (Stratagene, Houston, Texas, USA), using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. On day 3, the cells were washed with phosphate-buffered saline (PBS) and incubated for 16 hours (time taken to reach equilibrium; Appendix B2) at 37°C with 0.2 nM [^{3}H]-MIB (PerkinElmer Life and Analytical Science, South Africa) in the absence and presence of increasing concentrations of unlabelled MIB, R1881, DHT, Prog, MPA, NET-A or RU486. Working on ice at 4^{o}C, cells were washed three times with ice-cold 1X PBS containing 0.2% (w/v) bovine serum albumin (BSA) for 15 min. Cells were then lysed with 200 μl reporter lysis buffer (Promega, Madison, USA). Total binding ([^{3}H]-MIB in the absence of added unlabelled ligand) was determined by scintillation counting and expressed as 100%. Specific bound [^{3}H]-MIB was calculated as the difference between total and non-specific binding, the latter determined by incubating cells in the presence of [^{3}H]-MIB plus 10 μM unlabelled MIB and expressed as a relative % of total binding. The β-galactosidase chemiluminescent reporter gene assay system (Tropix Inc., U.S.A.) was used to determine β-galactosidase activity, to correct for differences in transfection efficiencies. Binding data were analyzed using GraphPad Prism software, with nonlinear regression and assuming competitive binding to one class of binding sites. K_i values for DHT, R1881, Prog, MPA, NET-A and RU486 were determined from the heterologous displacement curves using the EC_{50}s, K_d for MIB and concentration of radiolabelled MIB, according to the method of Cheng and Prusoff (1973). The reported values are averages of at least three independent assays, with each condition performed in triplicate.

Transient transfection assays

For transactivation assays, the COS-1 cell line was maintained as above and seeded into 96-well tissue culture plates (Nunc, Denmark) at 1 x 10^4 cells per well. On day 2, cells in each well were transfected with 100 ng of the appropriate luciferase reporter constructs, 10 ng of the appropriate expression vectors for either a human steroid receptor or relevant fusion proteins, and 10 ng of the pCMV-β-gal expression vector (Stratagene, Houston, Texas, USA), using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. On day 3, cells were washed with PBS and induced with increasing concentrations of MIB, R1881, DHT, Prog, MPA, NET-A or RU486. After 24 hours, cells were lysed with 25 µl reporter lysis buffer (Promega, Madison, USA). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega, Madison, USA) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale CA, USA). The values obtained were normalised to expression of β-galactosidase, which was measured using the GalactoStar Assay Kit from Tropix (Bedford MA, USA).

For transrepression assays, the COS-1 cell line was seeded into 24-well plates at a density of 5 x 10^4 cells per well. The following day the cells were transfected with 0.045 µg of pSVARo, 0.09 µg of 5xNFκB-luc or 7xAP-1-luc plasmids, and 0.0225 µg of pCMV-β-Gal using the FuGENE6 transfection reagent (Roche Molecular Biochemicals) in accordance with the manufacturer's instructions. Twenty four hours after transfection the cells were washed with PBS and incubated with serum-free medium containing 10 ng/ml PMA and 1 µM of the test compounds. Luciferase activity in the lysate was measured as described above. The values obtained were

normalised to expression of β-galactosidase, which was measured using the GalactoStar Assay Kit from Tropix (Bedford MA, USA). The reported values are averages of at least three independent experiments, with each condition performed in triplicate. Where normalising to β-galactosidase is absent, transfection efficiency was previously determined to be highly reproducible. The reported values are averages of at least three independent experiments, with each condition performed in triplicate.

Mammalian two-hybrid assays

The COS-1 cell line was cultured in DMEM as described above. The cells were plated at a density of 5 x 10^4 cells/well in 24-well plates. Cells were transfected using FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. A total of 0.868 µg DNA was transfected per well as follows: 0.067 µg pSNATCH II-hAR (hAR-NTD), 0.067 µg of pSG5-hAR (DBD-LBD), 0.067 µg of pCMVβ-gal and 0.667 µg of pTAT-GRE-E1b-luc or 4xSC ARE1.2-luc or 4xSLP HRE2-luc, respectively. Twenty four hours after transfection the cells were washed with PBS and incubated for 24 hours with serum-free medium containing increasing concentrations of the test compounds. Luciferase activity in the lysate was measured as described above.

Data manipulation and statistical analysis

The Graph Pad Prism® software was used for data manipulations, graphical representations, and statistical analysis. One-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) posttests were used for statistical analysis. Non-linear regression and one site competition were used in whole cell binding assays, whereas non-linear regression and sigmoidal dose response were

used in transactivation (antagonist) experiments. Statistical significance of differences is indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, whereas no statistical significance is indicated by $p>0.05$. The letters a,b,c etc. are also used to denote statistically significant differences, where all those values which differ significantly from others, are assigned a different letter.

Results

MPA and NET-A have a similar binding affinity for the AR

The COS-1 cell line was transiently transfected with a full-length hAR expression vector, and in order to obtain accurate K_d and K_i values, a range of experiments were first performed to establish two important parameters, namely concentration of radiolabelled ligand to use and time to reach equilibrium. An explanation for the relevance of these parameters can be found in Appendix D1. An appropriate concentration of [^{3}H]-mibolerone ([^{3}H]-MIB), in the range two to ten times lower than the EC_{50}, was established as 0.2 nM (Appendix B1), and the incubation time required for equilibrium to be reached for 0.2 nM [^{3}H]-MIB binding to the AR was determined as sixteen hours (Appendix B2). Homologous/heterologous curves with unlabelled steroids show that R1881, DHT, Prog, MPA, NET-A and RU486 are able to compete with [^{3}H]-MIB for binding to the AR (figure 3). The curves for all the test compounds are consistent with competitive binding to the same site as mibolerone (MIB). These homologous/heterologous displacement curves were analysed and the K_d value determined for MIB, while K_i values were determined for R1881, DHT, Prog, MPA, NET-A and RU486 (Table 1). Both the synthetic androgens, MIB (K_d = 0.38 ± 0.15 nM) and R1881 (0.217 ± 0.62 nM), have similar affinity for the AR, which is significantly higher than that of DHT, MPA and NET-A Prog ($p<0.01$). The results

indicate that MPA (K_i = 19.4 ± 0.04 nM), NET-A (K_i = 21.9 ± 0.10 nM) and Prog (36.6 ± 0.20 nM) bind to the AR with a similar affinity as that of the natural androgen DHT (K_i = 29.4 ± 0.01 nM). DHT showed a 77-fold lower affinity for the AR than MIB whereas MPA and NET-A displayed a 51-fold and 57-fold lower affinity than MIB, respectively (Table 1). Interestingly, RU486 has a higher affinity for the AR than the other progestins, and the natural androgen, DHT. It displays an approximately 10-fold lower affinity than MIB and R1881, compared to about a 100-fold lower affinity shown by the other ligands in this study.

MPA and NET-A display androgen agonist activity that is similar to that of DHT for transactivation

The finding that both Prog, MPA and NET-A bind to the AR with the same affinity as the natural androgen, DHT, raised the question whether once bound, these three ligands could activate the AR in a manner similar to DHT. We thus investigated the relative agonist and antagonist potency and efficacy of the test compounds for transcriptional regulation via the classical androgen response element (ARE). Due to the high degree of structural and functional conservation within the DBD of steroid receptors, most steroid receptors can bind the same DNA response element (reviewed in Beato, 1989). The classical ARE thus also serves as a response element for the GR, PR and MR (reviewed in Beato, 1989), and is then termed glucocorticoid response element (GRE), progesterone response element (PRE) and mineralocorticoid response element (MRE), respectively. Thus, the COS-1 cell line was transiently transfected with a glucocorticoid response element (GRE)-driven reporter construct containing two copies of the rat TAT-GRE-luc (a promoter-reporter construct containing a simple promoter linked to the luciferase reporter gene, and

referred to as the “classical” ARE), a full-length hAR expression vector, as well as the pCMV-β-gal expression vector. The cells were subsequently exposed to increasing amounts of MIB, R1881, DHT, Prog, MPA, NET-A or RU486. Figure 4A clearly indicates that MPA and NET-A have similar, and relatively strong, agonist potencies compared to each other, and to the natural androgen DHT, for the AR. Although the levels of maximal induction displayed by DHT appear to be higher than that of MPA and NET-A, this difference is not statistically significant ($p>0.05$), indicating that these compounds probably have a similar efficacy for agonist activity via the hAR. The transcriptional activity of these compounds in the absence of transfected receptor was negligible (Appendix D3). The potencies and efficacies of these compounds for the AR are summarised in Table 2. Although Prog has a similar binding affinity as DHT, MPA and NET-A, it displays relatively weak agonist activity (EC_{50} = 601.2 nM, with maximal induction of ~34%). Similarly, despite the high binding affinity of RU486, it displays very weak agonist activity. In addition, we confirmed the previously reported AR antagonist activity of RU486 (Kemppainen *et al.,* 1992; Song *et al.,* 2004), and Prog (Fuhrmann *et al.,* 1996; Kemppainen *et al.,* 1999) (figure 4B). The determined antagonist potency (EC_{50} values) for RU486 (29.5 nM ± 0.12) and Prog (12.3 nM ± 0.17) were not significantly different ($p>0.05$). Thus, although Prog, MPA and NET-A bind to the AR with similar binding affinity, MPA and NET-A differ from Prog in their transcriptional activity.

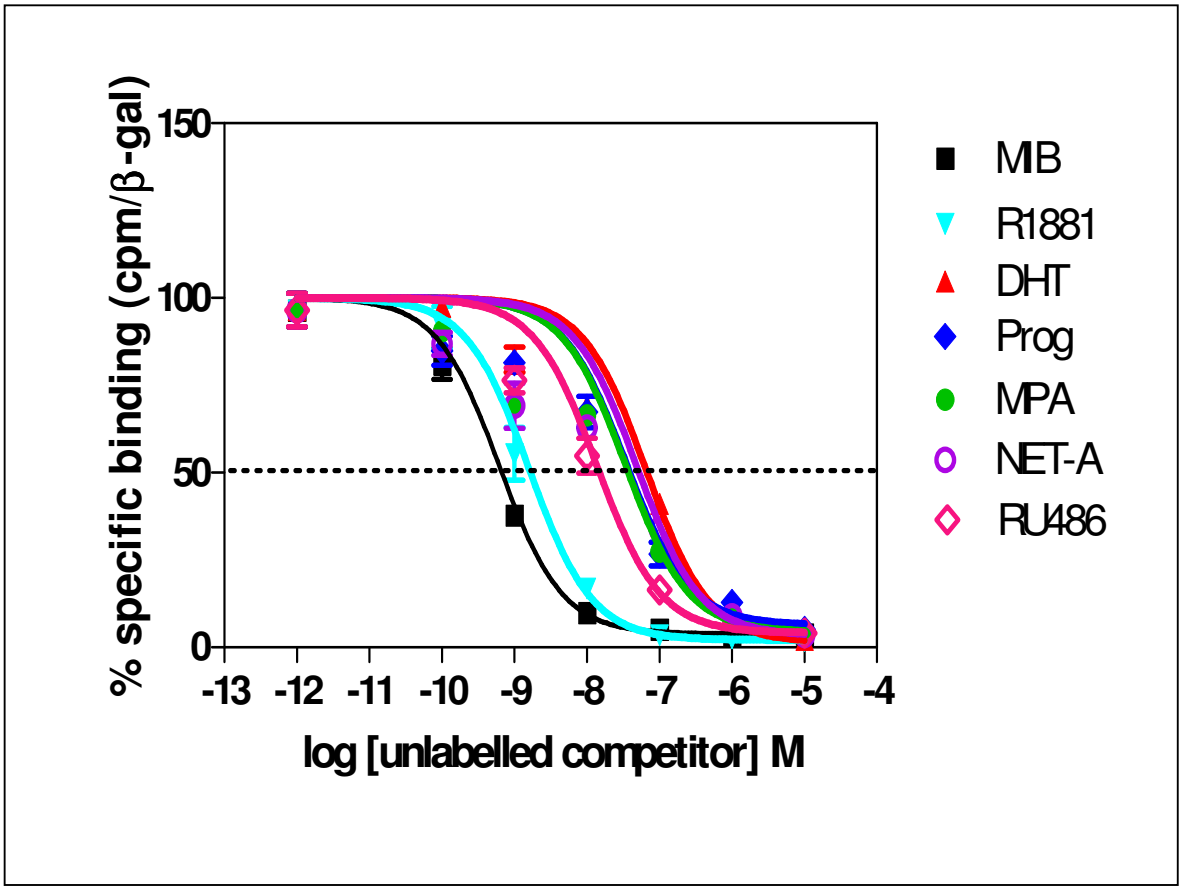


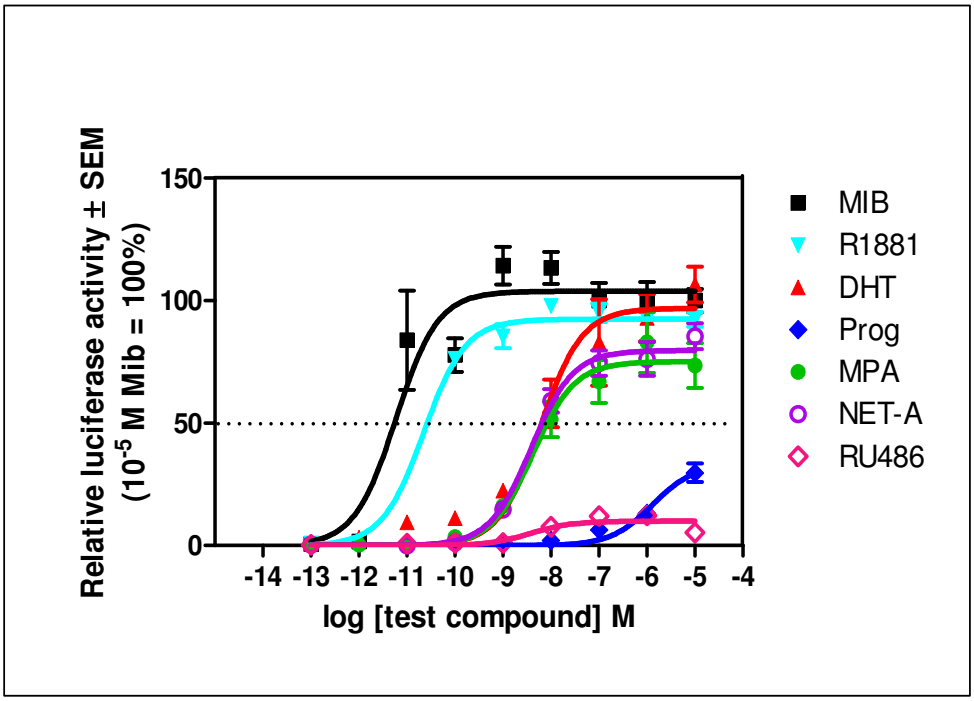
Figure 3: MPA and NET-A both compete with [^{3}H]-mibolerone ([^{3}H]-MIB) for binding to the human androgen receptor. The COS-1 cell line was transiently transfected with the pSVARo and pCMV-β-gal expression vectors. Twenty-four hours later the cells were incubated with 0.2 nM [^{3}H]-MIB in the absence and presence of increasing concentrations of either unlabelled mibolerone (MIB) (■), R1881 (▼), DHT (▲), Prog (♦), MPA (●), NET-A (○) or RU486 (◊) for 16 hours. Results are plotted as % specific binding where total specific binding of [^{3}H]-MIB in the absence of unlabelled ligand is set as 100% and binding of unlabelled ligand is set as a % binding relative to MIB, after normalization for transfection efficiency with β-galactosidase levels. Competition for binding is illustrated by the total percentage of [^{3}H]-mibolerone bound to the hAR. Result shown is representative of three independent experiments with each condition performed in triplicate (±SEM).

Table 1: Relative binding affinities of the ligands for the AR (K_d / K_i values)[a]

Ligand	K_d or K_i (M) ± SEM	Fold differences
MIB	$3.83 \times 10^{-10} \pm 0.15$	1
R1881	$2.17 \times 10^{-10} \pm 0.62$	0.57
DHT	$2.94 \times 10^{-8} \pm 0.01$	76.76
Prog	$3.66 \times 10^{-8} \pm 0.20$	95.56
MPA	$1.94 \times 10^{-8} \pm 0.04$	50.65
NET-A	$2.19 \times 10^{-8} \pm 0.10$	57.18
RU486	$4.66 \times 10^{-9} \pm 0.55$	12.17

[a] Binding data of three independent experiments were analyzed using GraphPad Prism software, with nonlinear regression and assuming competitive binding to one class of binding sites. K_i values ± SEM for R1881, DHT, Prog, MPA, NET-A and RU486 were determined from the heterologous displacement curves using the EC_{50}s, K_d ± SEM for MIB, and concentration of radiolabelled MIB, according to the method of Cheng and Prusoff (1973) that is described in Appendix D1. Fold differences calculated by setting the K_d for MIB in M as 1, and expressing the K_i in M for the other ligands as a fraction thereof. Statistical analysis of the K_d / K_i values using Bonferroni (compares all pairs of columns) posttests, indicated the following: MIB vs. DHT ($p< 0.01$); MIB vs. DHT, MPA, NET-A ($p< 0.01$); MIB vs. Prog ($p<0.001$); MIB vs. RU486, R1881 ($p>0.05$); DHT vs. Prog, MPA, NET-A, RU486 ($p>0.05$); DHT vs. R1881 ($p<0.01$); MPA vs. NET-A ($p>0.05$); MPA, NET-A vs. RU486)$p>0.05$); MPA, NET-A vs. R1881 ($p<0.01$); Prog vs. MPA, NET-A, RU486 ($p>0.05$); Prog vs. R1881 ($p<0.01$); RU486 vs. R1881 ($p>0.05$).

A **Agonist activity**



B **Antagonist activity**

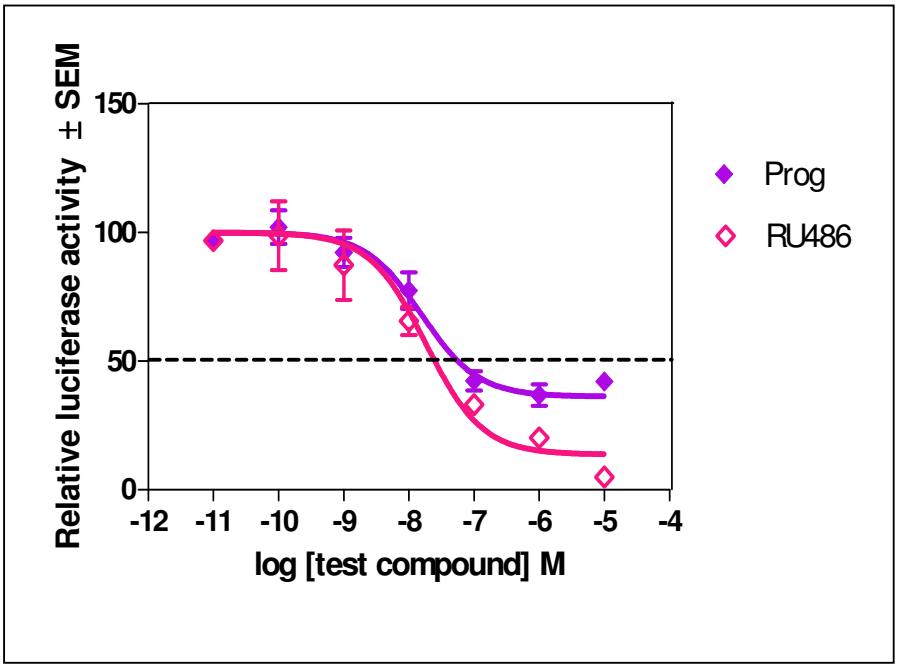


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Figure 4: (A) MPA and NET-A display androgen agonist activity similar to DHT. The COS-1 cell line was transiently transfected with the pTAT-GRE-E1b-luc reporter, pSVARo and pCMV-β-galactosidase expression vectors. Subsequently, the cells were incubated in the absence and presence of increasing concentrations of either mibolerone (MIB) (■), DHT (▲), R1881 (▼), Prog (♦), MPA (●), NET-A (○) or RU486 (◊) for 24 hours. Induction is shown as % luciferase (luc) activity expressed in relative light units (rlu). Result shown is one representative figure with each condition performed in triplicate (±SEM). **(B) Prog and RU486 display androgen antagonist activity**. The COS-1 cell line was transiently transfected as above. Subsequently, the cells were incubated with 0.1 nM MIB alone (100%) or with increasing concentrations of Prog (♦) or RU486 (◊) for 24 hours. Result shown is the average of at least four independent experiments with each condition performed in triplicate (±SEM).

Table 2: Potencies (agonist and antagonist activity) for transactivation of the ligands via the AR expressed as EC_{50} in nM, as well as efficacy (maximal agonist activity expressed as %.[b]

LIGAND	Agonist activity		Antagonist activity
	EC_{50} (M) ± SEM	MAX (%) ± SEM	EC_{50} (M) ± SEM
MIB	$1.318 \times 10^{-12} \pm 0.19$	100 ± 0.0	-
DHT	$1.416 \times 10^{-9} \pm 0.18$	98.77 ± 7.19	-
R1881	$5.012 \times 10^{-11} \pm 0.02$	90.46 ± 7.62	-
Prog	N/A	33.89 ± 6.45	$1.23 \times 10^{-8} \pm 0.17$
MPA	$1.365 \times 10^{-9} \pm 0.18$	86.54 ± 7.56	-
NET-A	$2.333 \times 10^{-9} \pm 0.19$	78.37 ± 5.82	-
RU486	N/A	6.97 ± 0.35	$2.95 \times 10^{-8} \pm 0.12$

[b] Data from experiments depicted in figure 4A and 4B were analysed to obtain EC_{50} **±** SEM values, and figure 4A to obtain maximal values **±** SEM for each test compound. Maximal values are expressed as a percentage of 10^{-5} MIB=100%. N/A = no activity up to 10^{-5} M test compound. Statistical analysis of the EC_{50} values for agonist activity, indicated MIB vs. DHT, MPA and NET-A ($p < 0.001$); DHT vs. MPA, NET-A ($p > 0.05$); MPA vs. NET-A ($p > 0.05$), whereas there were no statistically significant differences observed for the MAX values.

MPA, but not NET-A, induces the ligand-dependent interaction between the amino- and carboxyl-terminals of the androgen receptor in a promoter-dependent manner

The functional, *in vivo*, androgen-dependent interaction between the NTD and LBD of the AR, the so-called N/C-interaction, has been well-described for the AR (Langley *et al.,* 1995; Doesburg *et al.,* 1997; Kemppainen *et al.,* 1999; He *et al.,* 2000). It has been shown that the AR N/C-interaction is important for agonist activity for transactivation via the AR (He and Wilson, 2002). Here we used a mammalian two-hybrid assay to compare the potential of MPA and NET-A to induce the N/C-interaction of the hAR. The COS-1 cell line was co-transfected with a GRE-driven reporter construct, an expression vector encoding the DBD and LBD of the hAR, as well as an expression vector encoding the NTD of the hAR fused to a VP16 activation domain.

In figure 5A we show that NET-A has the ability to induce the N/C-interaction of the AR on the classical ARE with an efficacy similar to R1881, but weaker than MIB and DHT. Although DHT, MPA and NET-A have similar potencies for transactivation on the classical ARE, MPA is not able to induce the N/C-interaction of the AR. To investigate whether the inability of MPA to induce the N/C-interaction may depend on the DNA binding sequence, reporter assays were performed in parallel using the classical ARE construct (pTAT-GRE-E1b-luc) as well as constructs containing two AR-specific androgen response elements (AREs), 4xSC-ARE1.2 and 4xSLP-HRE2 (Schauwaers *et al.,* 2007). These binding motifs, which resemble a direct rather than a palindromic repeat of the 5'-TGTTCT-3' hexamer are termed AR-specific, as the AR but not GR is able to transactivate via these DNA motifs. Dose response curves

for all the ligands on the three individual promoters were plotted (figure 5), and the efficacies for each ligand (maximal response at 10 μM), are shown in figure 6 (A-C), and summarized in Table 3. The results indicate that when using the AR-specific sequences, the ability of all the ligands to induce the AR N/C-interaction increases. For example, MPA's ability increases from 7% to 36% (SLP-HRE2) and 59% (SC-ARE1.2), respectively. This suggests that MPA can induce the AR N/C-interaction in a promoter-specific way. In contrast, NET-A induces the AR N/C-interaction regardless of promoter context. This suggests that the induction of the AR N/C-interaction by MPA is promoter-dependent, while this is not true for NET-A. Interestingly, DHT, MPA and NET-A also have similar potencies for transactivation on the AR-specific SC-ARE1.2 promoter (figure 7).

As it is thought that the androgenic agonist potency of a ligand is dependent on the ability of the AR to induce the N/C-interaction (Alen *et al.,* 1999; Berrevoets *et al.,* 1998, He *et al.,* 2000), we correlated the agonist potencies (EC_{50} values) for transactivation on the classical ARE with the potencies for N/C-interaction on the same promoter. The results indicate a positive linear correlation between potency of N/C interaction and potency for transactivation, indicating a good ($r^2 > 0.59$) and significant ($p<0.05$) correlation (figure 8A). In contrast, no linear correlation was detected between efficacy (maximal responses) for transactivation and efficacy for N/C-interaction (figure 8B), indicating that the two processes are unrelated. This suggests that the induction of the AR N/C interaction by the ligands in this study does not determine efficacy for transactivation.

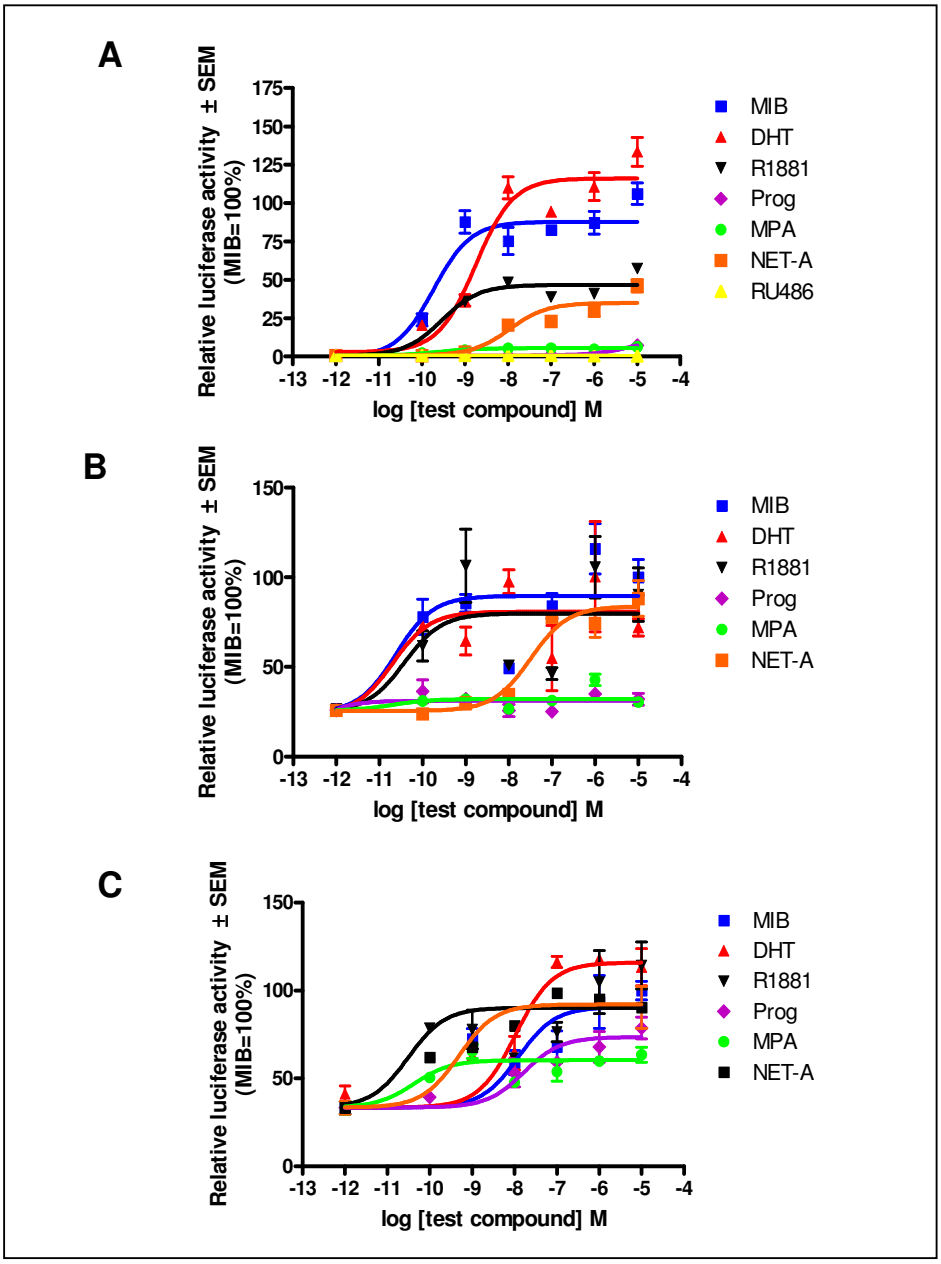

Figure 5: MPA induction of the AR N/C-interaction is promoter dependent. COS-1 cells were transiently transfected with the pSG5-hAR(DBD-LBD), the pSNATCH-II(hAR-NTD) and the pCMV-β-galactosidase expression vectors and **(A)** pTAT-GRE-E1b-luc or **(B)** 4xSLP HRE2-luc or **(C)** 4xSC ARE1.2-luc. Cells were incubated in the absence and presence of increasing concentrations of either mibolerone (MIB), DHT, R1881, Prog, MPA, or NET A for 24 hours. Induction is shown as % luciferase (luc) activity relative to MIB=100%. Result shown is one representative figure of three independent experiments with each condition performed in triplicate (±SEM). Higher basal activity is characteristic of these ARE-specifc constructs (Frank Claessens, personal communication).

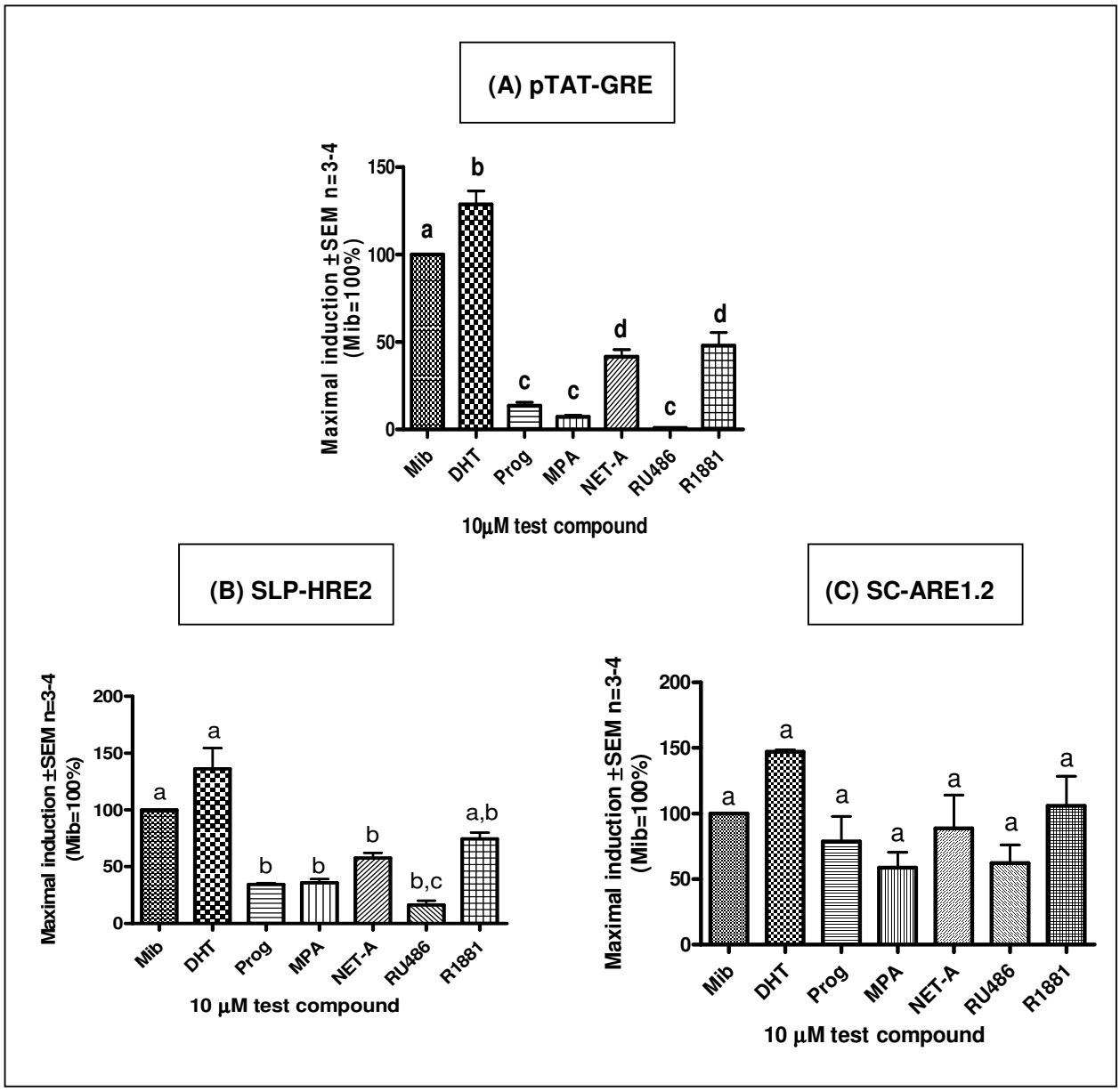

Figure 6: In contrast to DHT and NET-A, MPA does not induce the ligand-dependent N/C-interaction of the hAR on a pTAT-GRE. The COS-1 cell line was transiently transfected with the pSG5-hAR(DBD-LBD), the pSNATCH-II(hAR-NTD) and the pCMV-β-galactosidase expression vectors and **(A)** pTAT-GRE-E1b-luc or **(B)** 4xSC ARE1.2-luc or (**C)** 4xSLP HRE2-luc. Cells were subsequently exposed to 10 µM of MIB, DHT, R1881, Prog, MPA, NET-A or RU486 for 24 hours. Results are averages of at least three independent experiments with each condition performed in triplicate (±SEM).

Table 3: Maximal AR N/C-induction (at 10 μM of each ligand).[c]

LIGAND	MAXIMAL N/C-INDUCTION (%) ± SEM		
	(Classical) pTAT-GRE-E1b	(AR-selective) 4xSLP-HRE2	(AR-selective) 4xSC-ARE1.2
MIB	100 ± 0.0	100 ± 0.0	100 ± 0.0
DHT	128.70 ± 7.72	136.0 ± 18.37	147.2 ± 1.54
R1881	47.97 ± 7.42	74.6 ± 5.31	105.9 ± 22.73
Prog	13.51 ± 2.0	34.41 ± 1.19	78.77 ± 19.2
MPA	7.19 ± 0.10	35.89 ± 3.30	58.82 ± 11.58
NET-A	41.6 ± 4.10	57.59 ± 4.62	88.73 ± 25.48
RU486	0.81 ± 0.16	16.35 ± 3.67	62.19 ± 14.03

[c]Relative responses for AR transactivation on pTAT-GRE-E1b-luc and for induction of AR N/C-interaction on pTAT-GRE-E1b-luc or 4xSLP-HRE2 or 4xSC-ARE 1.2-luc reporter plasmids at 10 μM ligand, were determined in the transiently transfected COS-1 cell line. Values were obtained from at least three independent experiments, each performed in triplicate, after which the ± SEM was calculated for each ligand. MIB activity was taken as 100% and all other values are expressed as a % relative to this.

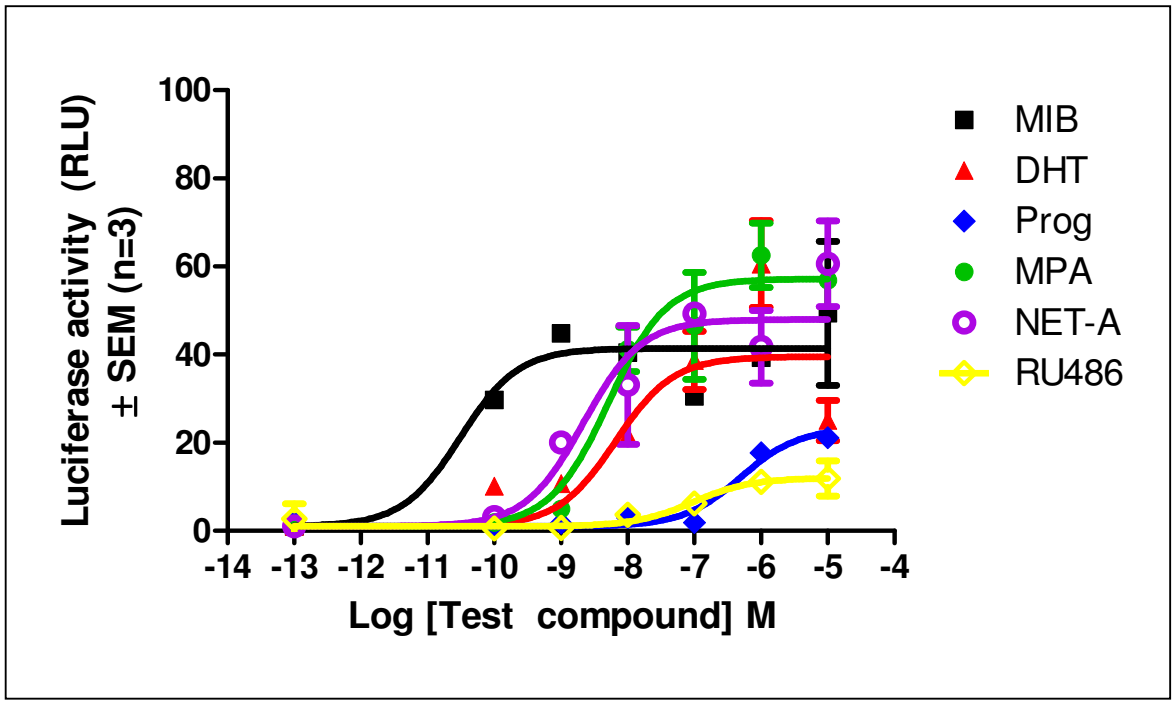

Figure 7: MPA and NET-A display androgen agonist activity similar to DHT on an AR-specific ARE. COS-1 cells were transiently transfected with the 4xSC ARE1.2-luc reporter, pSVARo and pCMV-β-galactosidase expression vectors. Subsequently, the cells were incubated in the absence and presence of increasing concentrations of either mibolerone (MIB) (■), DHT (▲), Prog (♦), MPA (●), RU486 (◊), or NET-A (○) for 24 hours. Induction is shown as % luciferase (luc) activity expressed in relative light units (rlu). Result shown is one representative figure with each condition performed in triplicate (±SEM).

MPA and NET-A display similar androgenic properties for transrepression.

Having shown that both MPA and NET-A can transactivate an ARE-containing promoter via the AR, albeit via a different mechanism, we were interested in comparing the androgenic properties of MPA and NET-A for transrepression. We thus evaluated the transrepressive ability of MPA and NET-A via the AR by transfecting the COS-1 cell line with luciferase reporter plasmids linked downstream of either five copies of a nuclear factor kappa-B (NFκB) site or seven copies of an activator protein-1 (AP-1) site, both in the absence and presence of a transfected AR expression vector (Appendix B3). Cells were stimulated with phorbol 12-myristate 13-

acetate (PMA) and incubated for 24 hours with 1 μM of each test compound. To correct for any low level responses via endogenous steroid receptors by the different test compounds, the response without AR was subtracted from the response with transfected AR. The resulting plots in figure 9, show the AR-dependent repression calculated as a percentage of the MIB-dependent repression via the AR (MIB=100%).

Using the NFκB promoter-reporter construct, results showed greatest, and similar, transrepression of PMA-mediated induction with DHT, MPA and NET-A, whereas Prog and RU486 showed virtually no repression (figure 9A). These responses are similar to those seen on the synthetic ARE promoter-reporter construct. In contrast to similar to responses by MPA and NET-A on the synthetic classical ARE and NFκB promoter constructs, AR-mediated repression on the AP-1 promoter-reporter construct (figure 9B) by both MPA and NET-A, was significantly different from that of DHT (summarized in Table 4). In addition, AR-mediated maximal repression of the AP-1 promoter-reporter construct by Prog and RU486 was 59% and 38% respectively, while on the NFκB promoter-reporter construct, it was only 7% and 13% respectively. This indicates promoter-specific repression by Prog and RU486. Interestingly, although the repression by MPA and NET-A on the NFκB promoter reporter is similar to MIB and DHT, it is significantly different to DHT on the AP-1 promoter-reporter. In summary, the transrepressive activities of Prog and RU486 are similar, while the transrepressive activities of Prog are significantly different to MPA and NET on both promoters.

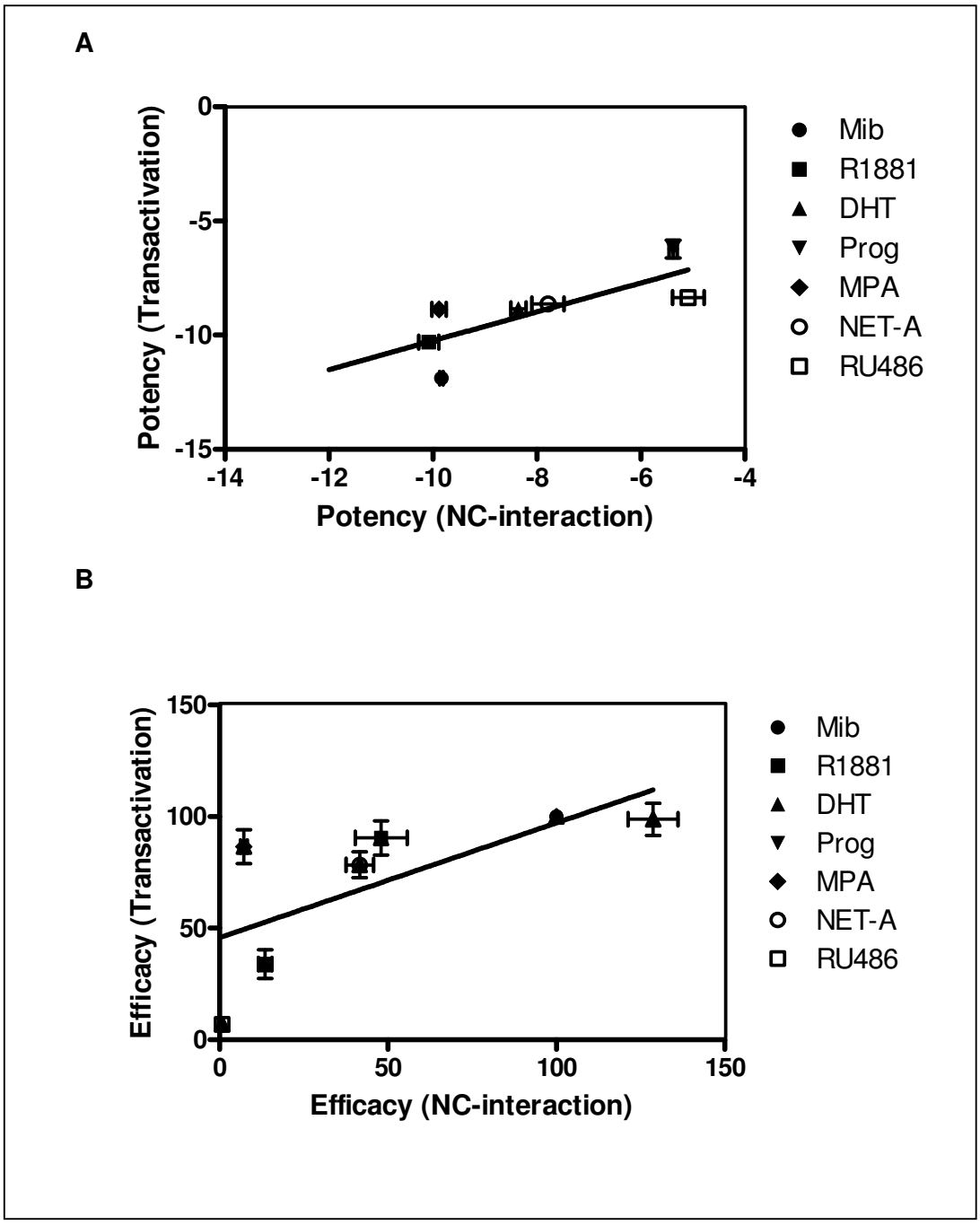

Figure 8: (A) Correlation analysis of potency for GRE transactivation versus GRE N/C-interaction. Relative potencies for transactivation (Table 2) were compared to relative potencies for N/C-interaction as determined by mammalian two hybrid assay (figure 5A) using Pearson correlations. Results showed $p<0.05$ and $r^2=0.59$. **(B) Correlation analysis for efficacy for GRE transactivation versus GRE N/C- interaction**. Relative efficacies for transactivation (Table 2) were compared to relative efficacies for N/C-interaction as determined by mammalian two hybrid assay (figure 5A) using Pearson correlations. Results showed no statistical significance and $r^2=0.49$.

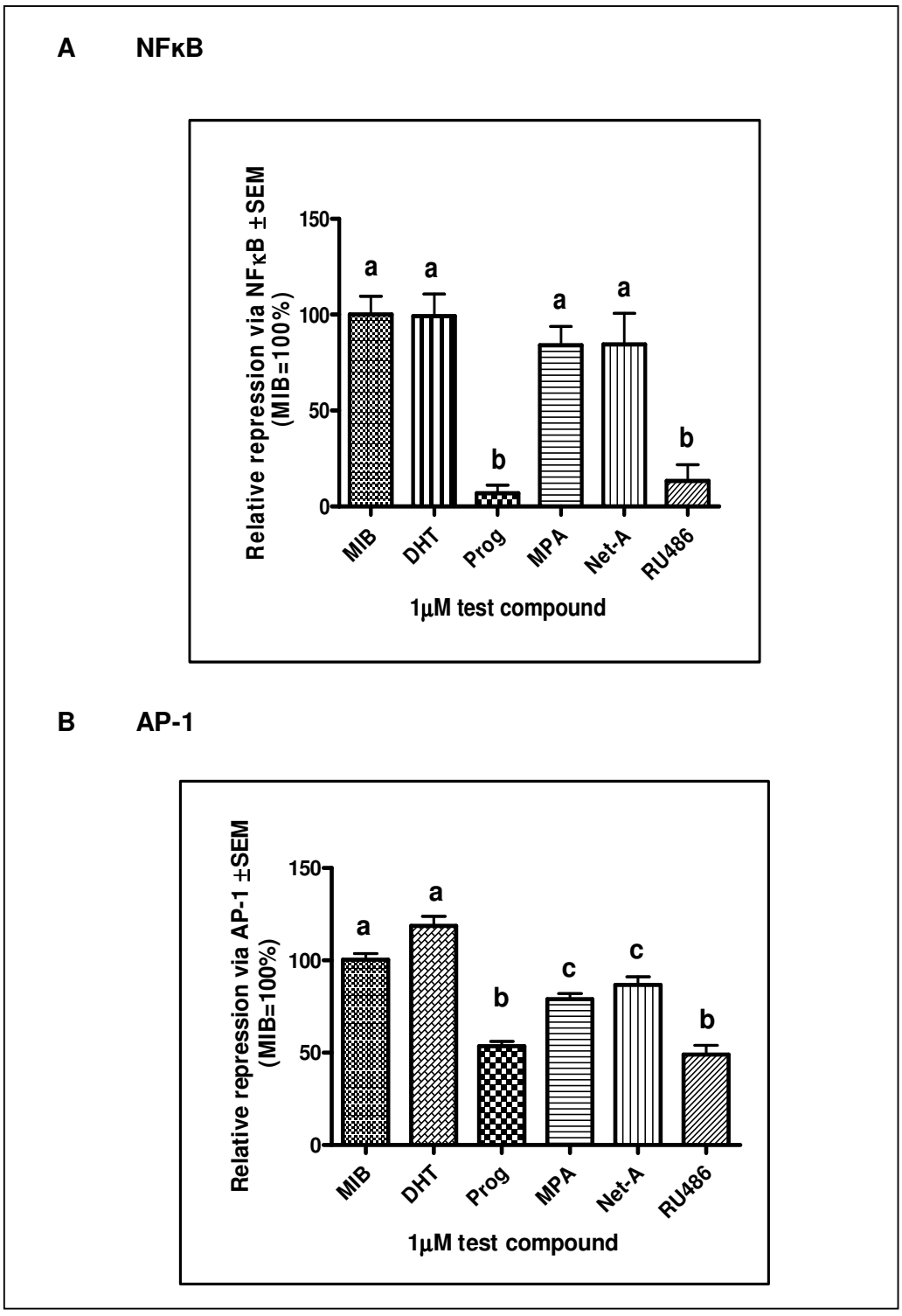

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Figure 9: Transrepression activity via the hAR. The COS-1 cell line was transiently transfected with pSVARo, pCMV-β-galactosidase expression vectors and **(A)** NFκB- or **(B)** AP-1-containing promoter-luciferase reporter constructs. Subsequently, the cells were stimulated with PMA and incubated with 1 µM of either MIB, DHT, Prog, MPA, NET-A or RU486 for 24 hours. Once the repressive abilities of the test compounds were determined, in the absence and presence of transfected AR (Appendix B3), repression in the absence of AR was subtracted from the repression in the presence of transfected AR. The resulting plots show repression of the individual ligands calculated as a percentage of repression by MIB (100%), thus indicate the transrepression dependent on the expression of AR. Result shown is the average of three independent experiments with each condition performed in triplicate (±SEM).

Table 4: Relative efficacies for transactivation and transrepression via the AR at 1 µM ligand[d]

Ligand	GRE (from Table 2) Relative response	NFκB Relative response	AP-1 Relative response
MIB	100	100	100
DHT	108 ± 11	99 ± 12	108 ± 12
Prog	13 ± 2	7 ± 4	59 ± 8
MPA	78 ± 3	84 ± 10	79 ± 3
NET-A	73 ± 7	85 ± 16	87 ± 4
RU486	12 ± 2	13 ± 9	38 ± 12

d Relative responses for transactivation via pTAT-GRE-E1b-luc and for transrepression via either NFκB or AP-1-luc reporter plasmids at 1 µM ligand, were determined in a transiently transfected COS-1 cell line. Values were obtained from at least three independent experiments, each performed in triplicate, after which the ± SEM was calculated for each ligand. MIB activity was taken as 100% and all other values are expressed as a % relative to this.

DISCUSSION

MPA and NET-A bind with similar relatively affinity as DHT to the hAR

To the best of our knowledge, a detailed comparison of the relative equilibrium dissociation constants, and relative efficacy for transactivation and transrepression, by Prog, MPA and NET-A (or NET) for the AR, in the same system, has not been reported previously. We show that these compounds bind to the same site as that of MIB, and that Prog, MPA and NET-A bind to the AR with a similar affinity to that of DHT, the endogenous ligand of the AR (figure 3), and that in the context of this series of experiments, there is no significant difference in the binding affinities of DHT, Prog, MPA and NET-A for the hAR (Table 1). Moreover, we determined accurate equilibrium dissociation constants for these compounds.

The determined K_d / K_i of MIB and R1881 for the AR in our system (K_d / K_i = 0.383 and 0.22 nM, respectively), is similar to that reported in a previous study for MIB (0.53 nM; Schilling *et al.,* 1984) and R1881 (0.6 nM; Bonne and Raynaud, 1975). Consistent with our results, a study by Kemppainen *et al.* (1999), also in the COS-1 cell line, showed that DHT and MPA have similar relative binding affinities. We also demonstrate, in accordance with Kemppainen and co-workers, that Prog binds with a similar relative binding affinity to the AR as DHT and MPA (Kemppainen *et al.,* 1999). In addition, a previous report in intact MCF-7 cells, showing that MPA and NET bind with a similar relative affinity to the endogenously expressed AR (Bergink *et al.,* 1983 and references therein), is in agreement with our results. In contrast, a study by Hackenberg *et al.* (1993) determined a K_d of MPA for the AR as 3.6 nM in the MFM-223 human mammary cancer cell line compared with a K_d of 0.18 nM for dihydrotestosterone (DHT). In addition, the K_d / K_i values reported in the literature for

DHT of 0.5 nM (Zhao *et al.,* 2000) differ from the values that we determined for DHT (K_i = 29.4 nM). As the cell lines used in the studies by Hackenberg *et al.* (1993) and Zhao *et al.* (2000), breast- and prostate cancer cells, respectively, also express steroid receptors other than the AR, to which these progestins may bind, the possibility exists that the results reported in these studies may be inaccurate. Thus, to determine accurate K_d values of different ligands, it is best to use a system like the one in the present study, where the AR was overexpressed in COS-1 cells, with negligible levels of endogenous steroid receptors present. This allows a direct comparison of the relative binding affinities of ligands for the AR within a system where only the AR is present. Thus, the K_i-values of these compounds for the AR reported in the present study may better reflect the relative affinities of these compounds.

To our knowledge, this study is the first to establish accurate K_i values for MPA, NET-A and RU486 towards the human AR. Interestingly, RU486 has a higher affinity for the AR than all the other progestins used in our study, including DHT, but lower than MIB and R1881. Song *et al.* (2004) previously reported that the relative binding affinities of R1881 and RU486 differ by a factor of 12, and by 32 from data from Kemppainen *et al.* (2002). Our result indicates a factor of 22, which is similar to the result of Kemppainen *et al.* (2002). Taken together, our findings show that Prog and the synthetic progestins, MPA, NET-A and RU486, all bind the AR with relatively high affinity.

Unlike Prog, MPA and NET-A are potent agonists for the AR

We subsequently demonstrated that, although Prog, MPA, NET-A and DHT bound the AR with similar binding affinities, Prog showed only weak partial agonist activity (figure 4A). In contrast, both MPA and NET-A are potent and efficacious AR agonists, with activity comparable to that of DHT (figure 4A). Notably, DHT, MPA and NET-A have a lower potency and efficacy in comparison to MIB. Kemppainen *et al.,* 1999, showed that 100-fold more MPA is required to achieve the same agonist activity as DHT, MIB and R1881. This AR agonist activity was determined in the CV-1 cell line transiently transfected with a mouse mammary tumor virus (MMTV)-luciferase reporter and full-length hAR expression vectors. In a separate study, using the Chinese hamster ovary (CHO) cell line stably transfected with the MMTV-luciferase reporter and hAR expression vectors, the relative agonistic activity of NET for the AR was found to be only about 1% of that of DHT (Deckers *et al.,* 2000). Thus, in these two aforementioned studies, due to the fact that MPA and NET both required 100-fold greater concentrations to achieve similar agonist activity as DHT, MPA was described as a weak AR agonist whereas NET was said to have low androgenic activity. The discrepancies between our results and the aforementioned studies could be attributed to the different systems in which the agonist activities were measured. If this is the case, then contributing factors could be the cell line used and/or the sequence or context of the hormone response element used in the reporter construct. In addition, a very recent study also performed in the COS-1 cell line using a PRE2-tk-luc construct, reported EC_{50} values for Prog, MPA and NET-A of approximately 3000, 56.8 and 19.6 nM, respectively (Sasagawa *et al.,* 2008), while we determined values of 601, 1.37 and 2.33 nM, respectively.

In accordance with our results indicating that MPA and DHT are equally potent AR agonists, a comparative study by Ghatge and co-workers (2005) recently showed that MPA is as potent as DHT in regulating AR-mediated gene expression in human breast cancer cell lines (Ghatge *et al.,* 2005). Furthermore, we demonstrate that Prog and RU486 are potent antagonists of MIB induced AR-mediated transcription (figure 4B), with minimal agonist activity (figure 4A). Similarly, Prog (Kemppainen *et al.,* 1999; Sasagawa *et al.,* 2008) and RU486 (Song *et al.,* 2004) have previously been reported to antagonize DHT-induced AR- mediated transcription. In contrast, MPA and NET-A did not antagonize MIB-induced AR-mediated transcription. Overall, these results indicate that Prog displays weak androgenic activity, while the synthetic progestins, MPA and NET-A, display potent androgenic activities similar to the natural androgen, DHT. In addition, Prog has anti-androgenic activities, while the synthetic progestins do not. Interestingly, the synthetic progestin, RU486, has similar activities as Prog.

MPA and NET-A activate the AR by different mechanisms

A number of parameters contribute to the potency of a ligand such as its affinity for the receptor, rate of nuclear translocation, elicited receptor conformation, stabilisation of receptor-ligand complex, the recruitment of coregulators and transcription machinery, as well as DNA binding and rate of ligand dissociation. As we had already addressed the binding of ligands to the AR, we next investigated the effect of all the ligands in our study on another parameter. Considering that the N/C-interaction of the AR is well-described, we decided to investigate the N/C-interaction of the AR in response to ligand, as a measure of receptor conformation and stabilisation. To the best of our knowledge, the capability of MPA (Kemppainnen *et al.,* 1999), but not

NET-A, to induce the N/C-interaction of the AR has been previously investigated. Our results indicate that MPA, in contrast to NET-A and DHT, does not induce the N/C-interaction on a classical ARE-promoter (figure 6A), suggesting that MPA and NET-A activate the AR by different mechanisms. This difference between MPA and NET in inducing the AR N/C-interaction was first observed by Tamzin Tanner in the Hapgood laboratory (MSc thesis). These results suggest that the conformation of the AR attained by the N/C-interaction is not imperative to render the receptor transcriptionally active, since MPA still has the ability to activate an androgen-responsive reporter. When comparing the ability of the two synthetic progestins to induce the AR N/C-interaction, it would appear that NET-A displays behaviour that is more characteristic of classical strong androgen agonists. An explanation for this difference in induction of AR N/C-interaction, could be the fact that MPA and NET-A are structurally different, with MPA being a 21-carbon series steroid, and therefore considered a true progestin, whereas NET-A is a 19-carbon series steroid structurally more closely related to the androgens. This difference in the structures could possibly account for the more typical androgenic profile of NET-A.

The crystal structure analysis of the AR bound to DHT showed a conformational arrangement of the ligand binding domain (LBD), and the formation of the conserved cleft, AF-2, which is critical for interaction with coregulators (Sack *et al.,* 2001). Recently, in a review by Birrell and co-workers (Birrell *et al.,* 2007), a molecular model was presented, in which MPA forces the displacement of a critical amino acid residue, Phe874, in the AR, thereby disrupting this well-defined AF-2 cleft observed for the DHT-bound receptor. The authors hypothesized that this could cause MPA and DHT to have differing effects on AR-regulated gene expression. A recent study

of AR-regulated genes in breast cancer cell lines supports this hypothesis (Ghatge *et al.,* 2005). This raises the possibility that NET-A-bound AR may have a similar conformation as the DHT-bound AR, and that MPA and NET-A, could therefore have divergent effects on AR-regulated genes.

The ability of a compound to bind to the AR but not induce the N/C-interaction has previously been reported for the AR antagonists hydroxyflutamide and cyproterone acetate (Langley *et al.,* 1995; Kemppainen *et al.,* 1999). In accordance with these previously reported results, the two antagonists in our system, Prog and RU486 (see figure 8B), also did not induce the N/C-interaction of the AR (Kemppainen *et al.,* 1999; Song *et al.,* 2004). Consistent with the previous finding that the degree of agonist potency at low physiological concentrations parallels the ability of a compound to induce the N/C-interaction (Kemppainen *et al.,* 1999), we show that potency for transactivation via a synthetic GRE correlates with the potency for the induction of N/C-interaction (r^2 = 0.59; $p<0.05$) for most of the ligands (figure 8A), barring MPA. Correlation of efficacy for transactivation versus efficacy for induction of N/C-interaction, showed no statistical significance (r^2 = 0.49). In addition, it was previously reported that MPA is a potent antagonist of a DHT-induced N/C-interaction (Kemppainen *et al.,* 1999; Birrell *et al.,* 2007). This result was corroborated in our lab (MSc thesis of Tamzin Tanner), while we found that NET-A could not antagonize the DHT-induced N/C-interaction (Appendix B5), highlighting a potentially significant difference in the mechanism of action of the two progestins. Altogether, the findings indicate that Prog, MPA and NET-A activate the AR by different mechanisms. Notably, the mechanism of AR activation by RU486 is comparable to Prog.

Induction of the AR N/C-interaction is promoter-dependent

The steroid receptor family has a high degree of structural and functional conservation. Thus, it is not surprising that they can recognize virtually the same DNA motifs, e.g. some androgen response elements (AREs), also function as glucocorticoid response elements (GREs) or progesterone response elements (PREs), or vice versa (Evans, 1988; Beato, 1989; Mangelsdorf *et al.,* 1995). However, the AR can also interact with binding motifs that resemble direct rather than palindromic repeats of the 5'-TGTTCT-3'-hexamer. These motifs are termed AR-specific, and have been described in several androgen-responsive genes, such as the sc-ARE1.2 in the far upstream enhancer of the human secretory component (SC) gene (Haelens *et al.,* 1999), and the SLP-HRE2 from the upstream enhancer of the mouse sex-limited (SLP) gene (Adler *et al.,* 1991). In addition, it has been shown that the AR N/C interaction is important for AR activation of the promoters of some androgen-regulated genes, but not others (He *et al.,* 2002). We thus hypothesized that the inability of MPA to induce the N/C-interaction is dependent on the nature of the response element i.e. the DNA binding sequence. To this end, we tested whether MPA could induce the ligand-dependent N/C-interaction using two androgen-selective AREs. We observed an overall increase in the ability of all ligands to increase the AR N/C-interaction on both promoters. These results support our hypothesis that the induction of the N/C-interaction by MPA is promoter-dependent (figure 5A-5C and figure 6A-C). He and co-workers (2002b) have previously shown that the AR N/C-interaction is important for some androgen-regulated promoters, but not others. Our finding that NET-A shows similar potency and efficacy to MPA in inducing transcription via the AR, but has a different effect on the N/C-interaction, even on selective AREs, suggests that the N/C-interaction is not essential for

transactivation activity on these synthetic promoters. This has previously been suggested by other groups (Shen *et al.,* 2005). Other factors such as the dissociation of heat-shock proteins, nuclear translocation, DNA binding affinity and rate of ligand dissociation may also play a role in the transactivation activity.

MPA and NET-A display similar androgenic properties for transrepression on synthetic promoters

Many steroid receptors have been suggested to exert inhibitory effects on inflammatory response genes (transrepression) through direct interactions with transcription factors such as NFκB and AP-1, thereby attenuating inflammatory responses. NFκB and AP-1 have been shown to be crucial for the regulation of the inflammatory process through their direct activation of proinflammatory cytokines and chemokines (Karin, 2009; Xiao and Ghosh, 2005). By far the most best-studied in terms of transrepression is the GR (De Bosscher *et al.,* 2003; Koubovec *et al.,* 2005), but transrepression has also been reported for the ER (reviewed by Pascual and Glass, 2006). Interestingly, not much is known about transrepression mediated via the AR. On these grounds, we investigated the comparative effects of all the ligands on transrepression via both NFκB and AP-1 on synthetic promoter-reporter constructs.

The results of the transrepression assays (at 1 μM of each ligand) (figure 9A and 9B) as well as the effects observed for transactivation (figure 4A), are summarized in Table 4. Similar relative activity of the ligands on all three promoters was observed. Notably, both the weak partial agonist/potent antagonists, Prog and RU486, could transrepress more effectively via the AP-1 promoter than via NFκB. This increase in

transrepression on AP-1 via the AR, at least for Prog, was also observed for this ligand on the same promoter via the GR (Ronacher *et al.,* 2009). Briefly, unlike Prog, the transrepressive activities of MPA and NET-A via the AR is similar to the natural androgen, DHT.

Conclusion

Although Prog, MPA and NET-A are structurally similar, relatively minor differences can cause profound alterations in their biochemical activity (Darney, 1995). The findings of our study highlight the fact that, although these compounds are designed to mimic the biological activity of natural Prog, they do not act similarly to Prog at the molecular level. Moreover, the ability of MPA and NET-A to exert similar effects through the AR, but via different mechanisms, implies that such compounds could then also exhibit very different effects via different steroid receptors in some target tissue. Additionally, MPA could also exert different effects on endogenous genes via the AR, in the same cell. Indeed, we have observed such differential effects by MPA and NET-A on the RANTES gene in a human ectocervical cell line (Ect1/E6E7) (Chapter 2).

This finding that MPA acts differently to NET-A, and moreover does not mimic the effects of Prog, may have important implications for women's health. By elucidating the mechanisms by which MPA and NET-A exert their effects, we could facilitate improved drug design for contraceptive and HRT agents, as well as help to unravel molecular mechanisms by which the compounds exert their side effects.

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Chapter 4

Investigating the anti-mineralocorticoid properties of synthetic progestins used in hormone replacement therapy

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Abstract

Progesterone (Prog) has a high affinity for the mineralocorticoid receptor (MR) and is a potent MR antagonist. The synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone derivatives, widely used as progestin-only injectable contraceptives and in hormone replacement therapy (HRT), have been reported to lack this anti-mineralocorticoid activity. This suggests that when they are used in combination with estrogen during HRT, they are unable to antagonize the salt-retaining effects of estrogen, which may lead to weight gain and increased blood pressure. As the latter are causes of cardiovascular disease, this may explain the mechanism for the observed adverse cardiovascular outcomes associated with estrogen plus progestin therapy. In this study, we compared the binding affinity of Prog for the MR with that of MPA and NET-A, as well as with the known glucocorticoid and progesterone receptor antagonist, RU486. We also compared the relative antagonist potencies of the ligands for transactivation on a synthetic GRE/MRE via the MR, as well as their ability to transrepress MR-mediated NFκB and AP-1 promoter-reporter genes. In addition, the effect on an endogenous gene, Orm-1, shown to contain a GRE/MRE, was investigated. Competitive binding revealed that MPA and NET-A have similar binding affinities, but that their affinity is a 100-fold lower than that of Prog for the human MR (K_d of 0.153 nM for aldosterone (Ald) and K_i's of 1.69, 197, and 229 nM for Prog, MPA and NET-A, respectively). Interestingly, RU486 has a very low affinity for the hMR (K_i of 2980 nM). Furthermore, on a synthetic mineralocorticoid response element (MRE)-containing promoter, we showed that MPA, NET-A and RU486 are all antagonists of the hMR, but that unlike Prog, they have weak antagonistic activity. However, on the endogenous MRE-containing Orm-1 (α-glycolytic protein or orosomucoid-1) gene expressed in a rat

cardiomyocyte cell line, NET-A, but not MPA, has similar antagonistic activity as Prog, suggesting that NET-A may be partially cardioprotective while MPA is not. Interestingly, RU486 also has similar antagonist activity as Prog on the Orm-1 gene. In addition, we highlight the underappreciated fact that dexamethasone (Dex), the so-called GR-specific agonist, is a partial agonist for MR transactivation, and show for the first time, that it is a full agonist for MR transrepression. Consistent with agonist-induced interactions between the N- and C termini of steroid receptors (N/C-interaction), our findings show that efficacy for transactivation via a synthetic GRE correlates with the efficacy for the induction of MR N/C-interaction (r^2 = 0.9179; $p<0.01$), indicating that there is a relationship between transactivation and MR N/C-interaction. Moreover, this study is the first to show that NET-A and RU486, but not MPA, can dissociate between transrepression (AP-1 promoter-reporter) and transactivation (GRE promoter-reporter) via the MR. These differences indicate that NET-A and RU486 may be promising therapeutics for the treatment of MR-mediated inflammation.

INTRODUCTION

Synthetic progestins have been used for decades in female reproductive health. Although used in the treatment of a wide range of gynaecological disorders such as endometriosis, the two most frequent uses of progestins are for contraception, and for hormone replacement therapy (HRT) in postmenopausal women. These compounds are used to mimic the actions of the endogenous hormone progesterone (Prog), but have a longer plasma half-life and higher bio-availability than Prog (Speroff, 1996). Medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) are two widely used progestins which are chemically derived from 17α-hydroxy-progesterone and from 19-nortestosterone derivatives, respectively. MPA itself, rather than its metabolites, is the major progestogenic compound, while NET-EN and norethisterone acetate (NET-A) are hydrolysed to norethisterone (NET) and its metabolites, which together have progestogenic action (Stanczyk and Roy, 1990).

Both MPA and NET are reported to have side-effects for users. The side-effect profile when used as contraception includes irregular bleeding patterns, breast tenderness, headache, dizziness, and weight gain (Haider and Darney, 2007; Nur-Isterate package insert reg. no. J/21.8.2/136, Shering (Pty) Ltd). Recent studies suggest that MPA and NET used in HRT may increase the incidence of breast cancer (Rossouw *et al.,* 2002; Beral, 2003). In addition to an increased risk of breast cancer, the Women's Health Initiative (WHI) study on combined estrogen and MPA used in healthy postmenopausal women also showed increased risk of cardiovascular complications (Rossouw *et al.,* 2002). As these progestins are designed to mimic the actions of progesterone, it is assumed that their biological effects, like progesterone, are mediated by the progesterone receptor (PR). However, these progestins cross-

react with other members of the steroid receptor family and it is speculated that the observed side-effects are mediated in this way.

The PR, androgen receptor (AR), glucocorticoid receptor (GR), estrogen receptor (ER) and mineralocorticoid receptor (MR) constitute the steroid receptor family. They are ligand-dependent transcription factors that display a high level of similarity with regards to their structure and mechanism of action, and they regulate gene expression of target genes by activating (transactivation) or repressing (transrepression) transcription (Evans, 1988; Beato, 1989). In general, for transactivation, the liganded-receptor translocates to the nucleus and binds hormone response elements (HREs) to activated genes, while during transrepression, the liganded-receptor binds to other transcription factors such as NFκB and AP-1, thereby inhibiting gene activation (Evans, 1988; Beato, 1989). Both MPA and NET-A have been shown to have similar affinities for the AR, and are both potent agonists for transactivation like dihydrotestosterone (DHT), the native androgen (Chapter 3). Interestingly, MPA has a greater affinity than NET-A for the GR, and acts as a partial agonist for transactivation via the GR (Koubovec *et al.,* 2005), while NET-A is a GR antagonist (supplementary material in Appendix C6). Interestingly, the activity of MPA and NET via the GR and AR differs from that of Prog, in that Prog is a weak partial agonist for the GR, and an antagonist for the AR. Collectively, this demonstrates that MPA and NET do not have the same activity, nor do they act the same as Prog, via different steroid receptors.

Prog has a high affinity for the MR, displaying weak agonist transactivation activity, but potent antagonist activity (Wambach *et al.,* 1979; Rafestin-Oblin *et al.,* 1992;

Quinkler *et al.,* 2002). In contrast, both MPA and NET-A have been reported to bind to the MR with low relative affinity and do not display any agonist or antagonist properties towards the MR (Winneker *et al.,* 2003, Palacios *et al.,* 2006). The difference in the activity of MPA and NET as compared to Prog, via the MR, may have serious implications for post-menopausal women using estrogen/progestin treatment for HRT. This is because the progestin used in HRT should be able to antagonize the salt-retaining and blood pressure raising effects of estrogens. Estrogen acts on the renin-angiotensin-aldosterone system (RAAS), leading to increased synthesis of angiotensinogen, which subsequently increases aldosterone levels thereby promoting sodium and water retention (Oelkers, 1996). Thus estrogen indirectly leads to increases in weight and blood pressure. Thus, if progestins used in combined HRT lack anti-mineralocorticoid activity, it could lead to side-effects such as weight gain and an increase in blood pressure. Since high blood pressure is a cardiovascular disease risk factor, using synthetic progestins, such as MPA and NET-A, that lack anti-mineralocorticoid activity in HRT, could lead to cardiovascular complications, as observed in the WHI study (Rossouw *et al.,* 2002). However, the role of progestins such as MPA and NET-A, in cardiovascular disease remains poorly defined.

Recently, Fejes-Tóth and Náray-Fejes-Tóth (2007) showed that Aldosterone (Ald), the natural MR ligand, regulates the expression of several genes that may contribute to the development of cardiovascular damage. Furthermore, it is known that Ald plays an important role in hypertension and heart failure, and MR antagonists are frequently prescribed to block MR activity in hypertensive patients (Pitt *et al.,* 1999; Pitt *et al.,* 2003). Thus, blocking Ald regulation of these genes by MR antagonists

could alleviate cardiovascular damage, and may be beneficial to patients with chronic heart failure. We were particularly interested in the Orm-1 (α-glycolytic protein or orosomucoid-1), an inflammation/acute phase-related gene, whose increased expression is associated with increased risk of cardiovascular disease (Engström *et al.,* 2003). The promoter of Orm-1 contains a functional glucocorticoid response element/mineralocorticoid response element (GRE/MRE) (Klein *et al.,* 1988). It has been reported that the effect of Ald on Orm-1 is direct, mediated by the binding of the liganded-MR to MREs in the promoter region (Fejes-Tóth and Náray- Fejes-Tóth, 2007).

In the current study, we determined the equilibrium constants (K_d or K_i's) for the MR of Ald, Prog, MPA, NET-A, as well as RU486, the known PR and GR antagonist, and probable MR antagonist (www.nursa.org/10.1621/datasets.02001). We also compared the relative antagonist potencies of the ligands for transactivation on a synthetic GRE/MRE, as well as their ability to transrepress NFκB and AP-1 promoter-reporter genes via the MR. To further investigate the anti-mineralocorticoid activity of these compounds, we used rat cardiomyocytes transiently transfected with MR (Fejes-Tóth and Náray- Fejes-Tóth, 2007), and measured the expression of the endogenous Orm-1 gene in response to Ald, in the absence and presence of the synthetic progestins. Specifically, we hypothesized that MPA and NET-A, unlike Prog, would not antagonize the Ald-mediated MR transactivation.

MATERIALS AND METHODS

Plasmids

The plasmid, pTAT-GRE-E1b-luc, driven by the E1b promoter containing two copies of the rat TAT-GRE, has been described previously (Sui *et al.,* 1999). The cytomegalovirus (CMV)-driven-β-galactosidase expression vector (pCMV-β-gal) was obtained from Prof. G. Haegeman (University of Gent, Belgium). The 5xNFκB-luc and 7xAP-1-luc plasmids were from Stratagene (Houston, Texas, USA). A plasmid expressing the human mineralocorticoid receptor, pRShMR, was a kind gift from Prof. R. Evans and previously described (Arriza *et al.,* 1987). Plasmids expressing human glucocorticoid receptor, pRS-hGRα were a kind gift from Prof. R. Evans (Howard Hughes Medical Institute, La Jolla, USA). A plasmid expressing the human androgen receptor, pSVARo (Brinkmann *et al.,* 1989) was obtained from Frank Claessens, (University of Leuven, Leuven, Belgium). The plasmid expressing the human progesterone receptor, isoform B, pSG5hPR-B (Kastner *et al.,* 1990), was obtained from Stoney Simons Jr (NIH, Bethesda, USA). The GAL4-MRC expression vector and the VP16-MRNT fusion protein expression vectors (provided by Prof's Peter Fuller (Prince Henry's Institute of Medical Research, Victoria, Australia) and Fraser Rogerson (University of Melbourne Department of Paediatrics, and Murdoch Childrens Research Institute, Royal Children's Hospital, Victoria, Australia), were previously described (Rogerson and Fuller, 2003). The reporter vector, pG5-luc, which contains the luciferase gene driven by a GAL4 responsive promoter, was obtained from Prof Douglas Stocco (Texas Tech University Health Sciences Center, Texas, USA). The pGL2basic empty vector was obtained from Promega, Madison, USA.

Inducing compounds

11β,21-Dihydroxy-3,20-dioxo-4-pregnen-18-al (Aldosterone; Ald), 11β-(4-Dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one (mifepristone; RU486), 7α-acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone (spironolactone), 4-pregnene-3, 20-dione (progesterone; Prog), 6α-methyl-17α-hydroxy-progesterone acetate (medroxyprogesterone acetate; MPA), 17α-ethynyl-19-nortestosterone 17β-acetate (norethindrone acetate; NET-A), (11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione (Dexamethasone; Dex) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich, South Africa. NET-A, the acetate, was used as it is soluble in water compared with the insoluble ester NET-EN. *In vivo*, both NET-EN and NET-A undergo hydrolysis and are converted to NET and its metabolites (Stanczyk and Roy, 1990). [^{3}H]-Aldosterone ([^{3}H]-Ald) (87.9 Ci/mmol), unlabelled 17β-hydroxy-7α,17α-dimethylestr-4-en-3-one (mibolerone; MIB), and unlabelled R5020 were purchased from PerkinElmer Life and Analytical Science, South Africa. All unlabelled test compounds were dissolved in absolute ethanol and stored at -20^{o}C. These compounds were then added to serum-free culturing medium such that the final concentration of ethanol was 0.1%. Control incubations (no test compounds) were performed in the presence of 0.1% ethanol.

Cell culture

COS-1 cell lines (monkey kidney cells) were maintained in high glucose (4.5 g/ml) Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa). Medium was supplemented with 10% fetal calf serum (FCS) (Delta Bioproducts, South Africa), 50 IU/ml penicillin and 50 µg/ml streptomycin (GibcoBRL, Paisley, UK). The H9C2 cell line (rat cardiomyocyte cells) was a kind gift from Dr. Rob Smith (Physiology

Department, University of Stellenbosch, South Africa) and was maintained in high glucose (4.5 g/ml) DMEM (Sigma-Aldrich, South Africa). Medium was supplemented with 10% FCS (Delta Bioproducts, South Africa), 50 IU/ml penicillin and 50 µg/ml streptomycin (GibcoBRL, Paisley, UK). All cultures were maintained in 75 cm^2 culture flasks (Greiner, Bio-One International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO_2, and were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney, 1987), and only mycoplasma-negative cell lines were used in experiments.

Whole cell binding assays

Competitive whole cell binding assays were performed essentially as described by Bamberger *et al.* (1995), with the following modifications. The COS-1 cell line was maintained in DMEM as described. Cells were seeded into 24-well tissue culture plates at 1 x 10^5 cells per well. On day 2, cells were transfected with 0.375 µg pRShMR expression vector and 0.0375 µg of the pCMV-β-gal expression vector (Stratagene, Houston, Texas, USA), using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. On day 3, the cells were washed with phosphate-buffered saline (PBS) and incubated with 0.2 nM [^{3}H]-Ald (PerkinElmer Life and Analytical Science, South Africa) in the absence (total binding) or presence of increasing concentrations of unlabelled Ald, Prog, MPA, NET-A or RU486 for 16 hours (time taken to reach equilibrium; Appendix C2) at 37°C. Working on ice at 4^{o}C, cells were washed three times with ice-cold 1X PBS containing 0.2% (w/v) bovine serum albumin (BSA) for 15 min. Cells were then lysed with 200 µl reporter lysis buffer (Promega, Madison, USA). Total binding ([^{3}H]-Ald in the absence of added unlabelled ligand) was

determined by scintillation counting and expressed as 100%. Specific bound [^{3}H]-Ald was calculated as the difference between total and non-specific binding, the latter determined by incubating cells in the presence of [^{3}H]-Ald plus 10 µM unlabelled Ald and expressed as a relative % of total binding. The β-galactosidase chemiluminescent reporter gene assay system (Tropix Inc., U.S.A.) was used to determine β-galactosidase (β-gal) activity, to correct for differences in transfection efficiencies. Binding data were analyzed using GraphPad Prism software, with nonlinear regression and assuming competitive binding to one class of binding sites. K_i values for Prog, MPA, NET-A and RU486 were determined from the heterologous displacement curves using the EC_{50}s, K_d for Ald, and concentration of radiolabelled Ald, according to the method of Cheng and Prusoff (1973) and described in Appendix D1. The reported values are averages of at least three independent assays, with each condition performed in triplicate.

Luciferase reporter assays

For transactivation assays in the COS-1 cell line: COS-1 cells were maintained as above and seeded into 96-well tissue culture plates at 1 x 10^4 cells per well. On day 2, cells in each well were transiently transfected with 50 ng of the pTAT-GRE-E1b-luc construct, 5 ng of the expression vector for the human mineralocorticoid receptor (pRShMR), and 5 ng of the pCMV-β-gal expression vector, using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. On day 3, the cells were washed with PBS and induced with 1 or 10 µM of either Ald, Prog, MPA, NET-A, RU486 or Dex to investigate agonist activity, while for antagonist activity, the cells were incubated with 1 nM Ald in the absence and presence of increasing concentrations of Prog, MPA,

NET-A or RU486. After 24 hours, the cells were lysed with reporter lysis buffer (Promega, Madison, USA). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega, Madison, USA) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale CA, USA). The values obtained were normalised to expression of β-gal, which was measured using the GalactoStar Assay Kit from Tropix (Bedford MA, USA).

For transactivation assays in the H9C2 cell line: H9C2 cells were maintained as above and seeded into 24-well tissue culture plates at 5 x 10^4 cells per well. On day 2, cells in each well were transiently transfected with 330 ng of the pTAT-GRE-E1b-luc construct and 33 ng of the expression vector for the hMR, hGR, hAR, hPR-B or the empty vector pGL2basic, using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. On day 3, the cells were washed with PBS and induced with 10 μM of either Ald, Dex, MIB, or R5020. After 24 hours, the cells were lysed with reporter lysis buffer (Promega, Madison, USA). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega, Madison, USA) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale CA, USA).

For transrepression assays, the COS-1 cell line was seeded into 24-well tissue culture plates at a density of 5 x 10^4 cells per well. The following day the cells were transfected with 0.045 μg of pRShMR, 0.09 μg of 5xNFκB-luc or 7xAP-1-luc plasmids respectively, and 0.0225 μg of pCMV-β-Gal using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. Twenty four hours after transfection the cells were washed with PBS and

incubated with serum-free medium containing 10 ng/ml PMA and 1 µM of each test compound. Luciferase activity in the lysate was measured as described above. The values obtained were normalised to expression of β-gal, which was measured using the GalactoStar Assay Kit from Tropix (Bedford MA, USA). Where normalising to B-galactosidase is not performed, transfection efficiency was previously determined to be highly reproducible. The reported values are averages of at least three independent experiments, with each condition performed in triplicate.

Mammalian two-hybrid assays

The mammalian two-hybrid assay was performed in the COS-1 cell line. The cells were maintained as described, and then plated in 24-well tissue culture plates at a density of 5 x 10^4 cells per well. On day 2, the cells were transfected with 0.1 µg/well GAL4-MRC, 0.5 µg/well VP16-MRNT and 0.5 µg/well pG5-luc, using the FuGENE6 transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. Twenty four hours after transfection, cells were induced with 1 or 10 µM Ald, Prog, MPA, NET-A, RU486 or Dex for 24 hours. Luciferase activity in the lysate was measured as described above. The reported values are averages of at least three independent experiments, with each condition performed in triplicate. The values obtained were normalised to expression of β-gal, which was measured using the GalactoStar Assay Kit from Tropix (Bedford MA, USA).

Isolation of total RNA and real-time quantitative RT-PCR analysis of representative genes

The H9C2 cell line was maintained as described above. Cells were seeded into 12-well plates, transiently tranfected with the MR, and subsequenlty grown in the

absence of FCS for 48 h before treatment with test compounds. Cells were induced with 1 nM Ald in the absence and presence of 1 µM Prog, MPA, NET-A, RU486 or Spironolactone for 24 hours. Total RNA was isolated from cells using Tri-reagent (Sigma-Aldrich, South Africa) according to the manufacturer's instructions. The integrity of the RNA (presence of intact 18S and 28S ribosomal bands) was confirmed by agarose gel electrophoresis. Total RNA (1 µg) was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. Real-time PCR was performed by using a Light-Cycler, rapid thermal cycler system (Roche Applied Science, South Africa) according to the manufacturer's instructions. Nucleotides, TaqDNA polymerase, and buffer used in the reaction were those included in the LightCycler-FastStart DNA Master^{PLUS} SYBR Green I system (Roche Diagnostics, South Africa). β-actin was used as an internal control. Primer sets are as described in Table 1. The thermal cycling parameters were: initial denaturation at 95^{o}C for 3 min, followed by 40 cycles at 95^{o}C for 15 sec and 57^{o}C for 30 sec.

Table 1: QPCR primer sets (Orm-1 and β-actin sequences were obtained from Prof. Anikó Náray-Fejes-Tóth) (Fejes-Tóth and Náray- Fejes-Tóth, 2007).

Gene	Primers	Strand	Annealing temperature (^{o}C)
Orm-1	TGCCCATTTGATAGTGCTGAAG CAGGCTGTCAAAGATGTGGGCA	Forward Reverse	60
β-actin	TTGTAACCAACTGGGACGATATGG GATCTTGATCTTCATGGTGCTAGG	Forward Reverse	60

Western blotting

H9C2 cell lines were plated at 1 x 10^6 cells per well in 6-well plates and allowed to grow to confluency. Cells were washed with PBS and lysed in 100 µl SDS-sample buffer (Sambrook *et al.,* 1989). Lysates were boiled and subjected to electrophoresis on a 8% SDS polyacrylamide gel using β-actin expression as a loading control. Following electrophoresis, proteins were transferred to nitrocellulose membranes. Blots were probed with an anti-MR (H300, 1:1000), anti-GR (H300, 1:3000), anti-AR (H-280, 1:1000), or anti-PR-AB (sc-810, 1:500) antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), as well as the anti-β-actin (1:1500) antibody from Cell Signalling Technology, Massachusetts, USA, for 16 hours at 4 ^oC. Blots were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10000, NA934VS, Amersham, South Africa) for 1 hour. Proteins were visualized using enhanced chemiluminescence (ECL) from Amersham, followed by exposure to Hyperfilm MP high performance autoradiography film (Amersham, South Africa).

Data manipulation and statistical analysis

The Graph Pad Prism® software was used for data manipulations, graphical representations, and statistical analysis. One-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) posttests were used for statistical analysis. Non-linear regression and one site competition were used in whole cell binding assays, whereas non-linear regression and sigmoidal dose response were used in transactivation (antagonist) experiments. Statistical significance of differences is indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, whereas no statistical significance is indicated by $p>0.05$. The letters

a,b,c etc. are also used to denote statistically significant differences, where all those values which differ significantly from others, are assigned a different letter. Fractional occupancy of the MR for each compound was calculated using the equation:

Fractional occupancy = [ligand] / ([ligand]+K_i), using the K_i values summarized in Table 2.

RESULTS

MPA and NET-A have a similar binding affinity for the MR

Competitive whole cell binding assays in the COS-1 cell line transiently transfected with a full-length human mineralocorticoid receptor (hMR) expression vector, were performed to determine K_d and K_i values. In order to obtain accurate K_d and K_i values using homologous and heterologous displacement curves, respectively, a range of experiments was first performed to establish an appropriate concentration of radiolabelled ligand and the time to reach equilibrium. An appropriate concentration of [^{3}H]-Ald, in the range two to ten times lower than the EC_{50}, was established as 0.2 nM (discussed in Appendix D1), and the incubation time required for equilibrium to be reached for 0.2 nM [^{3}H]-Ald binding to the MR, was determined as sixteen hours (discussed in Appendix D1).

Homologous/heterologous curves with unlabelled steroids were then obtained and results show that MPA and NET-A bind to the MR with a similar affinity, but approximately 100-fold lower than that of Prog and 1000-fold lower than that of the natural mineralocorticoid, Ald (figure 1). Interestingly, RU486 also binds the MR but with an approximately 20 000-fold lower affinity than Ald. The curves for each competitor steroid indicate competitive binding to the same site as Ald (R^2 values for

each MPA, NET-A, Prog and RU486 were 0.9453, 0.9452, 0.9837 and 0.9431 respectively, for one site competitive binding curve). The K_d for Ald and K_i values for Prog, MPA, NET-A and RU486 are summarised in the Table 2. The K_i for Prog was determined as 1.69 nM, which is similar to a previously reported value of 1.2 nM (Quinkler *et al.,* 2002).

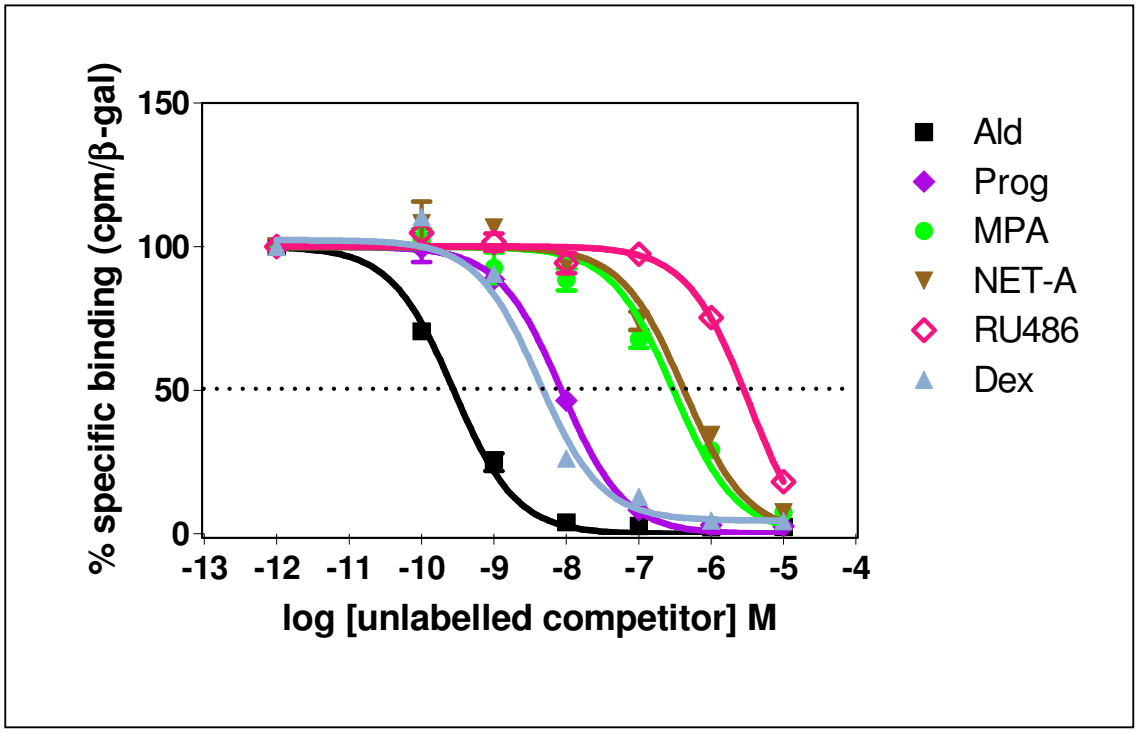


Figure 1: MPA and NET-A both compete with [^{3}H]-Ald for binding to the human mineralocorticoid receptor. The COS-1 cell line was transiently transfected with the pRShMR and pCMV-β-galactosidase expression vectors.Twenty-four hours later the cells were incubated with 0.2 nM [^{3}H]-Ald in the absence or presence of increasing concentrations of either unlabelled Ald (■), Prog (♦), MPA (●), NET-A (▼), RU486 (◊) or Dex (▲) for 16 hours. Results are plotted as % specific binding where total specific binding of [^{3}H]-Ald in the absence of unlabelled ligand is set as 100% and binding of unlabelled ligand is set as a % binding relative to Ald, after normalization for transfection efficiency with β-galactosidase levels. Result shown is representative of three independent experiments with each condition performed in triplicate (±SEM).

Table 2: Relative binding affinities of the ligands for the MR (K_d or K_i values)[a]

Test compound	K_d or K_i (M) ± SEM	Fold differences
Ald	1.53×10^{-10} ± 0.04	1
Prog	1.69×10^{-9} ± 0.07	11.05
MPA	1.97×10^{-7} ± 0.04	1287.58
NET-A	2.29×10^{-7} ± 0.07	1496.73
RU486	2.98×10^{-6} ± 0.08	19477.12
Dex	5.14×10^{-9} ± 0.07	33.59

[a] Binding data of three independent experiments were analyzed using GraphPad Prism software, with nonlinear regression and assuming competitive binding to one class of binding sites. K_i values ± SEM for Prog, MPA, NET-A, RU486 and Dex were determined from the heterologous displacement curves using the EC_{50}s, K_d ± SEM for Ald, and concentration of radiolabelled Ald, according to the method of Cheng and Prusoff (1973) that is described in Appendix D1. Fold differences calculated by setting the K_d for MIB in M as 1, and expressing the K_i in M for the other ligands as a fraction thereof. Statistical analysis of the K_d / K_i values, indicated the following: Ald vs. Prog, MPA, NET-A, RU486, Dex ($p< 0.001$); Prog vs. MPA, NET-A, RU486, Dex ($p< 0.001$); MPA, NET-A vs. RU486, Dex ($p<0.001$); MPA vs. NET-A ($p>0.05$); Prog vs. Dex ($p<0.05$).

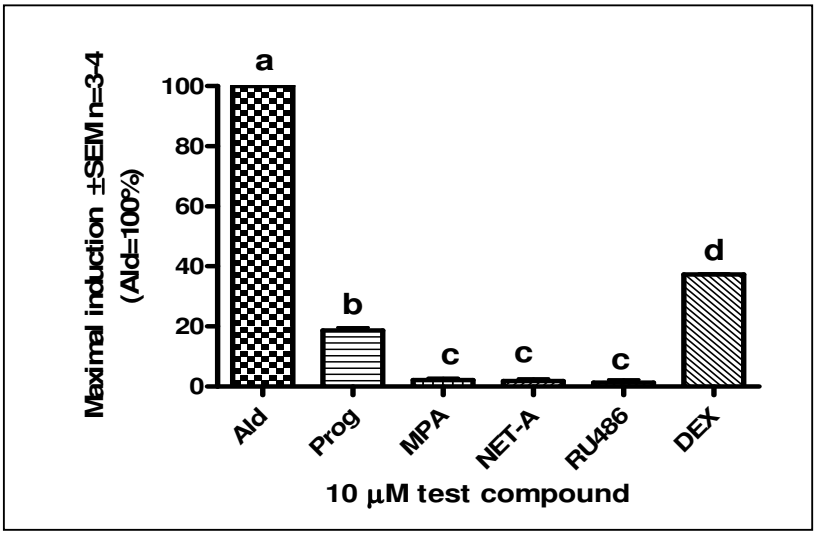
Unlike Prog, MPA and NET-A display weak MR antagonist activity and no mineralocorticoid agonist activity for transactivation

Having shown that MPA, NET-A and RU486 bind to the hMR, albeit with low affinity, we directly compared their mineralocorticoid properties by transiently transfecting the COS-1 cell line with a glucocorticoid response element (GRE)/mineralocorticoid response element (MRE)-driven reporter construct containing two copies of the rat TAT GRE, a hMR expression vector and the pCMV-β-gal expression vector. The

cells were subsequently exposed to either 1 or 10 µM Ald, Prog, MPA, NET-A, RU486 or Dex. The results clearly show that 10 µM MPA, NET-A and RU486, unlike 10 µM Prog, do not display agonist activity for the MR (figure 2A). A similar profile was observed at 1 µM of each compound (Appendix C4). The transcriptional activity of these compounds in the absence of transfected receptor was negligible (Appendix D3). Interestingly, Dex, the well-described potent GR-specific agonist (Honer *et al.,* 2003; Yu *et al.,* 2004), displays partial agonist activity of about 37% of maximal activity relative to Ald via the MR, showing that Dex is a partial agonist for transactivation via the MR. Whole cell binding experiments showed that Dex binds to the MR with a significantly higher affinity than Prog (Table 2).

In order to obtain additional information about the pharmacological profiles of these compounds, we investigated their antagonist activity by transfecting the COS -1 cell line as above, and incubating the cells with 1 nM Ald in the absence or presence of increasing concentrations of Prog, MPA, NET-A and RU486 (figure 2B). These results indicate that although, MPA, NET-A and RU486 can similarly antagonise the effects of Ald, they are much less potent than Prog (approximately 87-216 fold less potent). We determined potencies (EC_{50} values) for antagonist activity of 3.1, 310, 270 and 670 nM for Prog, MPA, NET-A and RU486, respectively. Interestingly, our EC_{50} value for antagonist activity of Prog (3.1 nM) indicates that Prog is a more potent at antagonizing the effects of Ald than the well-known MR antagonist spironolactone (EC50 of 14 nM) (reviewed in Lu *et al.,* 2006). Whether Dex has MR antagonist activity was not investigated.

A **Agonist activity**



B **Antagonist activity**

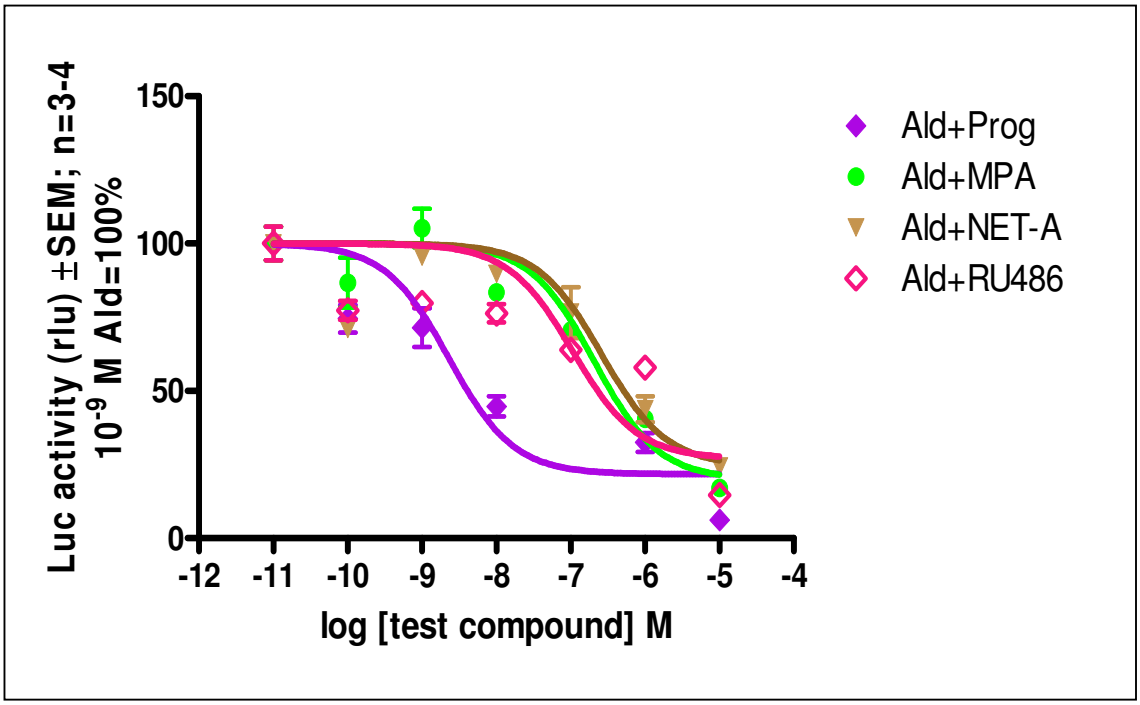


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Figure 2: (A) Unlike Ald, Prog and Dex, MPA, NET-A and RU486 do not display mineralocorticoid agonist activity. The COS-1 cell line was transiently transfected with the pTAT-GRE-E1b-luc reporter plasmid plus, pRShMR and pCMV-β-galactosidase expression vectors. Subsequently, the cells were incubated with 10 μM of either Ald (100%), Prog, MPA, NET-A, RU486 or Dex for 24 hours. Results shown are the average of at least three independent experiments with each condition performed in triplicate (±SEM). **(B) Prog, MPA, NET-A and RU486 can antagonize the agonist activity of Ald via the MR.** The COS-1 cell line was transiently transfected as above. Subsequently, the cells were incubated with 1 nM Ald alone (100%) or with increasing concentrations of Prog (♦), MPA (●), NET-A (▼) or RU486 (◊) for 24 hours. Result shown is a representative experiment of at least three independent experiments with each condition performed in triplicate (±SEM). Data from these experiments was analysed to obtain EC_{50} ± SEM values for each test compound. Statistical analysis of the EC_{50} values, indicated that Prog vs. MPA, NET-A and RU486 ($p< 0.001$); MPA vs. NET-A, RU486 ($p>0.05$); NET-A vs. RU486 ($p>0.05$).

Unlike MPA and NET-A, Prog and Dex induce the ligand-dependent interaction between the amino- and carboxyl-terminals of the mineralocorticoid receptor

Interaction between the N-terminal domain and the C-terminal domain (N/C-interaction) of liganded steroid hormone receptors has been described for other steroid receptors, and is best-described for the AR (Langley *et al.,* 1995; Doesburg *et al.,* 1997; Kemppainen *et al.,* 1999; He *et al.,* 2000). Recently, an Ald-dependent N/C-interaction was also reported for the MR using a mammalian-two-hybrid assay (Rogerson and Fuller, 2003). We thus used a similar approach to compare the capability of Prog, MPA, NET-A, RU486 and Dex to induce the N/C-interaction of the hMR. In transient transfection assays, the COS-1 cell line was co-transfected with GAL4-MRC (vector encoding amino acids 672-984 in the C-terminal region of the MR, containing the hinge region and LBD), VP16-MRNT (expression vector encoding amino acids 1-597 in the N-terminal domain of the MR) and the GAL4-responsive luciferase reporter vector pG5-luc. In figure 3 we show that the partial agonists, Prog and Dex, were able to induce the MR N/C interaction. However, Prog induced a very

weak N/C-interaction of about 5% (compared to 100% for Ald), while the observed transactivation activity was about 20% (figure 2A).

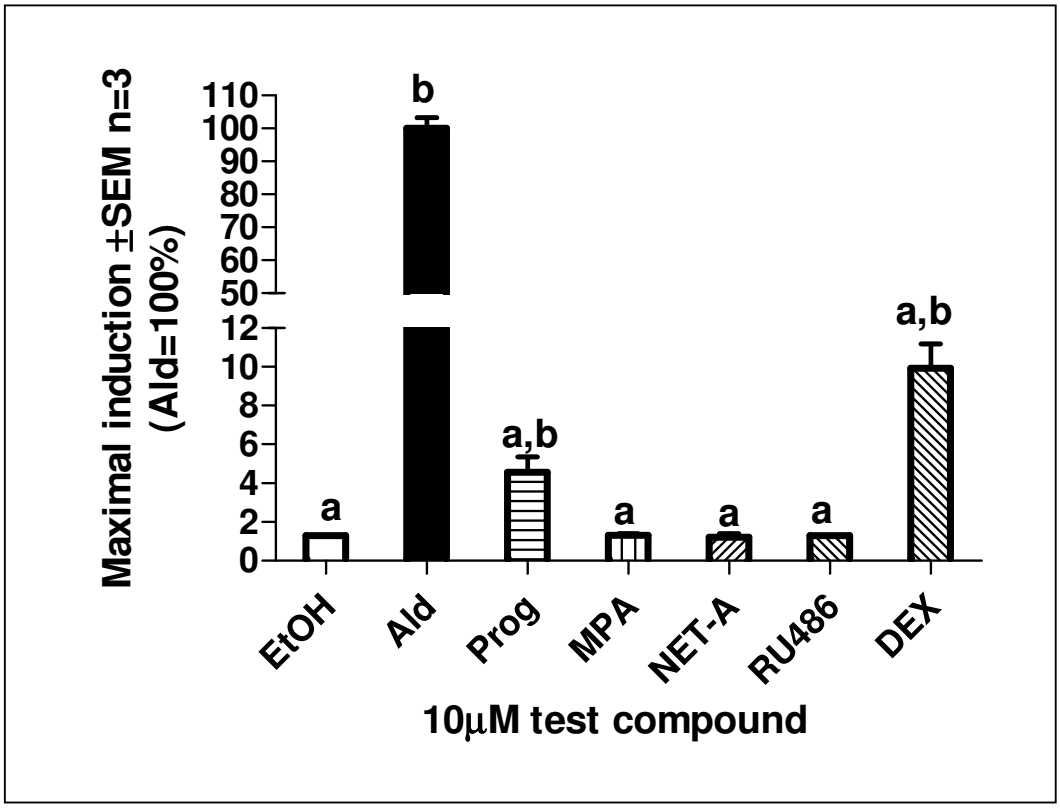


Figure 3: In contrast to Ald, Prog and Dex, MPA, NET-A and RU486 do not induce the N/C-interaction of the hMR. The COS-1 cell line was transiently transfected with the GAL4-MRC and VP16-MRNT expression vectors, as well as the GAL4-responsive luciferase reporter vector pG5-luc. The cells were exposed to 10 µM Ald (100%), Prog, MPA, NET-A, RU486 or Dex for 24 hours. Results are averages of at least three independent experiments with each condition performed in triplicate (±SEM).

Interestingly, no N/C-interaction was observed for Prog at 1 µM (data not shown), even though it had transactivation activity of 6% at this concentration (Appendix C4). In contrast, Dex could induce the MR N/C-interaction at 1 and 10 µM (data not shown; figure 3). Nevertheless, the transactivation efficacy of the group of compounds (maximal induction at 10 µM ligand) showed a good (r^2=0.9179), and significant ($p<0.01$) correlation with their efficacy for inducing the N/C-interaction

(figure 4). The effect of a given ligand depends on the fraction of receptors that are in fact occupied by the specific ligand. Thus, fractional occupancy describes the relative receptor occupancy for any ligand at equilibrium, as a function of the concentration of ligand and the K_d of the particular ligand. In Table 3, it is shown that 10 µM Ald, Prog and Dex saturates the MR, while MPA and NET-A both have a fractional occupancy of ~ 98%. In contrast, only 77% of the MR is occupied at 10 µM RU486.

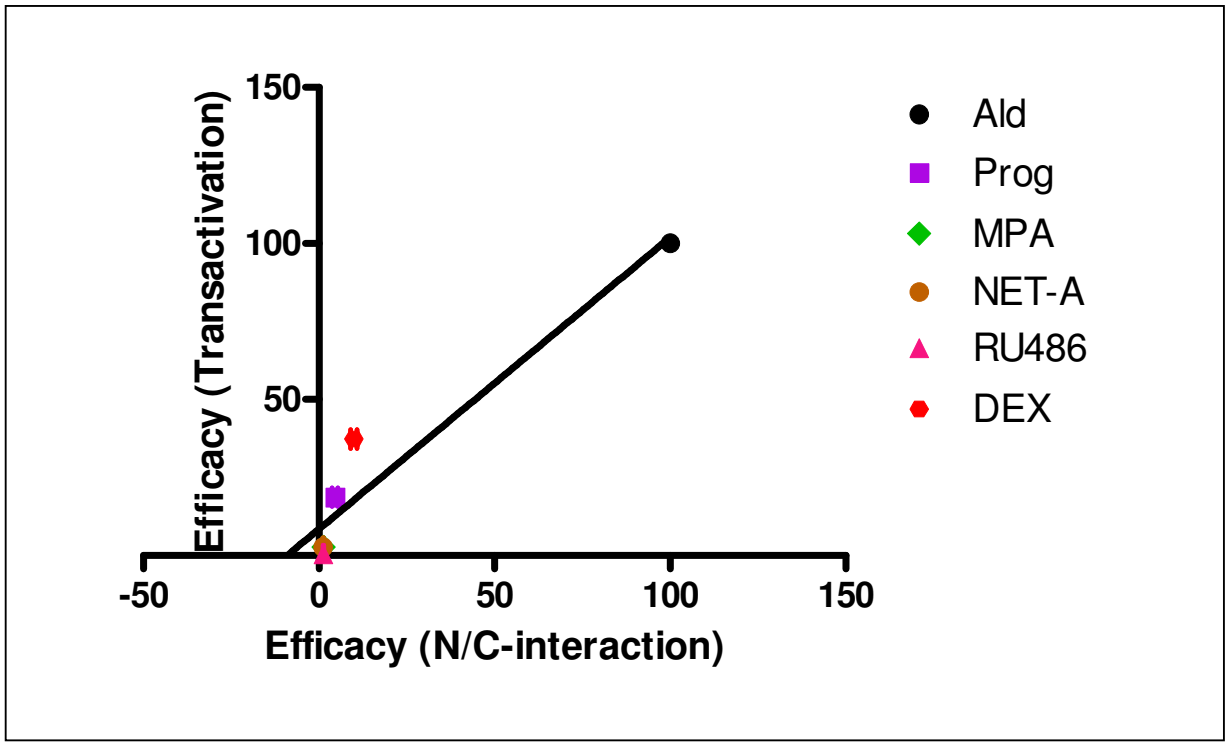


Figure 4: Correlation analysis of efficacy for transactivation versus N/C- interaction. Relative efficacies for transactivation (figure 2A) were compared to relative efficacies for N/C-interaction as determined by mammalian two hybrid assay (figure 3) using Pearson correlations. Results showed statistical significance ($p<0.01$) and $r^2 = 0.9179$. *Strictly, it is not correct to do correlation analysis on data that has only one point with a great efficacy, whilst the other points are either 0 or close to 0.

Table 3: Fractional occupancy of the MR at 1 µM and 10 µM ligand[c]

Ligand	Fractional occupancy (%) at 1 µM	Fractional occupancy (%) at 10 µM
Ald	99.98	100
Prog	99.83	99.98
MPA	83.54	98.07
NET-A	81.37	97.76
RU486	25.13	77.04
Dex	99.49	99.95

[c] The fractional occupancy was calculated according to the equation: Fractional occupancy = [ligand] / ([ligand] + Ki). K_d for Ald and K_i values for Prog, MPA, NET-A, RU486 and Dex were from Table 2.

MPA and NET-A display dissimilar mineralocorticoid properties for transrepression on an AP-1 promoter.

Having shown that both MPA and NET-A cannot transactivate a simple GRE-containing promoter via the MR, we were interested to compare their mineralocorticoid properties for transrepression. The COS-1 cell line was transiently transfected with luciferase reporter plasmids linked downstream of either five copies of a NFκB site or seven copies of an AP-1 site, both in the absence and presence of a transfected MR expression vector. Cells were stimulated with PMA and incubated for 24 hours with 1 µM of each test compound. Figure 5 shows % repression with Ald.

Surprisingly, results indicate that there is no statistically significant difference in the transrepression potential of Ald, Prog, NET-A, RU486 and Dex on the AP-1 promoter via the MR. However, the results indicate that unlike Prog and NET-A, MPA does not

transrepress the PMA-induced response on the AP-1 promoter via the MR (figure 6A).

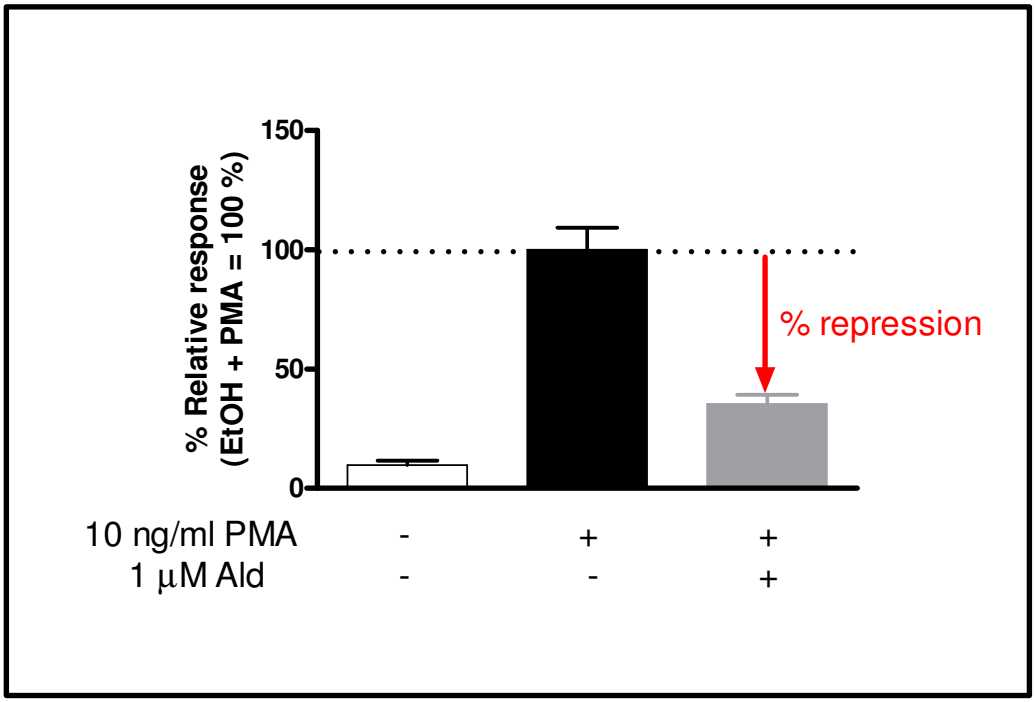


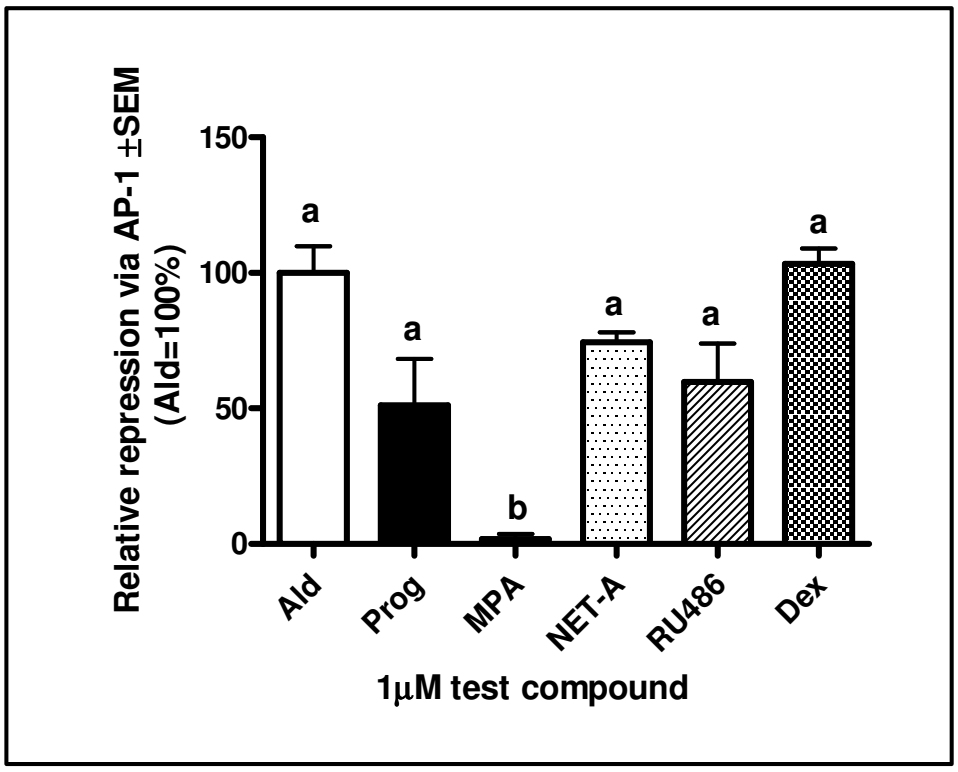
Figure 5: Transrepression activity of Ald on the AP-1 promoter in the presence of hMR. The COS-1 cell line was transiently transfected with the pRShMR, pCMV-β-galactosidase expression vectors and AP-1-containing promoter-luciferase reporter constructs. Subsequently, the cells were incubated with vehicle (EtOH), EtOH in the presence of 10 ng/ml PMA, or 10 ng/ml PMA in the presence of 1 µM Ald. β-galactosidase (β-gal) values were used to normalize for transfection efficiency. EtOH + PMA was set as 100% and Ald response calculated as a percentage of EtOH + PMA (100%). The % repression (indicated in red) was calculated by subtracting the % relative response of Ald from the % relative response of EtOH + PMA (100%). These calculations were also done for the Ald response on the AP-1 promoter in the absence of MR. Similar calculations were performed for MR-mediated responses on the NFκB-promoter in the absence and presence of transiently transfected MR.

Using the NFκB promoter-reporter construct, results showed similar but weak levels of transrepression of PMA-mediated induction with Prog, MPA, NET-A, RU486 and Dex (figure 6B). Although not statistically significantly different, MPA and RU486

appear to repress the NFκB promoter to a slightly better extent (26% and 23%, respectively) than Prog and NET-A (12% and 5%, respectively). When comparing the transrepression activity of the different ligands on the AP-1 versus the NFκB promoter-reporter, Prog, NET-A and RU486 all exhibit greater transrepression via AP-1 (51%, 74%, 60%, respectively) than via the NFκB promoter (12%, 5%, 23%, respectively). The activities of the ligands (at 1 μM) on the three synthetic promoter constructs are summarized in Table 4. Given that MPA and NET-A bind to the MR with similar affinities, and that neither is an agonist for transactivation (GRE), it is noteworthy that they act differently on the AP-1 promoter.

The transrepression/transactivation (AP-1/GRE) ratios for all the ligands were calculated, and are summarized in Table 5 in the discussion. Transrepression induced by NET-A for example was 74% of that caused by Ald (100%), while NET-A-mediated transactivation is only 1.6%. The transrepression/transactivation ratio for NET-A is thus 46.3 (74% / 1.6%). It should be noted that at 1 μM of the ligands, the fractional occupancy for Ald, Prog and Dex are ~ 100%, while for MPA and NET-A it is ~84% and 81%, respectively (Table 3). Given that the fractional occupancy of 1 μM RU486 is only 25%, the 60% percent MR-mediated repression with RU486 on the AP-1 promoter is surprising, and difficult to explain. It may indicate that the affinity of a ligand for the MR, does not necessarily determine the efficacy of the ligand for transrepression.

A AP-1



B NFκB

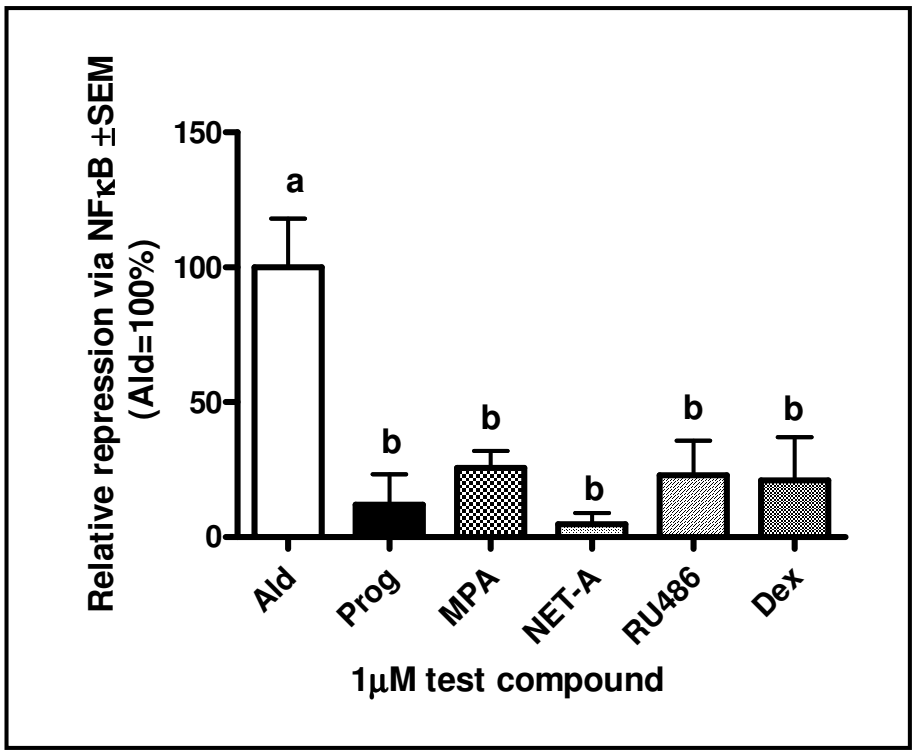


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Figure 6: Transrepression activity via the hMR. The COS-1 cell line was transiently transfected with the pRShMR, pCMV-β-galactosidase expression vectors and **(A)** NFκB- or **(B)** AP-1-containing promoter-luciferase reporter constructs. Subsequently, the cells were stimulated with 10 ng/ml PMA and incubated with 1 µM Ald, Prog, MPA, NET-A, RU486 or Dex for 24 hours. β-gal values were used to normalize for transfection efficiency. Once the repressive abilities of the test compounds were determined, in the absence and presence of transfected MR (Appendix C4), repression in the absence of MR was subtracted from the repression in the presence of transfected MR. The % repression for the other ligands was expressed as a % of Ald response which was set at 100%. The result shown is the average of three independent experiments with each condition performed in triplicate (±SEM).

Unlike MPA, Prog, NET-A and RU486 do inhibit the aldosterone-induced upregulation of the endogenous Orm-1 gene

To investigate whether the results observed on a synthetic promoter could be mimicked on an endogenous promoter, we transiently transfected a rat cardiomyocyte cell line (H9C2) with the full-length hMR or hGR expression vector. Western blot analysis confirmed the expression of the transfected MR and GR in these cells, as well as endogenous expressed MR and GR (figure 7A and 7B). To determine whether the endogenous MR and GR, as well as the overexpressed receptors are transcriptionally active, we assessed their transactivation potential in the presence of receptor agonists, using a promoter-reporter assay. The results in figure 7C-F indicate that the endogenous MR and GR, as well as the overexpressed MR, GR, PR and AR, are transcriptionally active. Consistent with the lack of transcrptional activity for endogenous PR and AR, Western blot analysis did not detect endogenous AR or PR protein levels in these cells (data not shown).

We focussed our study on the inflammation/acute phase-related gene, Orm-1, known to be associated with increased risk of cardiovascular disease (Engström *et al.,* 2003), and previously reported to be upregulated by 1 nM Ald in a H9C2 cell line stably expressing the MR, via the suggested mechanism of binding of the liganded

MR to a MRE (Fejes-Tóth and Náray-Fejes-Tóth, 2007). In these cells, the investigators showed a 1000-fold induction of the Orm-1 gene by 1 nM Ald at 24 h, whereas we observed only a 6-fold induction at this time point (figure 8A and 8B). Although not significantly different to the control, MPA caused a 4.4-fold upregulation of the Orm-1 gene, while the other ligands displayed a 2-fold upregulation (figure 8A). We also investigated the antagonist activity of the progestins on the Ald-induced expression of Orm-1, by incubating the parent H9C2 cell line transiently transfected with the MR, with 1 nM Ald in the presence of 1 μM of each progestin, as well as the known MR antagonist, spironolactone, as a positive control. Our result on a synthetic GRE showing that Prog is a potent MR antagonist (EC_{50} = 3.1 nM) was corroborated on the endogenous Orm-1 gene. Although MPA, NET-A and RU486 showed a weak but similar antagonist potency for the MR on the synthetic promoter (EC_{50} of 310, 270 and 670 nM, respectively), they acted differently on the endogenous promoter of the Orm-1 gene (figure 8B). Interestingly, NET-A and RU486 antagonized the Ald-induced upregulation of the Orm-1 gene to similar extents as Prog. In contrast, MPA did not antagonize the upregulation of the Ald-induced Orm-1 gene (figure 8B). In fact, although not significant, MPA seemed to enhance the upregulation of the Ald-induced Orm-1 gene (figure 8B).

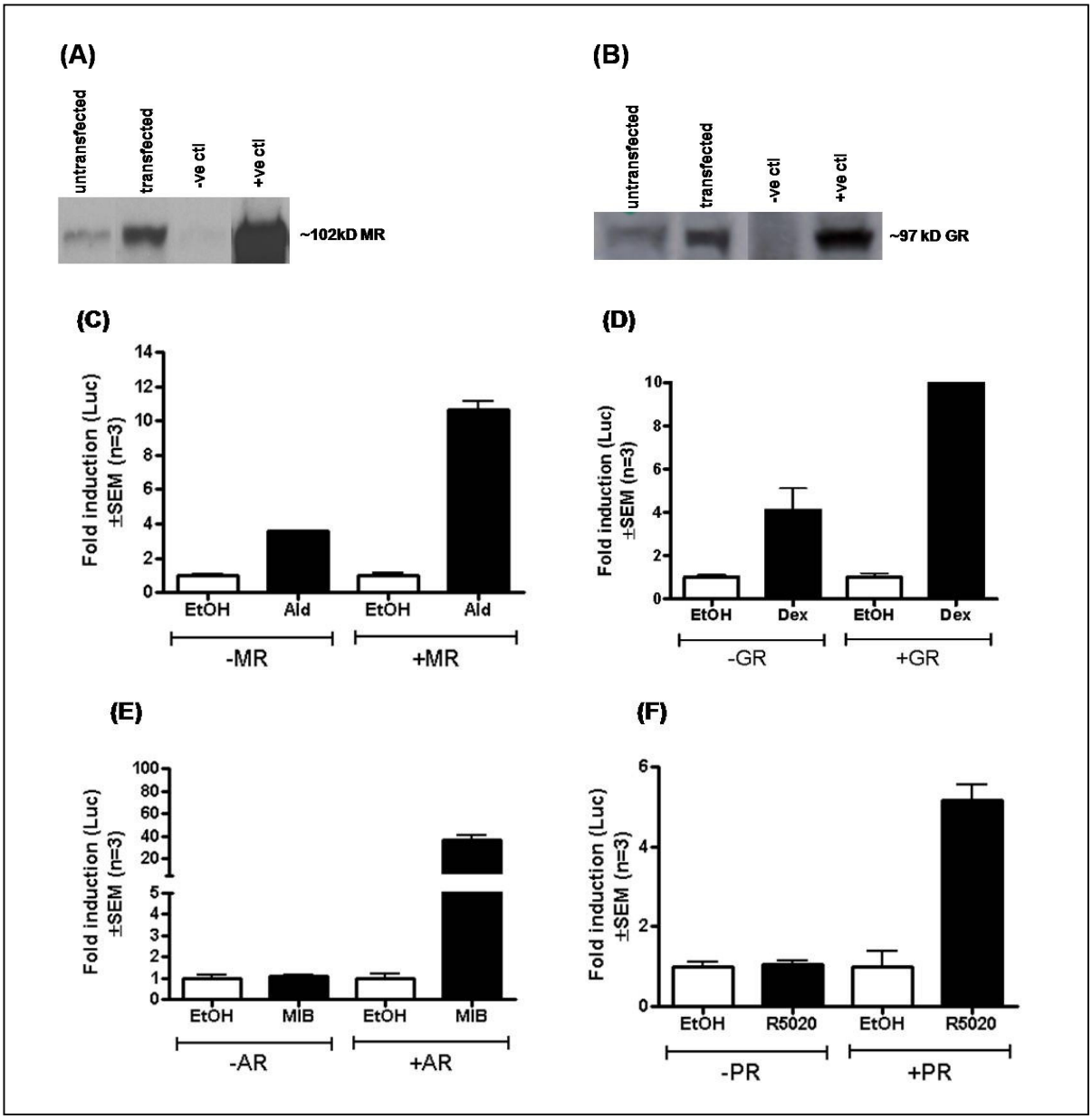
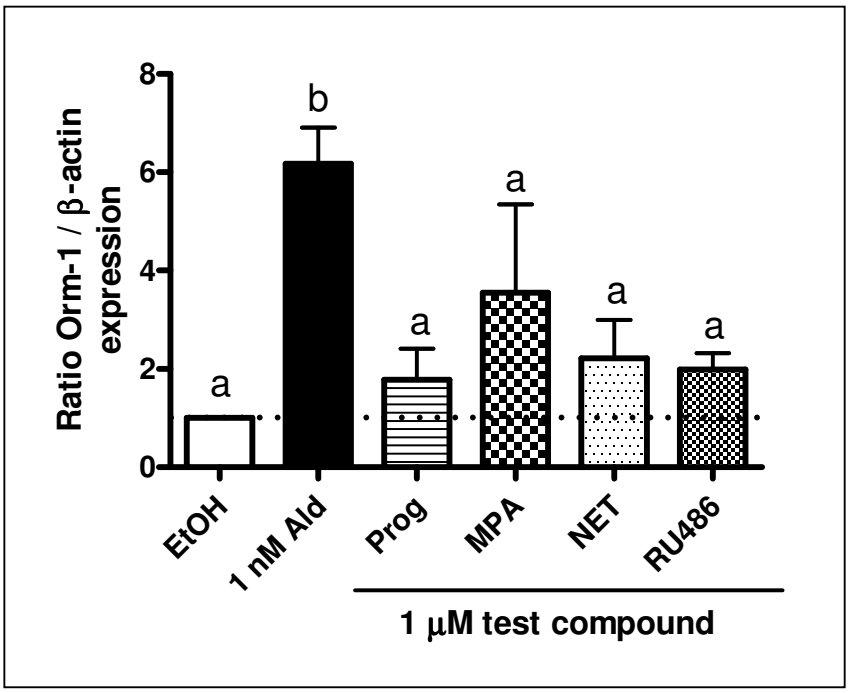


Figure 7: MR (A) and GR (B) are expressed in the rat cardiomyocyte (H9C2) cell line. Whole cell extracts were prepared from the untransfected H9C2 cell line, the H9C2 cell line transfected with pRShMR or pRS-hGRα expression vectors, untransfected COS-1 cell line (negative control (-ve ctl)), COS-1 cell line transfected with pRShMR and pRS-hGRα expression vectors (positive controls (+ ctl)). Equal amounts of protein (20 µg) were analyzed by Western blotting with the MR or GR antibodies. **(C) - (F) Transactivation of a transiently transfected GRE-containing promoter-reporter construct via MR, GR, AR, or PR.** H9C2 cells were transiently transfected with pTAT-GRE2-Elb-luc and pRS-hMR, pRS-hGRα, pSVARo, pSG5hPRB, or pGL2basic (empty vector control) as indicated, with Fugene 6 transfection reagent according to the manufacturer's instructions. Twenty-four hrs after transfection cells were induced for 24 hrs with solvent (ethanol), or 10 µM Ald (for MR), Dex (for GR), MIB (for AR) or R5020 (for PR).

A



B

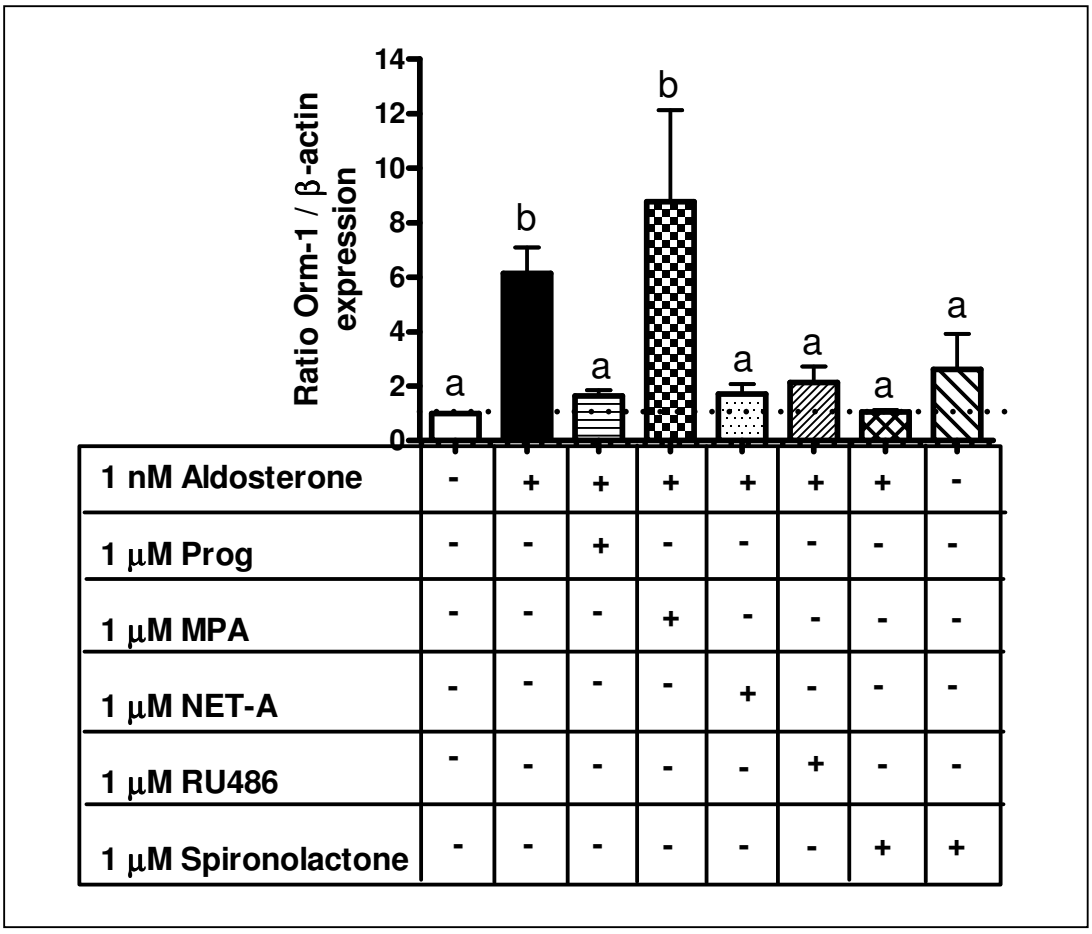


Figure 8 legend on next page.

Figure 8: (A) Agonist and (B) antagonist activity of Prog, MPA, NET-A and RU486 on the endogenous MRE-containing Orm-1 gene in the rat cardiomyocyte cell line transiently transfected with the MR. (A) Ald and MPA upregulate the Orm-gene to similar extents. Cells were incubated with 1nM Ald or 1 μM Prog, MPA, NET-A, or RU486, for 24 hours. **(B) Prog, NET-A and RU486 antagonise the aldosterone-induced upregulation of the Orm-1 gene.** Cells were incubated with 1 nM Ald in the absence and presence of 1 μM Prog, MPA, NET-A, RU486 or Spironolactone. Total RNA was extracted, cDNA synthesized and expression levels of Orm-1 and β-actin mRNA were determined by QPCR. Ratios of the Orm-1/β-actin gene were calculated using the Fit Points method described by Pfaffl (2001). Results shown are the averages of three independent experiments.

DISCUSSION

For women with an intact uterus, the inclusion of progestin in continuous combined HRT is needed to counteract the proliferative effects of estrogen on the uterine endometrium, thereby decreasing the risk of cancer associated with unopposed estrogen therapy (Gambrell Jr *et al.,* 1980; Palacios *et al.,* 2006). However, the estrogen component in HRT can also cause water and sodium-retention mediated via the RAAS (Oelkers, 1996), leading to weight gain and increased blood pressure. Since hypertension (high blood pressure) increases cardiovascular risk in both men and women (Calhoun and Oparil, 1997), there may be an association between the use of menopausal HRT and an elevated risk for cardiovascular disease. Endogenous progesterone, however, has anti-MR effects on the RAAS (Oelkers, 1996; Oelkers, 2004), thereby counterbalancing the estrogen-related water and sodium-retention and providing positive effects on body weight and also blood pressure. Thus, progestins used in HRT should ideally mimic these anti-mineralocorticoid properties.

The MR belongs to the family of steroid hormone receptors, comprising structurally similar proteins such as the GR, AR, PR and ER. In general, they are ligand-activated transcription factors that comprise four different domains namely the highly variable NH_2-terminal domain (NTD), the highly conserved and centrally located DNA-binding domain (DBD), which directly interacts with the DNA response elements, the hinge region and the COOH-terminal (C-terminal) domain. The C-terminal domain contains the ligand binding domain (LBD) to which the ligand binds. Furthermore, these receptors also possess transcriptional activation functions located in the NTD (AF1 domain) and the LBD (AF2 domain). Mineralocorticoids (or ligands binding to the MR) bind to the cytoplasmic MR in target tissues. Upon ligand binding, the receptor translocates to the nucleus where it regulates gene expression (Evans, 1988; Beato, 1989). An increase in gene transcription or positive regulation of target genes is termed transactivation, and occurs when ligand-activated MR binds to specific DNA sequences, called mineralocorticoid response elements (MREs) in the promoter regions of specific genes, resulting in gene activation (Evans, 1988; Beato, 1989). The steroid receptor family can also negatively regulate genes (transrepression), and this is mediated via the ligand-activated receptor not directly binding to DNA but via protein-protein interactions with other transcription factors such as nuclear factor-kappa B (NFκB) and activator protein-1 (AP-1) (Lu *et al.,* 2006). AP-1 can be composed of either homodimers or heterodimers between members of the Jun and Fos families. This mechanism of transrepression is well-described for the GR, but as yet the MR has not been shown to modulate AP-1- and NFκB-induced transcription by direct protein-protein interactions.

One of the aims of the present study was to evaluate the interactions with the MR of two synthetic progestins, MPA and NET-A, frequently prescribed for HRT and to provide a comparative biochemical profile of these progestins, as well as progesterone (the natural hormone), in the same model system. Although MPA and NET-A have been used clinically for more than 40 years (Westhoff, 2003), their apparent K_i values for the human MR in the same model system, have not been established. Furthermore, to our knowledge, our study is the first to investigate their ability to induce the MR N/C interaction, as well as their activity for transrepression of transcription via the MR.

The K_d for Ald binding to the hMR determined in our system (0.153 nM) is similar to previously reported K_d's of ± 0.8-3 nM (Arriza *et al.,* 1987; Lombes *et al.,* 1994; Lim-Tio *et al.,* 1997). We established K_i's for Prog, MPA and NET-A of 1.69, 197 and 229 nM, respectively (Table 1). In addition, we determined a K_i for RU486 (mifepristone), a known antagonist of the PR, GR and AR (Spitz and Bardin, 1993; Song *et al.,* 2004), of 2980 nM. RU486 has previously been reported to have no affinity for the MR (Leonhardt and Edwards, 2002). When comparing the binding affinities of MPA and NET-A as percentages relative to the native ligand Ald (100%), our values for MPA and NET-A (0.078% and 0.067%, respectively), are slightly lower than previously reported values of 3.1% and 2.7%, respectively (Philibert *et al.,* 1999; Winneker *et al.,* 2003). Furthermore, we show, as have others have (Arriza *et al.,* 1987; Rupprecht *et al.,* 1993b; Grossmann *et al.,* 2004), that the potent synthetic GR agonist, Dex, also binds the MR with relatively high affinity, although it does not activate the MR with the same potency as it does the GR (Arriza *et al.,* 1987; Rupprecht *et al.,* 1993b; reviewed in Rogerson *et al.,* 2003; Grossmann *et al.,* 2004).

In addition, we determined the agonist and antagonist properties of MPA and NET-A for transactivation via an MRE using promoter-reporter assays in the COS-1 cell line. Our results show that, while both MPA and NET-A are devoid of mineralocorticoid agonist activity via an MRE, Prog has very weak partial agonist activity at 10 µM (approximately 20% activity) (figure 2A), and only about 6% at 1 µM (Appendix C4). In contrast, a previous study reported agonist activity of Prog for transactivation via the MR as 27% at 1 µM (Quinkler *et al.,* 2002). The discrepancy between our results and that study could be due to the different promoter and cell lines used, as they used a synthetic reporter gene containing an MMTV promoter in the CV-1 cell line, while we used the pTAT-GRE-E1b-luc vector in COS-1 cells. However, the observed differences could also be attributed to differences in MR levels. It has previously been shown that receptor levels affect the potency and efficacy of full GR agonists, partial agonists and antagonists (Zhao *et al.,* 2003). Interestingly, a very recent study showed no agonist activity for up to 3 µM Prog in the COS-1 cell line co-transfected with a MR-expression vector and PRE2tk-luc promoter-reporter (Sasagawa *et al.,* 2008), which is more consistent with our results.

To further investigate agonist potential of ligands via steroid receptors, much attention has focussed on agonist-induced interactions between the N- and C termini, an interaction termed the N/C-interaction. This N/C-interaction is best characterized for the AR and involves binding of the AF2 region of the LBD with specific sequences in the N-terminal domain (He *et al.,* 2000), and has been shown to play a major role in AR transcriptional activity (Kemppainen *et al.,* 1999; He *et al.,* 1999; He *et al.,* 2000). An Ald-dependent N/C-interaction was also recently shown for the MR

(Rogerson and Fuller, 2003). Although the other physiological ligand of the MR, cortisol (Cort), has a higher potency than Ald in transactivation assays (Rogerson *et al.,* 1999), a MR N/C-interaction was only weakly induced in its presence (Rogerson and Fuller, 2003). The authors hypothesize that their results showing that Ald, but not Cort, can induce the MR N/C-interaction, may explain why Ald and Cort have differential effects in some non-epithelial cells. However, that study only compared Ald and Cort up to 100 nM. At a concentration of 10 µM Ald and Cort, we find similar induction of the N/C-interaction by both ligands (supplementary material in Appendix C5). In addition, Prog, a very weak partial agonist for the MR in our system, could weakly induce the N/C-interaction at a concentration of 10 µM (figure 3), but not 1 µM (data not shown). Similarly to Rogerson and Fuller (2003) who showed no N/C-interaction for the MR antagonist spironolactone, our results indicate that the antagonists in our system, that do not display partial agonist activity, such as MPA, NET-A and RU486, could not promote an N/C-interaction (figure 3). We show that efficacy for transactivation via a synthetic GRE correlates with the efficacy for the induction of N/C-interaction (r^2 = 0.9179; $p<0.01$) (figure 4), indicating that there is a good relationship between transactivation and MR N/C-interaction.

We determined considerable anti-mineralocorticoid activity of Prog (EC_{50} = 3.1 nM), which is in agreement with previously reported EC_{50} values in the range 2-10 nM (Rupprecht *et al.,* 1993; Auzou *et al.,* 2000; Geller *et al.,* 2000), but lower than a recent study where the EC_{50} for Prog was determined as 11.4-18.1 nM (Sasagawa *et al.,* 2008). The molecular basis of Prog MR antagonistic activity still needs to be researched, but it is speculated that it may be due to the destabilization of helix 12 of the MR binding domain (Elger *et al.,* 2003). Surprisingly, although it has previously

been reported that MPA, NET-A and RU486 have no MR antagonist activity (Winneker *et al.,* 2003 and references therein, reviewed by Rowlands 2003, Palacios *et al.,* 2006; Zhang *et al.,* 2006), we showed MR antagonist activity of these ligands, albeit to a lesser extent than for Prog (figure 2B). We determined potencies (EC_{50} values) of 310, 270 and 670 nM for MPA, NET-A and RU486, respectively. Interestingly, in accordance with our results, a recent study also showed MR antagonist activity for MPA and NET-A, but reported much lower potencies (1108.6 and 1100.9 nM for MPA and NET-A, respectively) (Sasagawa *et al.,* 2008). The EC_{50} values obtained in our study are higher than the peak serum level reached with HRT (MPA = 0.2 nM and NET-A = 17.7 nM) (Ghatge et al., 2005; Activelle package insert reg. no. 33/21.8.2/0532, Novo Nordisk Inc.) or contraception (MPA = 3.9 nM; NET-A = 59 nM) (Mishell, 1996; Fotherby *et al.,* 1983), but the possibility that higher concentrations may be present *in vivo* in target tissues compared with concentrations that are usually measured in the blood, should not be excluded.

In general, not much is known about transrepression mediated via the MR. Early work by Pearce and Yamamoto (1993) showed that the MR could not repress AP-1 activity in response to Ald. In contrast to that study, we have shown that Ald can repress AP-1 activity via the MR. In agreement with the study by Liden *et al.* (1997) suggesting that the MR can repress transactivation of NFκB, we also show that Ald can repress NFκB activity via the MR. We investigated the comparative effects of all the ligands on transrepression via the MR on both NFκB and AP-1 promoter constructs. The results of the transrepression assays (at 1 μM) (figure 6A and 6B) as well as the effects observed for transactivation (figure 2A), are summarized in Table

4. Figure 6B indicates that none of the ligands, except Ald, could significantly transrepress the NFκB site via the MR.

Table 4: Relative responses for transactivation and transrepression via the MR at 1 µM ligand[c]

Ligand	GRE Relative response (%)	NFκB Relative response (%)	AP-1 Relative response (%)
Ald	100	100	100
Prog	19 ± 0.7	12 ± 11.3	51 ± 17.1
MPA	2 ± 0.7	26 ± 6.3	1.8 ± 1.8
NET-A	1.6 ± 0.8	5 ± 4.0	74 ± 3.7
RU486	1.3 ± 1.0	23 ± 12.8	60 ± 14.2
Dex	27 ± 0.2	21 ± 16.0	103 ± 5.9

[c] Relative responses for transactivation via pTAT-GRE-E1b-luc and for transrepression via either NFκB or AP-1-luc reporter plasmids at 1 µM ligand, were determined in the transiently transfected COS-1 cell line. Results are averages of at least three independent experiments, each performed in triplicate. Ald activity was taken as 100% and all other values are expressed as a % thereof.

One of the most important and novel findings of our study, is that both NET-A and RU486 can transrepress the PMA-induced response on the AP-1 promoter via the MR, without exhibiting agonist activity for MR transactivation via a GRE. MPA, on the other hand, cannot transrepress the PMA-induced response on the AP-1 promoter via the MR, or induce agonist activity for MR transactivation via a GRE. Ligands that can transrepress, but not transactivate, are referred to as dissociated ligands, and are well described for the GR (Vaysierre *et al*., 1997; De Bosscher *et al.,* 2005). The transrepression by the ligands in this study is consistent with a MR-dependent

mechanism as the repression without transfected MR, was subtracted from the repression with transfected MR. However, elucidation of the mechanism behind this dissociative activity will require additional studies. Interestingly, Vaysierre *et al*. (1997) reported a similar ability for synthetic glucocorticoids that could differentiate between transactivation and AP-1 transrepression via the GR. Similarly, we show that while Dex is a partial agonist for MR transactivation on a GRE promoter, it is a full agonist for transrepression on the AP-1 promoter via the MR. An AP-1 transrepression/transactivation ratio was calculated for all the ligands (Table 5). NET-A and RU486 had an AP-1 transrepression/transactivation ratio of 46.3 and 46.2, respectively compared to 1.0 for Ald, indicating that NET-A and RU486 did not transactivate a GRE via the MR, but was able to repress AP-1 transactivation via the MR. As the fractional occupancy of the MR at 1 μM is only 25%, it is intriguing that RU486 could repress the AP-1 activity by 60%. This result may indicate that the affinity of a ligand for the MR, does not necessarily determine the efficacy of the ligand for transrepression, as has been shown for GR ligands via both NFκB and AP-1 (Ronacher *et al.,* 2009). In contrast, MPA could not transactivate a GRE or repress AP-1 activity via the MR. The transrepression/transactivation ratio for MPA was thus 0.9 indicating that MPA does not distinguish between transactivation and transrepression via the MR. In fact, MPA does not display any mineralocorticoid agonist properties. This ratio for Prog and Dex was 2.7 and 3.8, respectively, indicating that these ligands are better at transrepression than transactivation. MR actions via these transcription factors, NFκB and AP-1, may play an important role in inflammation, typically associated with MR-mediated cardiovascular injury.

It has been reported that MR antagonism protects the cardiovascular system from damaging inflammation (Fejes-Tóth and Náray-Fejes-Tóth, 2007). For this reason, we next determined whether MPA, NET-A and RU486 could antagonize Ald-induced effects on a risk factor of cardiovascular disease. Using a rat cardiomyocyte cell line transiently transfected with the MR as a model for cardiovascular disease, we investigated the antagonistic properties MPA, NET-A and RU486, on the expression of the endogenous Ald-responsive gene, Orm-1. This gene is involved in inflammation, and elevated levels are considered a cardiovascular risk factor (Engström *et al.,* 2003). Interestingly, patients with metabolic syndrome have elevated plasma Orm-1 levels, which may account for the increased prevalence of cardiovascular problems in this syndrome. Although MPA, NET-A and RU486 acted as weak antagonists of the MR in a GRE-reporter assay, we showed that NET-A and RU486, but not MPA, inhibit the effect of Ald on the expression of the endogenous MRE-containing gene, Orm-1, to a similar extent as Prog. MPA increased the expression of Orm-1 (figure 8A and 8B). However, the H9C2 cell line also expresses the GR (figure 7B) and it is known that glucocorticoids can induce Orm-1 (Vannice *et al.,* 1984). Taking this into account, together with the fact that we show no MR agonist properties for MPA in a GRE-reporter assay, it is probable that MPA, known to have glucocorticoid agonist properties at a concentration of 1 µM (Koubovec *et al.,* 2004; Koubovec *et al.,* 2005; Ronacher *et al.,* 2009), would preferentially act via the GR, and it is thus likely that the agonist activity of MPA on the endogenous Orm-1 gene is mediated by the GR rather than the MR.

Table 5: Transrepression/transactivation ratios (AP-1/GRE) via the MR at 1 µM ligand[d]

Ligand	GRE Relative response) (%)	AP-1 Relative response (%)	Ratio
Ald	100	100	1.0
Prog	19 ± 0.7	51 ± 17.1	2.7
MPA	2 ± 0.7	1.8 ± 1.8	0.9
NET-A	1.6 ± 0.8	74 ± 3.7	46.3
RU486	1.3 ± 1.0	60 ± 14.2	46.2
Dex	27 ± 0.2	103 ± 5.9	3.8

[d] Relative responses for transactivation via pTAT-GRE-E1b-luc and for transrepression via AP-1-luc reporter plasmids at 1 µM ligand, were determined in the transiently transfected COS-1 cell line. Results are averages of at least three independent experiments, each performed in triplicate. Ald activity was taken as 100% and other values are expressed as a % thereof. Ratios were calculated as % relative response on a GRE / % relative response on AP-1. This ratio is 1.0 for the standard mineralocorticoid Ald (100% transrepression / 100% transactivation = 1.0). A ratio of > 1 indicates that transrepression is preferred, while a ratio of < 1 indicates that transactivation is preferred.

In summary, our results indicate that on an endogenous gene, considered to be a cardiovascular disease risk factor, MPA and NET-A act differently, which has implications for women using these progestins in HRT. As NET-A acts similarly to Prog, it may be the preferable progestin for women at risk of cardiovascular disease. Further investigation into the anti-mineralocorticoid properties of MPA, NET-A and RU486 is required, especially *in vivo* in animal experiments. In the meantime, however, it is important that physicians understand that progestins differ widely in their chemical structure, pharmacokinetics, and biological activity, and take cognizance of this when prescribing the optimum type and dose of progestin for HRT.

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Chapter 5

CONCLUSIONS AND FUTURE PERSPECTIVES

5. CONCLUDING DISCUSSION

Although MPA and NET-A are widely used in female reproductive therapy, not much is known about their mechanism of action at the molecular level. Clinical evidence indicating that the use of MPA and NET-A in HRT increases the risk of breast cancer (Rossouw *et al.,* 2002; Beral, 2003), and also that MPA increases the risk of CVD, has highlighted the importance of investigating these mechanisms. In addition to these studies implicating MPA in effects on cardiovascular function and breast cancer in HRT users, use of MPA as a contraceptive has also been implicated in effects on local immune function (Ildgruben *et al.,* 2003). Little is known about the effects of MPA and NET-A, as compared to Prog, in the cervicovaginal environment, and whether they interfere with local epithelial immune function. In the first part of this thesis (Chapter 2), we thus investigated the regulation of cytokine genes by MPA and NET-A, as compared to the endogenous hormone Prog, in Ect1/E6E7 and Vk2/E6E7 cell lines with a view to understanding the possible steroid receptor mediated effects of these synthetic progestins on immunity in the female genital tract. In addition, although it is known that both MPA and NET-A can bind the PR, GR, AR and MR, no thorough comparison exists on the binding affinity of MPA and NET-A, as compared to Prog, towards the AR and MR. Moreover, an accurate pharmacological characterisation of the relative efficacy and potency of the two progestins on gene regulation via these receptors is lacking. In the second and third part of the experimental work in this thesis (Chapter 3 and 4), we thus examined the mechanism of action of MPA and NET-A, relative to Prog, at the molecular level via overexpressed AR and MR in the COS-1 cell line. Additionally, we investigated the MR-mediated transcriptional regulation of the endogenous Orm-1 gene, a marker of

CVD, in a rat cardiomyocyte cell line, with a particular focus on the antagonistic role of MPA and NET-A, as compared to Prog.

5.1 Comparing the regulation of endogenous pro-inflammatory cytokine genes, by MPA and NET-A, in the female reproductive tract

In Chapter 2, specific genital tract cell types for the induction or upregulation of protective molecules were investigated. As a model for cervicovaginal mucosal immunity, we used epithelial cell lines derived from the female ectocervical and vaginal mucosa, and showed that ligand-specific and cell-specific regulation of the pro-inflammatory cytokine (IL-6) and chemokines (IL-8 and RANTES) gene expression occurs by Prog, MPA and NET-A. Chemokines initiate the immune response by controlling leukocyte migration and lymphocyte development. All three cytokines have been shown to be expressed and secreted by Ect/E6E7 and Vk2/E6E7 cell lines in response to activation by TNFα (Fichorova *et al.,* 1999). Many inflammatory response genes containing NFκB response elements, like the IL-6, IL-8 and RANTES genes, are upregulated in response to proinflammatory cues initiated by cytokines like TNFα. IL-8 and RANTES are involved in the early inflammatory response, by recruiting specific leukocytes to sites of ongoing inflammation, while IL-6 is responsible for neutrophil priming to chemotactic factors (Kunkel *et al.,* 1997). These specific cytokines were chosen because they have previously been shown to be repressed by MPA in other cell types. For example, MPA has previously been shown to repress IL-6 and IL-8 promoter–reporter constructs at the transcriptional level (Koubovec *et al.,* 2004), as well as the RANTES gene in endometrial cells (Zhao *et al.,* 2002). Moreover, IL-6, IL-8 and RANTES have been reported to have distinct roles in HIV-1 pathogenesis (Fichorova *et al.,* 2004).

Results showed that unlike Prog which upregulated TNFα-stimulated IL-6 gene expression in both the ectocervical and vaginal cell lines, MPA and NET-A had no effect. This is inconsistent with previous studies that have shown the repression of IL-6 by MPA in other cell types (Mantovani *et al.,* 1997; Koubovec *et al.,* 2004; Wessel Kriek, MSc thesis). Similarly, NET-A has previously been shown to either upregulate (Zitzmann *et al.,* 2005) or downregulate (Wessel Kriek, MSc thesis) IL-6. In contrast, NET-A in combination with estrogen, like estrogen only, had no effect on IL-6 levels in healthy postmenopausal women (Žegura *et al.,* 2006).

In contrast to the observed effects on the IL-6 gene, we show that MPA and NET-A upregulate the TNFα-induced IL-8 gene expression to different extents in both cell types, indicating pro-inflammatory activity of these progestins which is similar to that of Prog. Similarly, Prog and MPA have previously been shown to upregulate IL-8 mRNA levels in human endometrial stromal cells (Arici *et al.,* 1996). As IL-8 is a chemotactic factor for neutrophils and lymphocytes, the local concentration of IL-8 is important in determining whether either neutrophils or lymphocytes are primarily recruited. Prog has previously been shown to induce a neutrophil response in the vagina of germ-free mice, even in the absence of bacterial infection (Beaver, 1960), indicating a protective role for Prog in the vagina. Thus, our results demonstrating upregulation of IL-8 by natural Prog, MPA and NET-A, may indicate that in these cell lines, the synthetic progestins are similar to Prog, and that this may provide an enhanced local defense mechanism against infections in the vaginal mucosa.

Interestingly, results indicated differential regulation of the TNFα-induced RANTES gene by MPA, NET-A and Prog in the Ect1/E6E7 cells, but similar regulation in the

Vk2/E6E7 cell line. As RANTES is a pro-inflammatory chemokine, the suppression of the RANTES gene by MPA indicates that MPA is anti-inflammatory. In epithelial cells, RANTES is responsible for recruiting immune cells into the reproductive tract (Kunkel *et al.,* 1997) and thus inhibition of RANTES would suppress inflammation, thereby blocking infiltration of immune cells. As the transmission of viruses such as HIV, HSV and HPV occurs at the cervicovaginal environment (Tjiong *et al.,* 2001), a lack of recruitment of immune cells into the reproductive tract may have negative implications for women using MPA, as they would not be able to effectively combat infections in the ectocervical environment. Consistent with this result, injectable hormonal contraceptive usage has previously been associated with increased risk of cervical cancer due to HPV (Castle *et al.,* 2005). In contrast, a South African study investigating the link between use of MPA and NET-A as progestin-only injectable contraceptives, and the risk for cervical cancer due to HPV, indicated that these progestins did not contribute to the risk of cervical cancer (Shapiro *et al.,* 2003).

Downregulation of RANTES gene expression by MPA has previously been reported in endometrial stromal cells (Zhao *et al.,* 2002), and the PR was shown to mediate the effect. In addition, MPA has previously been shown to repress the pro-inflammatory chemokine, IL-8, via the GR (Koubovec *et al.,* 2004). We thus investigated which steroid receptor is mediating the effects of MPA on RANTES in the Ect1/E6E7 cell line, by using receptor-specific antagonists. Our results using RU486, the antagonist for the PR and GR, was inconclusive as RU486 behaved as an agonist in this cell line. We were unable to exclude the involvement of the GR and PR, particularly the PR-A isoform, which is a weak activator of transactivation, but a strong activator of transrepression (Kalkhoven *et al.,* 1996). However, when using the

AR-specific antagonist, hydroxyflutamide, we showed that the repression of the RANTES gene by MPA is mediated by the AR. Consistent with this result, the androgenic properties of MPA have previously been proposed to be responsible for the anti-inflammatory effects of MPA on plasma inflammatory markers, such as C-reactive protein and serum amyloid A protein (Wakatsuki *et al.,* 2002).

Our study is the first to show ligand-specific and cell-specific effects on cytokine and chemokine genes in the cervicovaginal environment. In addition, we show that the observed repression of the TNFα-induced RANTES gene by MPA in the Ect1/E6E7 cell line is mediated by the AR. Considering that NET-A was shown to have similar androgenic properties to MPA for transactivation and transrepression of synthetic promoters (see Chapter 3), it was surprising that NET-A did not act via the AR on the endogenous RANTES promoter, that contains NFκB-binding sites, in the Ect1/E6E7 cell line. This difference indicates that the repression of the endogenous RANTES gene is more complex than repression on a synthetic NFκB-promoter, and possibly involves more factors than just NFκB tethering. It is thus possible that there are other regulators or elements determining the response of NET-A on the endogenous gene, which are not present in the promoter-reporter construct used. Furthermore, as it is known that NET-A can be metabolized (Stanczyk and Roy 1990), the possibility thus exists that NET-A is metabolized in this cell line, and that the metabolite(s), are less efficacious AR agonists than NET-A itself. This could be investigated in future, by transfecting an ARE-luc or NFκB-luc into the Ect1/E6E7 cells, and determining whether NET-A can transactivate or transrepress via the AR.

In addition, the repression of RANTES by MPA via the AR was not observed in the Vk2/E6E7 cell line, indicating that MPA acts in a cell-specific manner. As the levels of AR in the Ect1/E6E7 and Vk2/E6E7 cell lines are similar, it is unlikely that these differences in responses are due to different levels of steroid receptors. It may be due to other factors such as different extent of metabolism or even different levels of coregulators. Using whole cell binding experiments, we showed that the amount of GR and AR in the Ect1/E6E7 cells line are similar to those in the Vk2/E6E7 cell line. The expression of AR in the human vagina and cervix has previously been reported (Ruizeveld de Winter *et al.,* 1991), and it is known that the GR is ubiquitously expressed (reviewed in Gross and Cidlowski, 2008). A further complexity is the affinity of MPA and NET-A for the different steroid receptors, in particular the GR versus the AR, as there appear to be similar levels of these receptors in the Ect1/E6E7 cell line. MPA has a much higher affinity for the GR than NET-A (10.8 vs. 270 nM, respectively) (Koubovec *et al.,* 2005), while both MPA and NET-A have similar affinities for the AR (19.4 and 27.9 nM, respectively). Thus, in a cell where the AR and GR are expressed equally, it is assumed that MPA can act via either the GR or AR as its affinity for these receptors is similar. However, one would then assume that NET-A would act via the AR, which is not the case in this study.

Nevertheless, this study is the first to show that MPA suppresses the RANTES gene via the AR in a human ectocervical cell line. Further work is necessary to assess the impact of this novel finding. Moreover, the finding that MPA acts differently to NET-A, and does not mimic the effects of Prog, has important implications for women's health.

Finally, the immunoregulatory milieu of the genital mucosa is important in determining the response to antigenic challenge. Mucosal innate immune factors as well as proinflammatory cytokines are associated with elevated levels of HIV-1 shedding in the female genital tract (Gumbi *et al.,* 2008). During male-to-female transmission, it is thought that virus in the semen enters the stratified squamous epithelium of the vagina or the columnar epithelium of the endocervix to infect cells within or below the epithelium (reviewed in Pope and Haase, 2003). It is not clear which cells are the first to interact with, and to be infected with the virus, whether environmental factors in the reproductive tract tissues may influence the infection, or whether there are local immune-system responses directed at the virus. Thus, a greater understanding of the immune responses at these different anatomical locations in the genital tract, and how MPA and NET used as contraceptives and in HRT regulate genes relevant to the cervicovaginal mucosal immune response, may have implications for the development of efficacious topical microbicide products for the prevention of sexually transmitted HIV-1 infection, or for the development of HIV-1 vaccines. This may thus also have important implications for women's risk of susceptibility to infections. In addition, novel avenues may be identified both to protect against potential pathogens and to improve the quality of women's reproductive health.

5. 2. Comparing the androgenic properties of Prog, MPA and NET-A in the COS-1 cell line

In the second part of this thesis (Chapter 3), for the first time, equilibrium binding studies in the COS-1 cell line enabled the calculation of precise K_i values of Prog, MPA, NET-A and RU486 for the AR. RU486 was included as it is a synthetic progestin previously reported to bind the AR (Song *et al.,* 2004). The COS-1 cell line

does not contain significant levels of endogenous steroid receptors and is thus an appropriate model cell line for comparing the activities of ligands via a specific transiently transfected receptor. Although saturation binding experiments are the conventional way in which K_d's are determined, we determined K_d and K_i values by using the alternate method of homologous/heterologous competitive binding experiments, using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). We optimized the concentration of ^{3}H-[MIB] (two to ten times lower than the EC_{50}) to use, and found 0.2 nM to be optimal. The time for 0.2 nM ^{3}H-[MIB] to reach binding equilibrium was determined as 16 hours.

The results showed that Prog, MPA, NET-A and RU486 were able to compete with MIB for binding to the AR. Prog, MPA and NET-A displayed similar binding affinity to each other (K_i = 36.6 nM, 19.4 nM and 21.9 nM respectively), and the natural androgen DHT (K_i = 29.4 nM). MPA and NET have previously been shown to bind to the endogenously expressed AR in the MCF-7 breast cancer cell line with a similar relative affinity to each other, albeit with a much lower relative binding affinity than that found in our study (Bergink *et al.,* 1983 and references therein). In addition, our results for Prog, MPA and DHT are consistent with a previous study in the COS-1 cell line (Kemppainen *et al.,* 1999). Previous studies have reported that RU486 has low binding affinity for the AR in rat prostate cytosols (Philibert *et al.,* 1999; Cabeza *et al.,* 2007). Interestingly, the study by Cabeza and co-workers (2007) shows a 23-fold lower relative binding affinity of RU486 compared to MIB. Indeed, in our study when compared to MIB, RU486 has a 12-fold lower affinity, which is in the same order of magnitude as the results by Cabeza *et al.* (2007). However, when compared to the

natural androgen DHT, RU486 (synthetic progestin) has a 6-fold higher binding affinity for the hAR.

We demonstrated that unlike Prog, MPA and NET-A are potent and efficacious AR agonists, with activity comparable to that of DHT. Furthermore, consistent with other studies, we show that Prog (Kemppainen *et al.,* 1999; Sasagawa *et al.,* 2008) and RU486 are potent antagonists (Song *et al.,* 2004; Cabeza *et al.,* 2007) with minimal agonist activity. We show that in contrast to DHT and NET-A, MPA does not induce the N/C-interaction of the AR on the classical ARE, which also serves as a response element for the GR, PR and MR (reviewed in Beato, 1989). In addition, we show that MPA can induce the N/C-interaction on two selective AREs, suggesting a promoter-dependent induction of the AR N/C interaction by MPA. Indeed, it has been shown that the AR N/C interaction is important for AR activation of some androgen-regulated promoters, but not others (He *et al.,* 2002). However, we cannot exclude the possibility that the reason for these quantitative differences in the ability of MPA to induce the N/C interaction via the promoter-reporter-constructs used in this study is due to different expression levels of the promter-reporter constructs or the AR. Taken together, these results suggest that MPA and NET-A activate the AR by different mechanisms on the classical ARE, but by similar mechanisms on selective AREs.

It is well-known that the anti-inflammatory effects of glucocorticoids are mediated by the GR interacting with transcription factors, such as NFκB and AP-1, thereby repressing the activity of the transcription factor and the target genes (De Bosscher *et al.,* 2003). Considering that the AR and GR belong to the same superfamily and

are closely related, the question arose whether the AR could transrepress NFκB and AP-1 containing genes in a similar manner. Indeed all the AR agonists used in this study could transrepress NFκB and AP-1 synthetic promoter-reporter constructs via the AR. In addition, a comparison of the effects of all the ligands on transactivation on the classical ARE versus transrepression on both NFκB and AP-1 synthetic promoter-reporter constructs, showed similar activity of the ligands on all three promoters. Interestingly, the RANTES gene promoter has four potential NFκB response elements (Lebovic *et al.,* 2001), thus the possibility exists that the AR-mediated repression of the RANTES gene by MPA (Chapter 2) occurs via NFκB.

In summary, our results indicate that the synthetic progestins, MPA and NET-A, but not natural Prog, display binding affinities and biological activity comparable to the native androgenic ligand, DHT, via the AR. It has been proposed that the androgenic effects of MPA may reverse the protective effects of endogenous androgen signaling in the breast (Birrell *et al.,* 2007). Our results showing similar potent androgenic activity of MPA and NET-A suggest that NET-A would do the same. Indeed, both MPA and NET were shown to substantially increase the risk of breast cancer in long-term HRT in the 'Million Women Study' (Beral, 2003).

5. 3. Comparing the anti-mineralocorticoid properties of Prog, MPA and NET-A in the COS-1 and cardiomyocyte cell lines

In the third part of this thesis (Chapter 4), we used the same model system (COS-1 cell line) to evaluate MR activity induced by MPA and NET-A, relative to Prog. The results from our study are the first to determine accurate apparent K_i values for MPA, NET-A and RU486 towards the human MR in COS-1. As in Chapter 3, we first

optimized the appropriate concentration of ^{3}H-[Ald] to use and the time needed to attain binding equilibrium. The K_i's values established for Prog, MPA and NET-A indicate that MPA and NET-A bind to the MR with similar affinity, but with a significantly lower affinity than Prog (1288-fold, 1497-fold and 11-fold compared to Ald). In addition, the binding affinity of RU486 was 19 477-fold lower than Ald. Previous reports on whether RU486 binds the MR or not are contradictory, as it has been reported to have no affinity for the MR (Leonhardt and Edwards, 2002), while on the Nuclear Receptor Signaling Atlas webpage it is classified as an MR antagonist, the K_i of which still needs to be curated (www.nursa.org/10.1621/datasets.02001). In addition, we show that the synthetic GR agonist Dex also binds the MR with relatively high affinity (7-fold less compared to Ald). Although this is not a novel result (Arriza *et al.,* 1987; Rupprecht *et al.,* 1993b; Grossmann *et al.,* 2004), it is one that is under-appreciated in the current literature, which refers to Dex as a GR-specific ligand. In fact, in rabbit reticulocyte lysates, Dex has previously been shown to have a similar relative binding affinity as Ald (Hellal-Levy *et al.,* 1999). Thus, experiments performed in cell lines expressing both endogenous GR and MR, should not exclude a role for the MR on the basis that Dex is GR-specific.

Our results show that, while both MPA and NET-A are devoid of mineralocorticoid agonist activity via an MRE, Prog has very weak partial agonist activity at 10 µM. Other *in vitro* studies have demonstrated agonist activity of 1 µM Prog for transactivation via the MR (Quinkler *et al.,* 2002), while one recent study showed no agonist activity up to 3 µM (Sasagawa *et al.,* 2008). Consistent with previous reports

(Hellal-Levy et al. 1999; Grossmann *et al.,* 2004), Dex showed partial agonist activity for transactivation via the hMR.

As investigated and discussed in Chapter 3, studies have shown that the ligand-induced N/C-interaction of the AR plays a major role in AR transcriptional activity (Kemppainen *et al.,* 1999; He *et al.,* 1999; He *et al.,* 2000). Similarly, an Ald-dependent N/C-interaction has been shown for the MR (Rogerson and Fuller, 2003). When investigating the ability of the ligands used in this study to induce the MR N/C interaction, we show that both of the partial agonists for transactivation, Prog and Dex, could marginally induce the N/C-interaction at 10 μM. Consistent with previous results for the MR antagonist spironolactone (Rogerson and Fuller, 2003), we found no N/C-interaction for MPA, NET-A and RU486, the antagonists in our system. Our findings show that efficacy for transactivation via a synthetic GRE correlates with the efficacy for the induction of N/C-interaction on the same GRE, indicating that the MR N/C-interaction is important for MR transcriptional activity (r^2=0.9179; $p<0.01$). When investigating the MR antagonist activity of the ligands, we show that MPA, NET-A and RU486 are weak antagonists (EC_{50} values of 310 nM, 270 nM, and 670 nM, respectively) relative to the reported values for the well-known MR antagonist spironolactone (EC50 of 14 nM) (reviewed in Lu *et al.,* 2006). This is in contrast to previous reports showing no MR antagonist activity (Winneker *et al.,* 2003 and references therein, reviewed by Rowlands 2003, Palacios *et al.,* 2006; Zhang *et al.,* 2006).

MR antagonism has been reprted to protect the cardiovascular system from damaging inflammation (Fejes-Tóth and Náray-Fejes-Tóth, 2007). Orm-1, an Ald-

responsive gene, is involved in inflammation, and high levels are associated with increased risk of CVD (Engström *et al.,* 2003). Our results investigating the antagonist properties of MPA, NET-A and RU486, relative to Prog, on the expression of the endogenous Orm-1 gene in a rat cardiomyocyte cell line transiently transfected with the MR, showed that Prog, NET-A and RU486 inhibited the Ald-induced upregulation on Orm-1 mRNA expression. In contrast, MPA increased the expression of the Ald-induced upregulated Orm-1 gene. Moreover, when examining the expression of endogenous Orm-1, an MRE-containing gene, in response to Prog, MPA, NET-A and RU486 on their own, we showed that, in contrast to MPA Prog, NET-A and RU486 could not increase Orm-1 expression significantly. However, since we show that the H9C2 cell line also expresses endogenous GR, it may be that MPA, at a concentration of 1 μM, would act preferentially via the GR. Thus we cannot exclude the possibility that the agonist activity of MPA on the endogenous Orm-1 gene (figure 8A) is mediated, at least in part, by the GR. Future studies to delineate the role of the GR would include studies in the presence of a GR antagonist and/or knockdown of the GR by siRNA technology.

Given that MR antagonism is known to protect the cardiovascular system from damaging inflammation, it is interesting to see that, not only does MPA not antagonize the Ald-effects on Orm-1, but it may increase its expression. These results may provide an explanation for the observed adverse effects of MPA on CVD in the WHI study. On the other hand, NET-A partially inhibits the Ald-upregulated Orm-1 expression, indicating that NET-A could have cardioprotective properties.

Little is known about MR-mediated transrepression of genes, and similarly to the AR discussed in Chapter 3, this receptor is very closely related to the GR with a similar mechanism of action. Our findings show that only the natural ligand, Ald, and none of the progestins tested, could significantly elicit MR transrepression via NFκB. In contrast, this study demonstrates that Prog, NET-A and RU486 cause transrepression via AP-1 tethering. In addition, we show that even though MPA and NET-A are similar in most assays, NET-A but not MPA, can transrepress the PMA-induced response on the AP-1 promoter via the MR. The transrepression/transactivation ratio for MPA is 0.9, which is similar to that of Ald used as the standard (transrepression / transactivation = 1.0) indicating that MPA cannot dissociate between transrepression and transactivation. In fact, under these experimental conditions, MPA is not an agonist for transactivation or transrepression via the MR. In contrast, to our knowledge, our study is the first to show that NET-A and RU486 can discriminate between transrepression and transactivation (transrepression/transactivation ratio for is 46.3 and 46.2, respectively) via the MR. For the GR, such compounds that can dissociate between transrepression and transactivation mechanisms are called dissociated glucocorticoids. These compounds are thought to retain the beneficial anti-inflammatory activities (transrepression mechanism) without the negative side-effects (transactivation mechanism) (Vayssière *et al.,* 1997). Thus NET-A and RU486 may be referred to as dissociated mineralocorticoids. This result indicates that NET-A and RU486 may be promising agents for the treatment of certain inflammatory conditions, such as the inflammation associated with MR-mediated cardiovascular injury. However, elucidation of the mechanism behind this dissociative activity will require additional studies.

Taken together, the results from this study indicate that MPA and NET-A act differently to each other and Prog for transactivation and transrepression of synthetic promoters (GRE vs. AP-1) via overexpressed hMR in COS-1 cells. In addition, differential effects are also observed on an endogenous GRE/MRE-containing gene considered to be a cardiovascular disease risk factor. Although these results elucidate only a single step in the complex pathophysiological process of CVD, it may have implications for women using these progestins in reproductive therapy. As NET-A antagonizes the Orm-1 gene to a similar extent as Prog, it may offer cardioprotection, and thus be the preferable progestin for women at risk of cardiovascular disease. Similarly, the dissociative mineralocorticoid property of NET-A, makes it a candidate for therapeutic use that would combat inflammation, while displaying few side-effects. However, it has been reported that progestins displaying high androgenic potency (as shown for MPA and NET-A in Chapter 3), may not protect women against heart attacks (Sitruk-Ware, 2000).

Collectively, the results from our study show that sometimes MPA and NET-A act in a similar manner, but different from Prog. For example, MPA and NET's agonist transactivation and transrepression activity via expressed AR in COS-1 cells (Chapter 3), their lack of regulation of the IL-6 gene in the Ect1/E6E7 and Vk2/E6E7 cell lines (Chapter 2), as well as their lower binding affinity for the MR, lack of agonist activity, and weaker antagonistic activity on a synthetic MRE promoter (Chapter 4), are similar, but different from Prog. However, sometimes they act like Prog, for example in having similar binding affinities to the AR (Chapter 3), and in upregulating the IL-8 and RANTES genes in the Vk2/E6E7 cell line (Chapter 2). Sometimes MPA and NET-A act differently from each other, and from Prog. For example, in the

regulation of the endogenous RANTES gene in the Ect1/E6E7 cell line (Chapter 2). Finally, there are circumstances under which MPA and NET differ in their actions, but one of them displays actions similar to that of Prog. These circumstances include similar activities of Prog and NET-A, but not MPA, for transrepression on the AP-1 promoter via the MR (Chapter 4), similar regulation of the IL-8 gene by Prog and MPA, but not NET, in the Ect1/E6E7 cells (Chapter 2), and similar antagonist activities by Prog and NET-A, but not MPA, on the Orm-1 gene (Chapter 4).

Our results clearly suggest that making an informed decision about the specific progestin to use clinically, is not a trivial matter. Finally, it is thus important that physicians understand that progestins differ widely in their chemical structure, pharmacokinetics, and biological activity, and take cognizance of this when prescribing the optimum type and dose of progestin for contraception or HRT.

5.4 Future Perspectives

In the first part of this thesis (Chapter 2), the differential regulation of pro-inflammatory cytokines by MPA and NET-A, as well as cell-specific effects were demonstrated, to our knowledge, for the first time in human ectocervical and vaginal epithelial cell lines. Previous reports indicate that different cells may respond differently to a particular ligand, depending on several factors, such as the relative levels and different isoforms of different steroid receptors, and the receptor conformation induced by the ligand which subsequently affects the recruitment of co-regulators (co-activators and co-repressors) (Turgeon *et al.,* 2004; reviewed by Hapgood *et al.,* 2004).

To gain insight into the differential and cell-specific effects of MPA and NET-A, a number of factors may thus be addressed. We have already determined the relative expression levels of PR, GR, AR, and ER (Renate Louw and Dewald Noeth, MSc students) in these cell lines. Moreover, it would be interesting to determine PR isoform is expressed in these cell lines, and whether they might be involved in any of the observed responses, as these isoforms are known to be transcriptionally different (Shyamala *et al.,* 1990; Kastner, *et al.,* 1990).

We hypothesized that the differential effects of MPA and NET-A on the RANTES gene is as a result of the ligands acting via different steroid receptors. Although experiments using the AR antagonist, hydroxyflutamide indicate a role for the AR in mediating the repression of RANTES in the Ect1/E6E7 cell line, the possible role for the other steroid receptors, particularly the GR, has not been excluded. Thus these possibilities remain to be investigated. Specific steroid receptors may be knocked

down by using siRNA technology, in the quest to determine which steroid receptors play a role, and to what extent, in the responses of MPA and NET-A on the IL-8 and RANTES genes in the Ect1/E6E7 versus the Vk2/E6E7 cell lines. Furthermore, as MPA and NET-A are also used in combination with estrogen in HRT, it would be of interest to determine not only the direct effects of MPA and NET-A on these genes, but also their modulation of the effect of estrogen.

In addition, we have compared the K_i for MPA and NET-A via overexpressed AR in the COS-1 cell line (Chapter 3). It would be interesting to perform binding experiments in the Ect1/E6E7 and Vk2/E6E7 epithelial cell lines, and compare the K_i values for MPA and NET-A via endogenous AR, GR or PR, so as to provide insights into the relationship between binding parameters and biological activity. Furthermore, the co-regulator (co-factor and co-repressor) expression and/or recruitment leading to gene transcription may be different in the Ect1/E6E7 versus Vk2/E6E7 cell lines. The expression levels can be investigated by preparing whole cell lysates from both cell lines, and assessing the levels of co-regulators such as SRC-1, GRIP-1, p300, NCor and Smrt using Western blotting, while the differential recruitment of co-regulators to promoters could be determined by chromatin immunoprecipitation (ChIP) assays.

In the literature, there is a paucity of data thoroughly comparing the binding affinities of MPA and NET-A via specific steroid receptors. To date, only one study has addressed this, specifically for the GR, and performed a thorough pharmacological characterization of the relative efficacy and potency of MPA versus NET-A on gene regulation (Koubovec *et al.,* 2005). Our study has now addressed this for the AR and

MR (Chapters 3 and 4). However, it would also be interesting to do these experiments for the PR isoforms, as well as isoforms of the GR, AR and MR. Work in this area has already been started in our laboratory, showing that both MPA and NET-A have a similar affinity for overexpressed human PR-A and PR-B in COS-1 cells (Renate Louw, BSc(Hons)). Further investigation into their PR isoform-specific agonist and antagonist activity are currently in progress.

The AR N/C-interaction was initially shown to be crucial for transactivation (Kemppainen *et al.,* 1999). However, recent evidence shows that the AR N/C-interaction enhances AR transactivation activity but that transactivation can proceed without N/C-interaction (Shen *et al.,* 2005). Indeed, we have also shown a disparity between the MPA-induced AR N/C-interaction and AR transactivation activity in the presence of MPA. In addition, it has recently been shown that the p160 co-activator, GRIP-1, mediates the DHT-induced AR N/C-interaction (Shen *et al.,* 2005). Thus, to provide insight into the reason for the lack of N/C-interaction on a classical ARE by MPA, but not NET-A, it would be interesting to determine whether GRIP-1 is involved in AR N/C-interaction and AR transactivation in the presence of MPA and NET-A. It is likely that GRIP-1 would also mediate NET-A induced N/C-interaction, while it would not interact with MPA-bound AR.

We characterized the interaction between the N-terminal domain of the AR and the ligand-bound ligand binding domain *in vitro* using a mammalian two-hybrid assay. A recent study however used the quantitative fluorescence resonance energy transfer (FRET) assay to investigate AR N/C-interaction in living cells, and showed that the N/C-interaction is initiated rapidly following the addition of DHT, before transport to

the nucleus (Schaufele *et al.,* 2005). To confirm our results obtained with the mammalian two-hybrid assay, we could perform similar experiments to look at the conformation of the AR in the presence of MPA and NET-A, using ARs double tagged at the N and C termini with the FRET couple cyano fluorescent protein (CFP) and yellow fluorescent protein (YFP).

A limitation of our study may be that we used only promoter-reporter constructs and overexpressed AR to investigate the mechanisms of transcriptional regulation of MPA and NET-A via the AR. However, our results for the repression by MPA on the RANTES gene via the AR suggest that the effects seen with synthetic promoter-reporter genes are reflected on endogenous genes. In contrast, the result with NET on the endogenous RANTES gene differs to the effect found with synthetic promoter-reporters, and it is thus possible that promoter-reporters may not reflect the regulation on full-length endogenous promoters in the context of the native chromatin structure. However, a study by Ghatge and co-workers (2005), showing that MPA and DHT are equally potent in regulating endogenous genes in breast cancer cell lines, supports our findings showing similar potent androgenic activity of MPA and DHT in the COS-1 cell line transiently transfected with an AR expression vector and promoter-reporter constructs. We thus conclude that the approach of using promoter-reporter constructs, as well as using the COS-1 cell line, devoid of steroid receptors, are appropriate methods for directly comparing the mechanism of action of MPA and NET via different steroid receptors. This approach is also useful as cells expressing physiologically relevant genes are often found in cells that contain several different steroid receptors, making it difficult to identify which receptor is mediating a specific response. However, it would still be interesting to demonstrate the relevance of our

results obtained with transfected promoter-reporter constructs by verifying these results on an endogenous promoter, particularly for NET-A.

In the third part of this thesis (Chapter 4), we show that MPA, NET-A and RU486 are devoid of MR agonist activity for transactivation and are weak MR antagonists. For the GR it has been shown that receptor density can change the biocharacter of MPA and RU486 (Zhao *et al.,* 2003). At high receptor density, both MPA and RU486 behaved as full agonists for transrepression, while at low receptor density, the ligands behaved as full antagonists. It would thus be interesting to see whether MPA, NET-A and RU486, can also change their agonist versus antagonist activities for both transactivation and transrepression at varying concentrations of MR, and also other steroid receptors. Furthermore, as we only determined the efficacy of ligands for MR transrepression on AP-1, it would be useful to also determine the potency of these ligands.

In addition, the MR N/C-interaction was shown only for the agonists used in this study. It is thus possible that disruption of the N/C-interaction is one mechanism by which antagonists inhibit activation of the MR. It would be interesting to see whether MPA, NET-A and RU486 can antagonize the Ald-induced MR N/C-interaction. Our results on the expression of the endogenous Orm-1 gene were determined in H9C2 cells with transiently transfected MR. Furthermore, the hypothesis that the MR plays a role in the antagonism of NET-A and RU486 may be strengthened by knocking down the MR using siRNA technology. Similar experiments may also be performed to exclude the role of the GR.

Finally, the studies with overexpressed AR and MR, as well as synthetic promoters, in the COS-1 cell line shed light on the putative role of steroid receptors in mediating the responses of MPA and NET-A in different cell types, such as the ectocervical and vaginal epithelial cell lines, as well as the cardiomyocyte cell line.

5.5 Conclusion

In summary, the results presented in this thesis show that in agreement with our hypothesis Prog, MPA and NET-A differentially regulate IL-8 and RANTES gene expression in the female genital tract, in a cell-specific manner. The differential effects of the progestins as compared to each other and Prog, was most striking on the RANTES gene. Prog upregulated TNFα-induced RANTES gene expression, NET-A had no effect, and MPA repressed this gene. This result implies that the use of MPA *in vivo* may either exert local immunosuppressive side-effects, or positive effects in terms of inflammation and HIV, while NET-A and natural Prog, will not exhibit these effects. Interestingly, both MPA and NET-A act differently to Prog, which had previously been shown to have a protective role in the vagina. This could in fact indicate that both MPA and NET-A may impact on the risk of susceptibility to infections in the cervicovaginal environment. It was further shown that the repression of TNFα-induced RANTES gene expression by MPA in the ectocervical cell line is mediated by the AR. In contrast, the effect of MPA in the vaginal cell line was not mediated by the AR. As hypothesized, the results investigating the activity of MPA and NET-A via overexpressed AR in COS-1 cells, show that both these synthetic progestins, unlike Prog, are potent androgens. This suggests that women using these progestins therapeutically could have androgenic side-effects such as negative impact on fetal or neonatal development when contraceptives are inappropriately

used (Kemppainen *et al.,* 1999) or effects on hepatic endocrine function in HRT users (Nugent *et al.,* 2003). In addition, the use of these potent androgens may reverse the protective effects of natural androgens in breast tissue, leading to increased risk of breast cancer in women, as was seen in the 'Million Women Study' (Million women study collaborators, 2003). Furthermore, our results show that both MPA and NET-A are weak partial antagonists of Ald-mediated MR transactivation on a synthetic GRE promoter. However, MPA, unlike Prog and NET-A, cannot antagonize the Ald-upregulated expression of the endogenous GRE-containing Orm-1 gene. This may have adverse implications for cardiovascular side-effects when MPA is clinically used. Moreover, our results indicate that MPA on its own may directly enhance the expression of genes that are cardiovascular risk markers. The combined results from this thesis, together with the existing physiological and molecular information available on MPA and NET-A, relative to natural Prog, may improve our understanding of the physiological outcomes of these progestins *in vivo*.

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APPENDIX A: DATA NOT INCLUDED IN CHAPTER 2

A1: Hydroxyflutamide does not inhibit the effects of Prog, MPA or NET-A on RANTES gene expression in the human vaginal cell line (Vk2/E6E7)

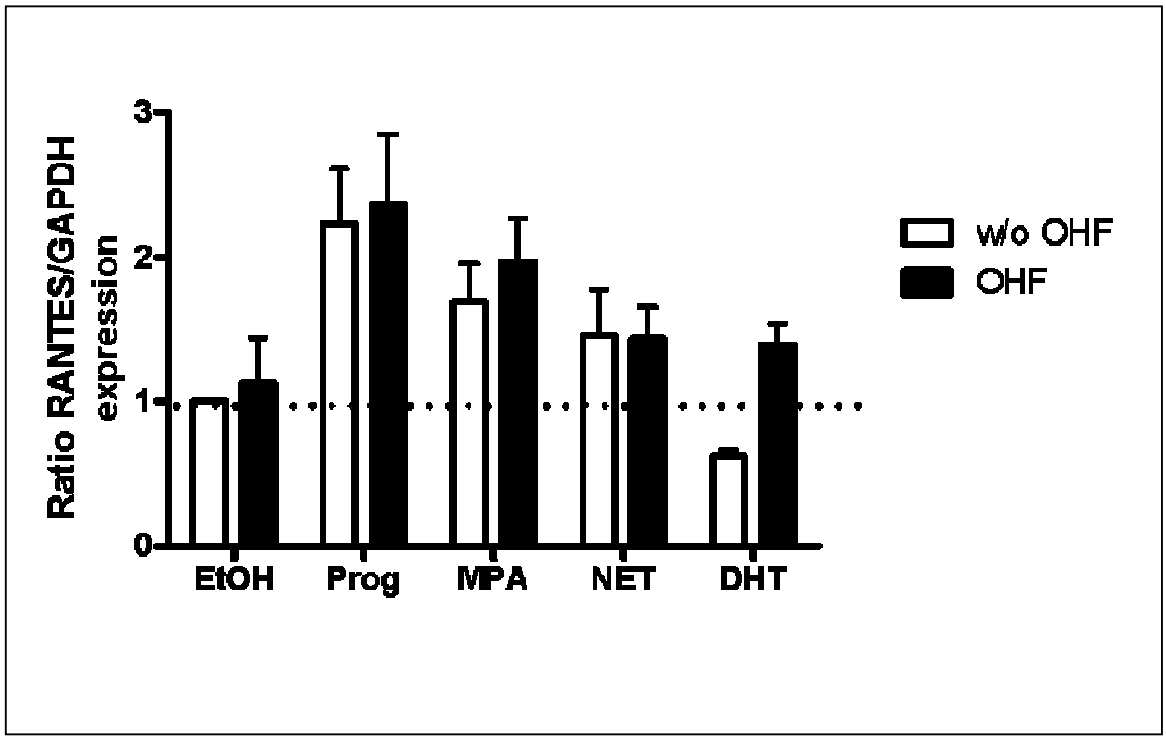


Figure A1: Effect of the androgen receptor antagonist (OHF) on the upregulation of the TNF-α-induced RANTES gene expression by Prog, MPA or NET-A. The human Vk2/E6E7 cell line was incubated with 0.02 µg/µl TNF-α in the presence of 1 µM Prog, MPA, NET-A, or DHT, and absence or presence of 10 µM hydroxyflutamide (OHF) for 24 hours. Total RNA was extracted, cDNA synthesized, and expression levels of RANTES and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are representative of two independent experiments. Open bars are compounds and vehicle only, while the black bars are compounds plus antagonist.

APPENDIX B: DATA NOT INCLUDED IN CHAPTER 3

B1: Optimisation of [^{3}H]-MIB concentration for the determination of K_d or K_i values of ligands via overexpressed hAR

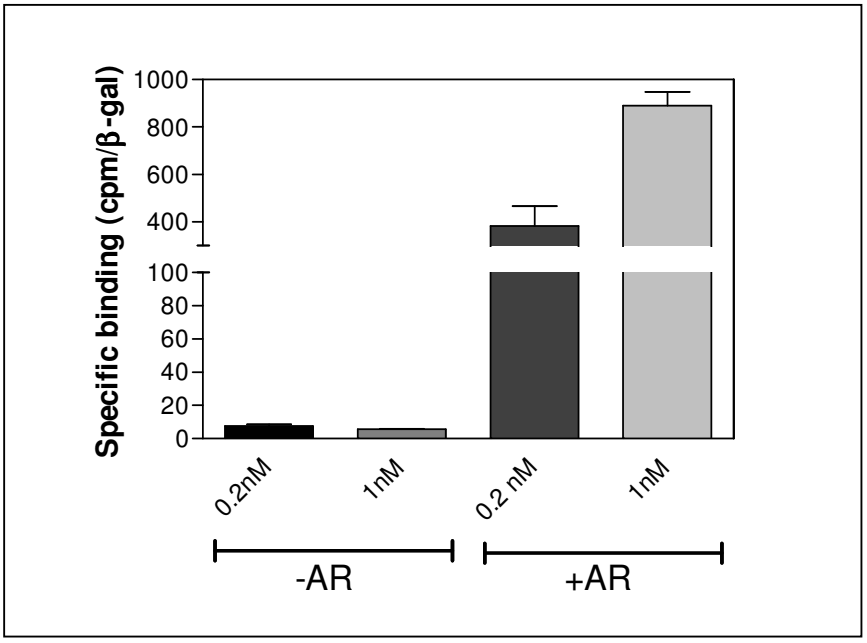


Figure B1: Determining the optimal concentration of [^{3}H]-MIB. COS-1 cells (1x10^5 cells per well in a 24-well plates) were transiently transfected with 0.375 µg pGL2 basic vector (-AR) or pSVARo and 0.0375 µg pCMV-β-gal expression vectors, using the FUGENE 6 transfection reagent according to the manufacturer's instructions. Twenty-four hours later the cells were incubated with 0.2 nM or 1 nM [^{3}H]-MIB in the absence or presence of 1000 fold excess unlabelled MIB for 90 minutes. Specific binding normalised to β-galactosidase expression is shown.

B2: Time course to establish equilibrium time for binding of 0.2 nM [^{3}H]-MIB to overexpressed hAR

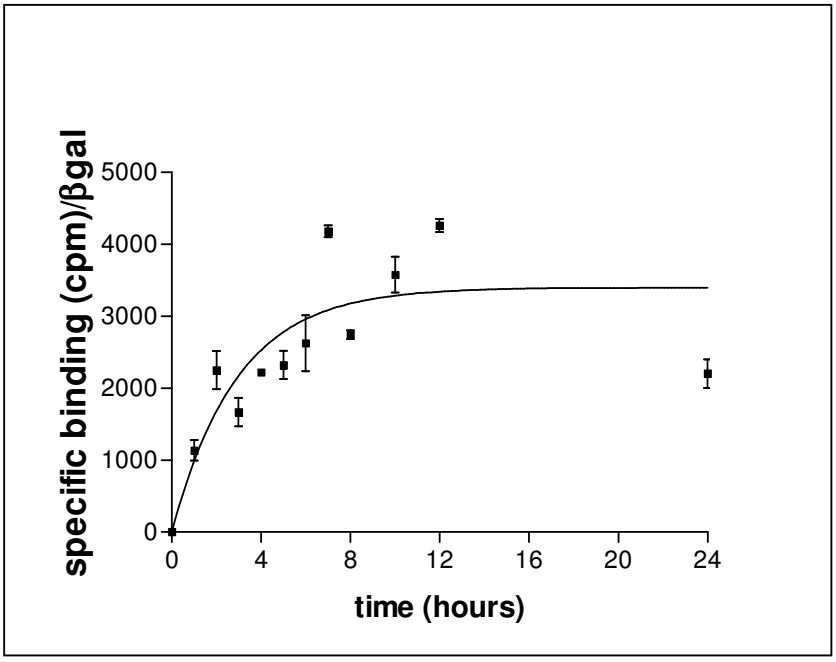


Figure B2: Equilibrium time for 0.2 nM [^{3}H]-MIB to bind to overexpressed AR is 16 hours. COS-1 cells (1x10^5 cells per well in a 24-well plate) were transiently transfected with 0.375 µg pSVARo and 0.0375 µg pCMV-β-gal expression vectors, using the FUGENE6 transfection reagent according to the manufacturer's instructions. Twenty-four hours later the cells were incubated with 0.2 nM [^{3}H]-MIB in the absence and presence of 10 µM unlabelled MIB for varying times. Specific binding normalised to β-galactosidase expression is shown.

B3: Transrepression assay in COS-1 cells in the absence and presence of overexpressed hAR

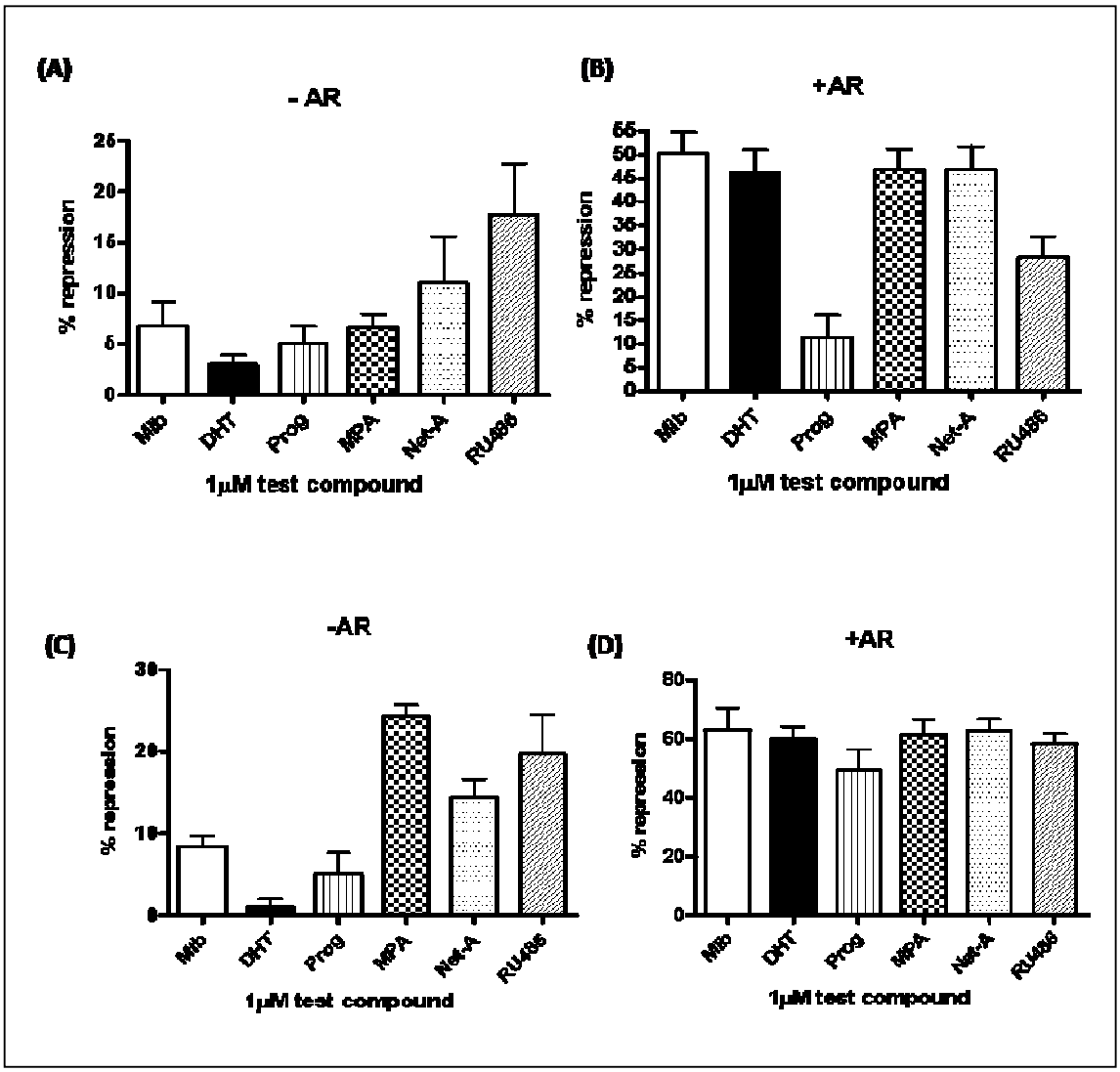


Figure B3: Transrepression activity in the absence and presence of overexpressed hAR. COS-1 cells were transiently transfected with pCMV-β-galactosidase expression vectors and **(A)** pGL2basic empty vector and NFκB-containing promoter-luciferase reporter construct or **(B)** pSVARo and NFκB-containing promoter-luciferase reporter construct or **(C)** pGL2basic empty vector and AP-1-containing promoter-luciferase reporter construct or **(D)** pSVARo and AP-1-containing promoter-luciferase reporter construct. Subsequently, the cells were stimulated with PMA and incubated with 1 µM of either MIB, DHT, Prog, MPA, NET-A or RU486 for 24 hours. The resulting plots show repression by the individual ligands calculated as a percentage of EtOH (100%). Result shown is the average of three independent experiments with each condition performed in triplicate (±SEM).

B4: MPA and NET-A lack AR antagonist activity

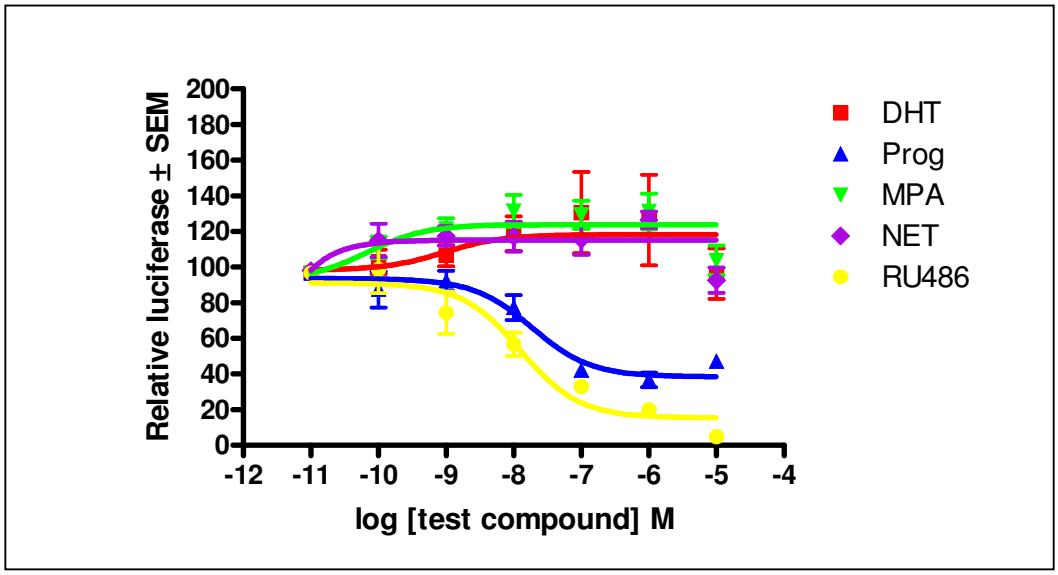


Figure B4: MPA and NET-A do no display androgen antagonist activity. COS-1 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter, pSVARo and pCMV-β-galactosidase expression vectors. Subsequently, the cells were incubated with 0.1 nM MIB alone (100%) or in the presence of increasing concentrations of DHT (■), Prog (▲), MPA (▼), NET-A (♦) or RU486 (●) for 24 hours. Result shown is the average of at least four independent experiments with each condition performed in triplicate (±SEM).

B5: MPA, but not NET-A, antagonizes the DHT-induced N/C-interaction of the AR

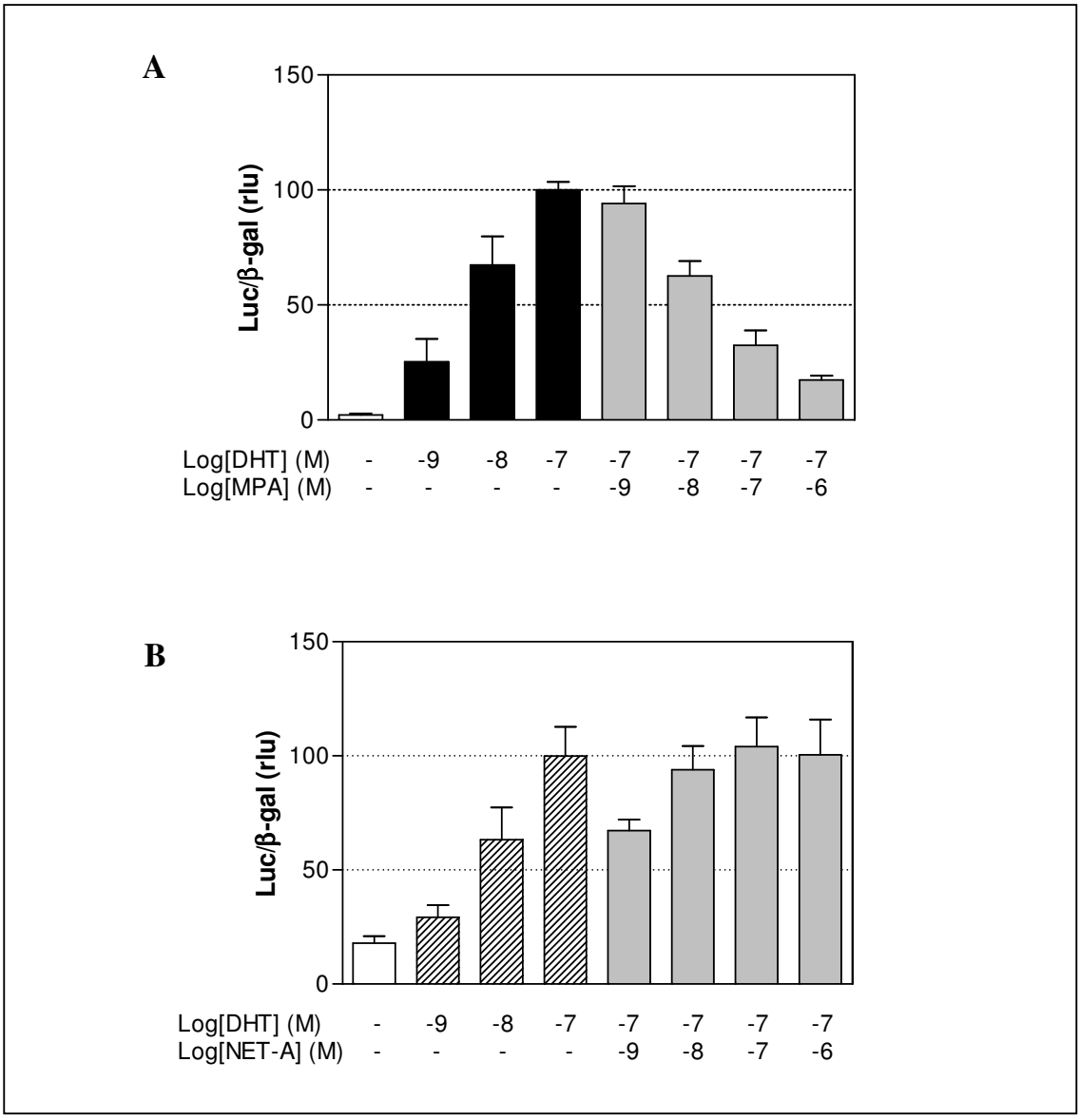


Figure B5: In contrast to MPA, NET-A does not antagonize the DHT-induced N/C-interaction of the hAR. COS-7 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter, the pSG5-hAR(DBD-LBD), the pSNATCH-II(hAR-NTD) and the pCMV-β-galactosidase expression vectors. Subsequently, the cells were exposed to increasing concentrations of DHT, or 10^{-7} M DHT in the presence of increasing concentrations of (**A**) MPA and (**B**) NET-A, for 24 hours. Result is shown as luciferase (luc) activity normalized to β-galactosidase (β-gal) activity. Results are averages of three independent experiments with each condition performed in triplicate (±SEM).

APPENDIX C: DATA NOT INCLUDED IN CHAPTER 4

C1: Optimisation of [^{3}H]-Ald concentration for the determination of K_d or K_i values of ligands via overexpressed hMR

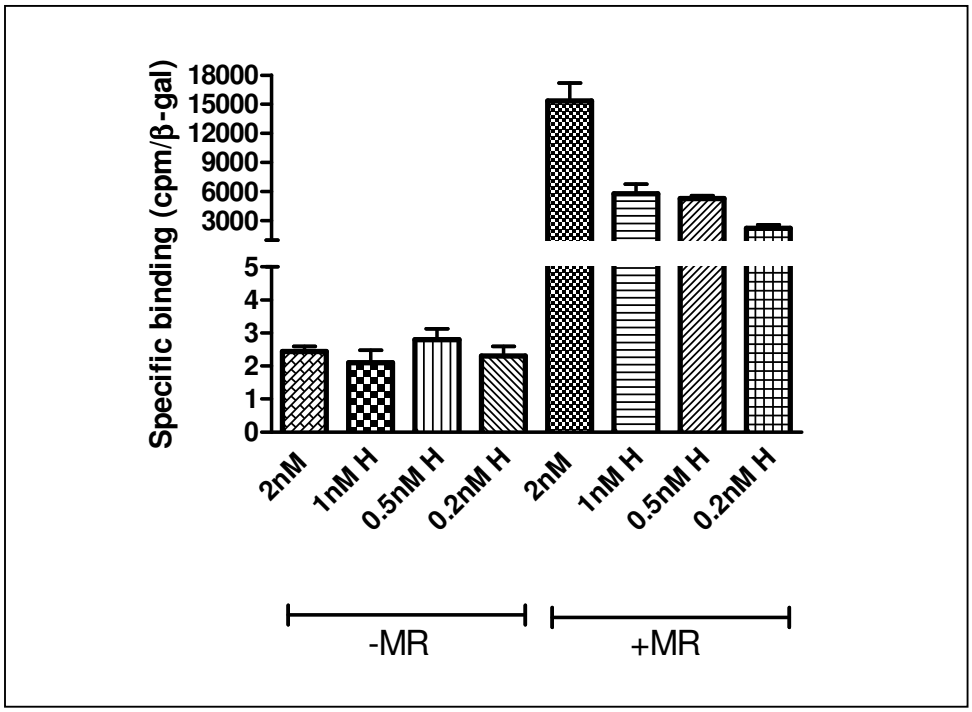


Figure C1: Determining the optimal concentration of [^{3}H]-MIB. COS-1 cells (1x10^5 cells per well in a 24-well plates) were transiently transfected with 0.375 µg pGL2 basic vector (-MR) or pRShMR and 0.0375 µg pCMV-β-gal expression vectors, using the FUGENE 6 transfection reagent according to the manufacturer's instructions. Twenty-four hours later the cells were incubated with 0.2 nM, 0.5 nM, 1 nM or 2 nM [^{3}H]-Ald in the absence or presence of 1000 fold excess unlabelled Ald for 90 minutes. Specific binding normalised to β-galactosidase expression is shown.

C2: Time course to establish equilibrium time for binding of 0.2 nM [^{3}H]-Ald to overexpressed hMR

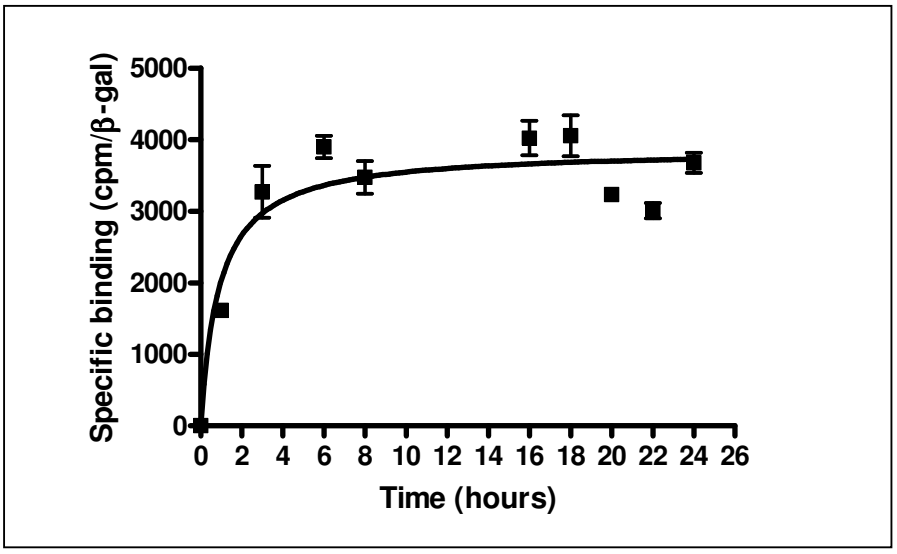


Figure C2: Equilibrium time for 0.2 nM [^{3}H]-Ald to bind to overexpressed MR is 16 hours. COS-1 cells (1x10^5 cells per well in a 24-well plate) were transiently transfected with 0.375 µg pRShMR and 0.0375 µg pCMV-β-gal expression vectors, using the FUGENE 6 transfection reagent according to the manufacturer's instructions. Twenty-four hours later the cells were incubated with 0.2 nM [^{3}H]-Ald in the absence and presence of 10 µM unlabelled Ald for varying times. Specific binding normalised to β-galactosidase expression is shown.

C3: Transrepression assay in COS-1 cells in the absence and presence of overexpressed hMR

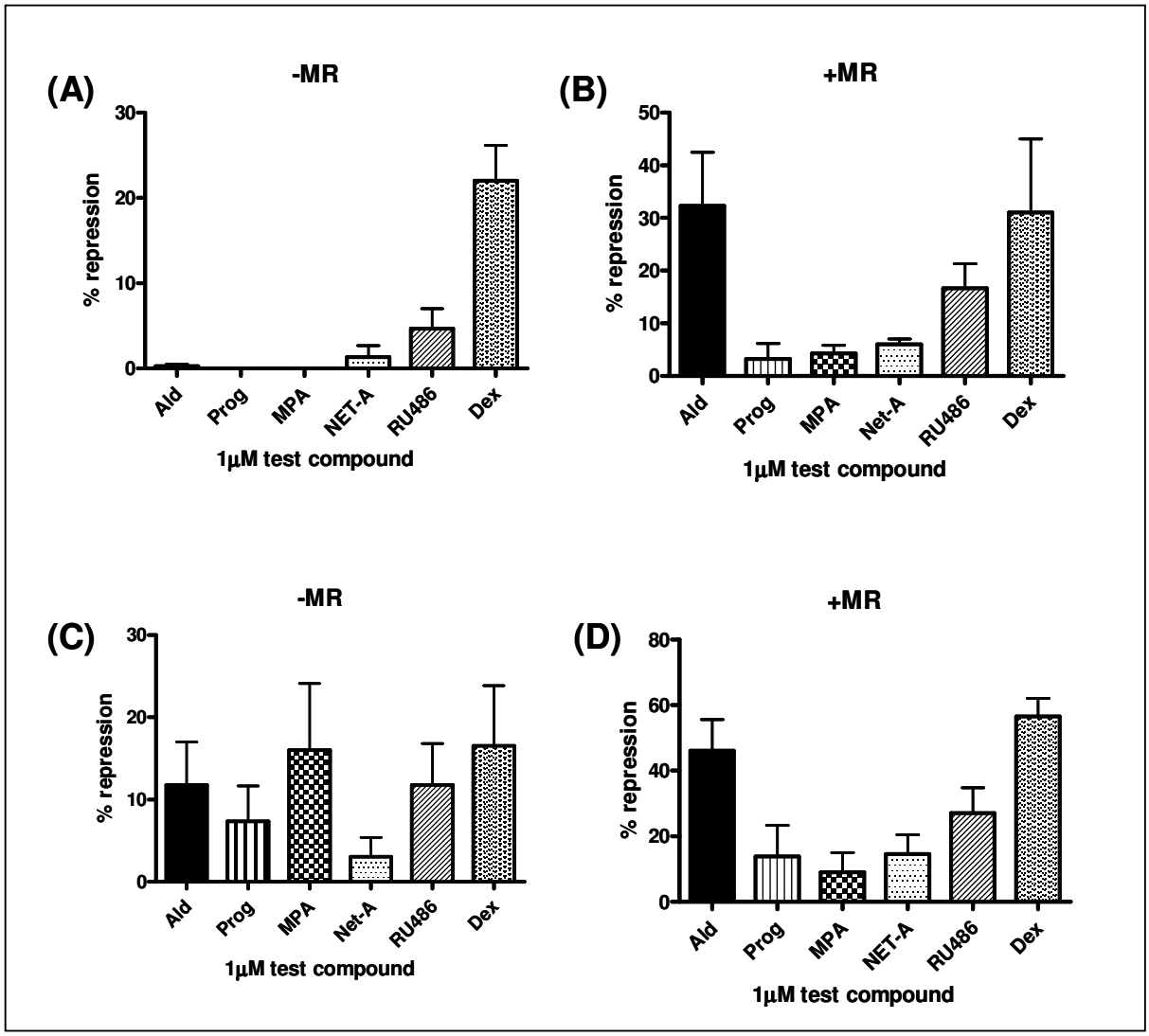


Figure C3: Transrepression activity in the absence and presence of overexpressed hMR. COS-1 cells were transiently transfected with pCMV-β-galactosidase expression vectors and **(A)** pGL2basic empty vector and NFκB-containing promoter-luciferase reporter construct or **(B)** pRShMR and NFκB-containing promoter-luciferase reporter construct or **(C)** pGL2basic empty vector and AP-1-containing promoter-luciferase reporter construct or **(D)** pRShMR and AP-1-containing promoter-luciferase reporter construct. Subsequently, the cells were stimulated with PMA and incubated with 1 μM of either Ald, Prog, MPA, NET-A, RU486, or Dex for 24 hours. The resulting plots show repression by the individual ligands calculated as a percentage of EtOH (100%). Result shown is the average of three independent experiments with each condition performed in triplicate (±SEM).

C4: Prog displays weak partial agonist activity for transactivation via overexpressed MR in COS-1 cells

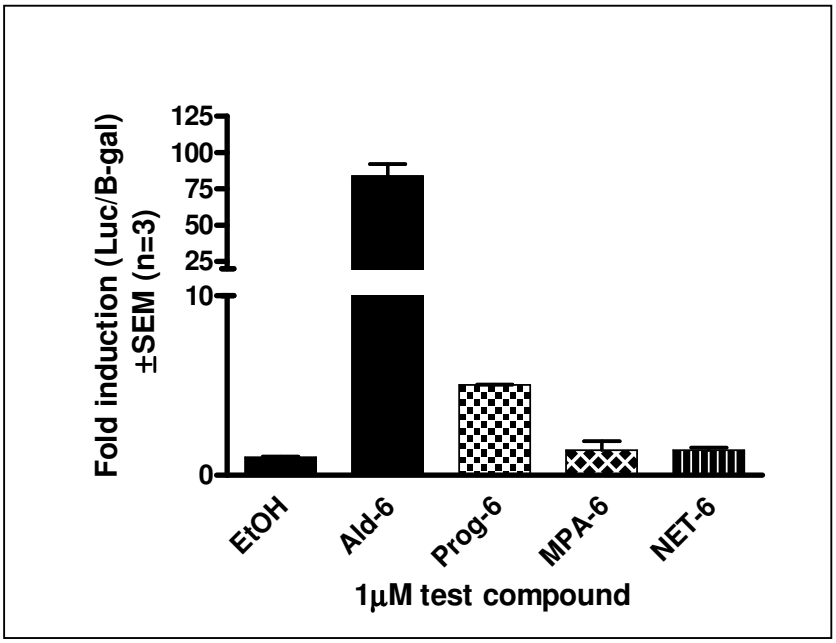


Figure C4: Unlike Ald and Prog, MPA and NET-A do not display mineralocorticoid agonist activity. COS-1 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter plasmid plus, pRShMR and pCMV-β-galactosidase expression vectors. Subsequently, the cells were incubated with 1 μM of either Ald, Prog, MPA or NET-A for 24 hours. Luciferase (luc) activity was normalized to β-galactosidase (β-gal) activity, and is shown as fold induction relative to EtOH set as 1. Results shown are the average of at least three independent experiments with each condition performed in triplicate (±SEM).

C5: Similar induction of the MR N/C-interaction by aldosterone and cortisol

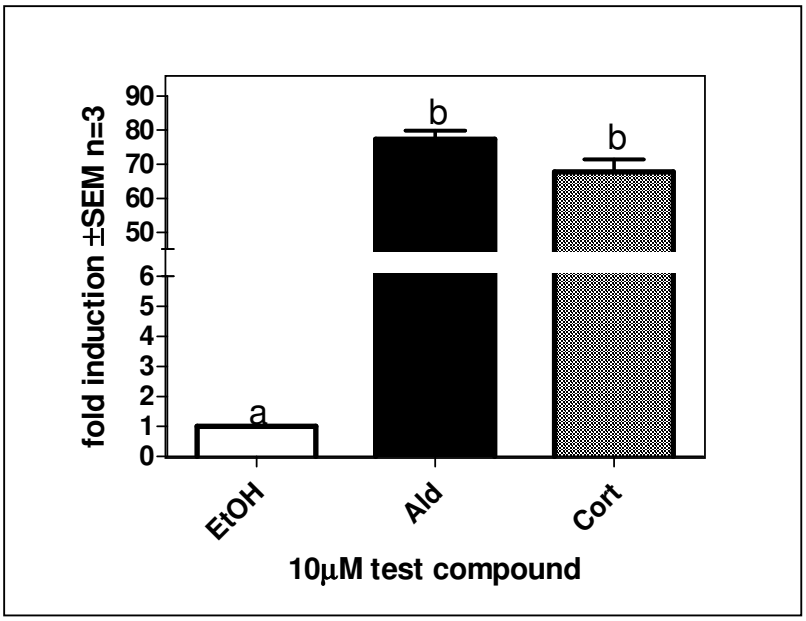


Figure C5: Ald and Cort induce the N/C-interaction of the hMR to a similar extent. COS-1 cells were transiently transfected with the GAL4-MRC and VP16-MRNT expression vectors, as well as the GAL4-responsive luciferase reporter vector pG5-luc. The cells were exposed to 10 µM Ald or Cort for 24 hours. Results are averages of at least three independent experiments with each condition performed in triplicate (±SEM), and are shown as fold induction relative to EtOH set as 1..

C5: Antagonist activity of NET-A via the hGR in COS-1 cells.

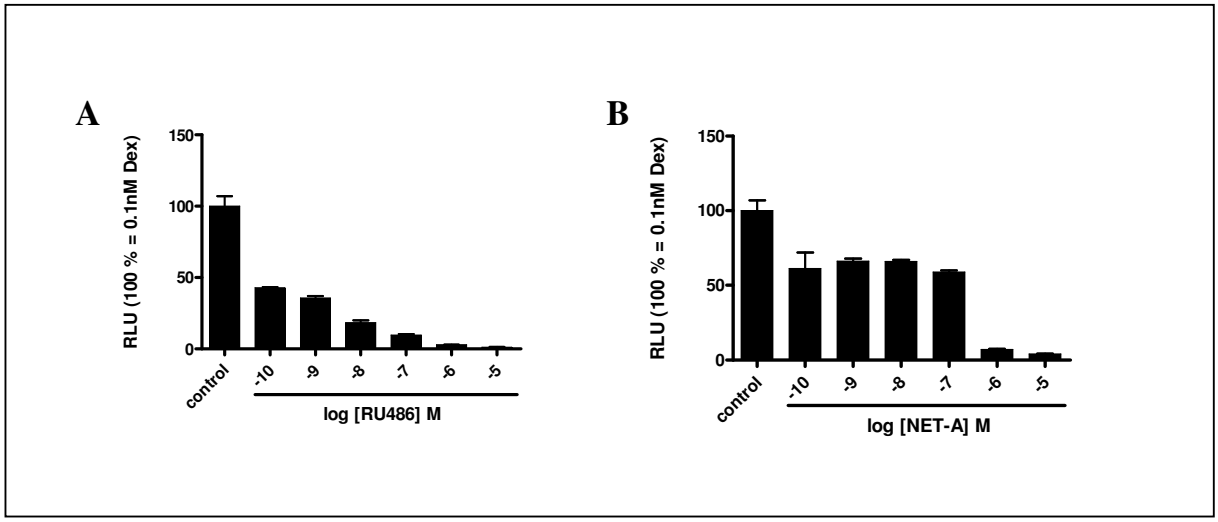


Figure C6: Relative antagonist activity of NET-A via the GR. COS-1 cells were transiently transfected with HA-hGR-pCMV and pTAT-GRE-E1b-luc. The cells were incubated with 0.1 nM Dex in the absence (100%) and presence of increasing concentrations of RU486 (A) or NET-A (B). Thereafter the lysates were assayed for luciferase activity. One representative experiment for each compound performed in triplicate (± S.E.M.) is shown.

APPENDIX D: DEFINITIONS AND EXTRA DATA NOT INCLUDED IN CHAPTERS

D1: Binding parameters and calculations

The relative binding affinity (RBA), also referred to as the EC_{50}, is a measure of the concentration of ligand that competes for half the specific binding. It is used to estimate the binding affinity of a ligand for a receptor. The EC_{50} values obtained from competitive binding curves however, are not equivalent to K_d or K_i (equilibrium dissociation constant) values. The EC_{50} is a relative term, which is influenced by different experimental conditions, such as receptor number or concentrations of radiolabelled ligand, while the K_d or K_i is a constant value for a particular receptor, which is not influenced by experimental variations (GraphPad Prism software).Thus, like the EC_{50}, the K_d or K_i, is a measure of the affinity of a ligand for a particular receptor, that reflects the concentration of ligand needed to bind to half the available receptors at equilibrium, but it represents a more accurate measure of binding affinity of a ligand for its receptor than EC_{50} values.

K_d is typically determined in a saturation binding assay using varying concentrations of a radiolabelled ligand. A low K_d or K_i value means that a low concentration of ligand is needed to bind half the available receptors. Thus, a low K_d or K_i value indicates that the receptor has a high affinity for the ligand. K_i refers to the equilibrium constant of a ligand determined in inhibition studies, and is usually determined by the alternative method for determining K_d or K_i, which is a homologous/heterologous competitive binding assay. A homologous binding assay is one where the radiolabelled and unlabelled are chemically identical, while in the heterologous assay, they differ. In these experiments, the radioligand concentration remains constant, and competes with varying concentrations of unlabelled competitor ligand for the receptor.

Analysis of radioligand binding experiments is based on the law of mass action, which assumes that binding is reversible. The number of binding events per unit of time = [Ligand]. [Receptor].K_{on}, where K_{on} is defined as the association- or on-rate constant (GraphPad Prism software). Following binding, the ligand remains bound to the receptor for a random amount of time, and the possibility of dissociation is very similar, at every instant of time. The rate of dissociation is the number of dissociation events per unit time= [ligand.receptor]. K_{off} where K_{off} is defined as the dissociation- or off-rate constant. Equilibrium is reached when the rate at which new ligand-receptor complexes are formed equals the rate at which the ligand-receptor complexes dissociate (GraphPad Prism software). Thus, at equilibrium, [Ligand].[Receptor].K_{on} = [Ligand.Receptor].K_{off}. If this equation is rearranged, the K_d is defined as K_{off} / K_{on}, which equals [Ligand].[Receptor] / [Ligand.Receptor] (GraphPad Prism software).

To accurately analyse and interpret the results from competitive ligand binding experiments, it is crucial to determine the time to reach binding equilibrium to the receptor with the concentration of radiolabelled ligand used in the experiment. The concentration of radiolabelled ligand used was in the range of two to ten times lower than the EC_{50}. In addition, the ligand depletion always needs to be considered. Ligand depletion measures the fraction of the ligand bound to the receptor (specific sites) vs. the fraction of the ligand bound to nonspecific sites. One would assume that only a small fraction of the added ligand binds to the receptor, so that the free concentration approximates to the concentration initially added. Less than 10% ligand depletion is considered acceptable.

As [^{3}H]-MPA and [^{3}H]-NET-A are not commercially available, K_d and K_i values were determined from pooled data of three independent experiments, by homologous and heterologous binding experiments, respectively (Chapter 3 and 4). Once the optimal concentration of radiolabelled ligand was established (0.2 nM for both [^{3}H]-MIB (Chapter 3) and [^{3}H]-Ald (Chapter 4)), the incubation time for equilibrium to be reached with 0.2 nM [^{3}H]-MIB and [^{3}H]-Ald was determined. The K_d was subsequently calculated from homologous binding experiments by GraphPad Prism software according to the following equation: Total binding = (B_{max}.[Hot] / [Hot] + [Cold] + K_d +NS, where NS is nonspecific binding and B_{max} is the maximum binding of ligand to receptors in cpm. Once the K_d for MIB was determined, heterologous binding experiments were performed, and the K_i values for Prog, MPA, NET-A, DHT, R1881, and RU486 were determined (Chapter 3) according to the Cheng- Prussoff equation: K_i = EC_{50} / 1 + ([ligand] / K_d), where the EC_{50} value is that determined for the competing ligand, the [ligand] is the radiollabelled igand concentration used, and the K_d determined from the homologous binding experiments. K_i can only be determined using the Cheng-Prussoff equation, provided that the EC_{50} is two to ten times lower than the concentration of radiolabelled ligand, and that binding equilibrium has been reached at that concentration. In the same manner, the K_d value for Ald, and K_i values for Prog, MPA, NET-A, RU486 and Dex were determined. All binding data were analysed using GraphPad Prism software, using the following parameters: Nonlinear regression curve fit, homologous competitive binding curve, one class of binding sites, weighting $1/y^2$ (this minimises relative distances squared), consider the mean y value of each point and a stricter (slower) criterion for convergence. In addition, run rest and residuals test were used to determine whether the curve differed systematically from the data.

D2: Pharmacological definitions

To characterize the effects elicited in terms of transactivation and transrepression of genes when a ligand binds to its receptor, two parameters need to be determined, namely efficacy and potency (figure D2(i)).

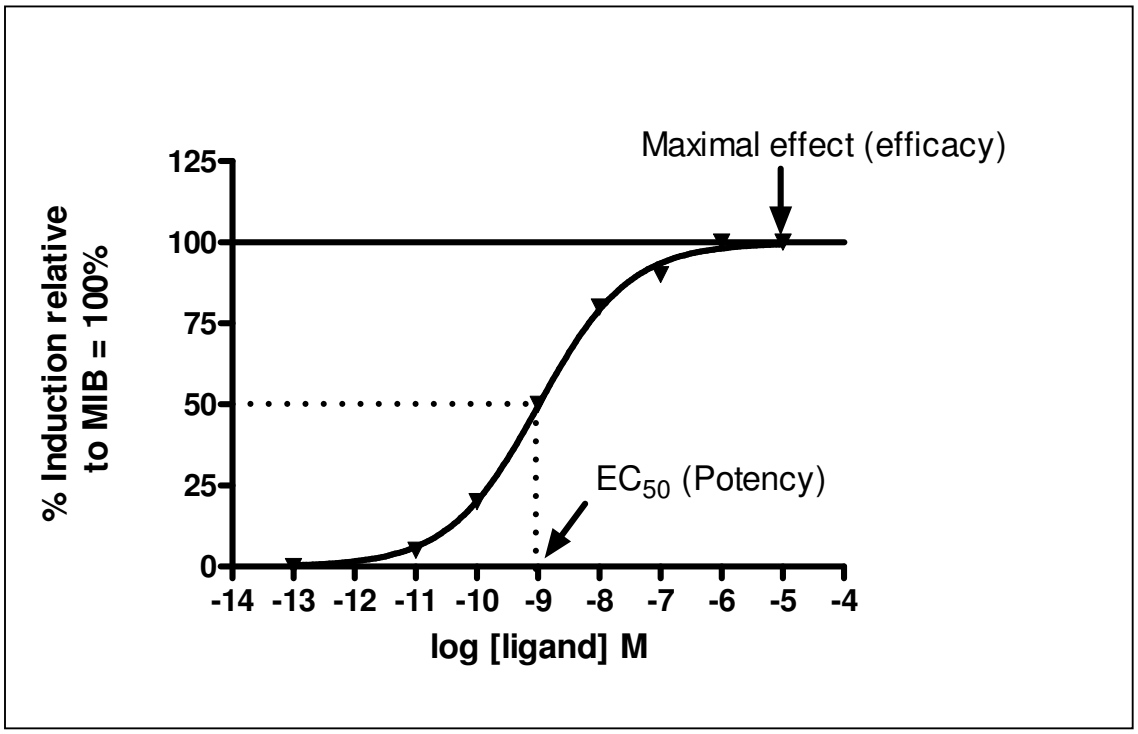


Figure D2(i): A schematic representation of a sigmoidal dose-response curve indicating potency (EC50 value) and efficacy (maximal effect).

The efficacy of a ligand refers the maximal effect it can elicit in a given cell under specific experimental conditions. Efficacy is characterised as either a full agonist or partial agonist. A full agonist is a ligand that produces a maximal response, while a partial agonist is a ligand eliciting a response less than the maximal response of that of a full agonist (figure D2(ii)). In contrast, an antagonist is a compound that binds to a receptor but that does not elicit a response itself, but inhibits agonist-mediated

responses (figure D2(ii)). The potency of a ligand is commonly quantified as the EC_{50}, which is the concentration that induces half the maximal response.

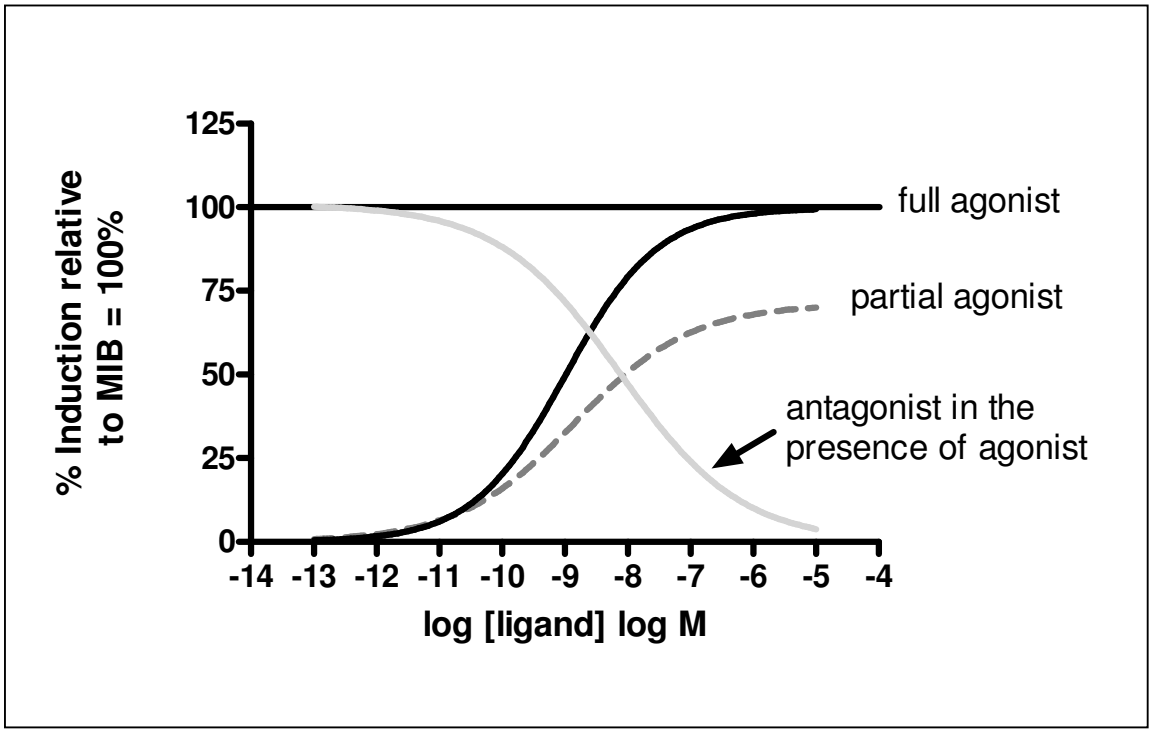
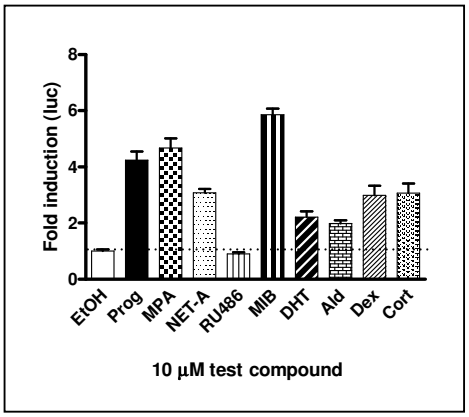


Figure D2(ii): A schematic representation of a sigmoidal dose-response curves indicating a full and partial agonist, as well as an antagonist. (for ligands binding to a single site, with no co-operativity)

D3: Transactivation in COS-1 cells in the absence and presence of overexpressed MR, AR or GR

(A) no transfected SR



(B) MR **C) AR** **(D) GR**

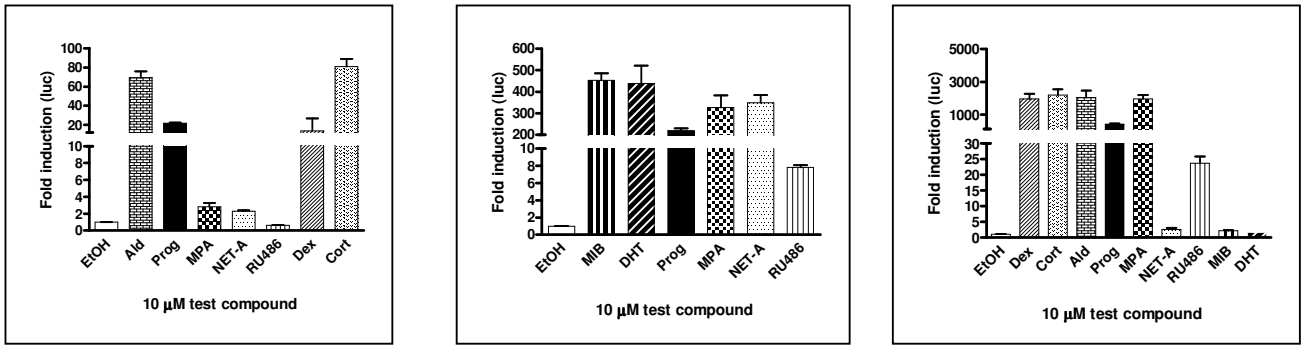
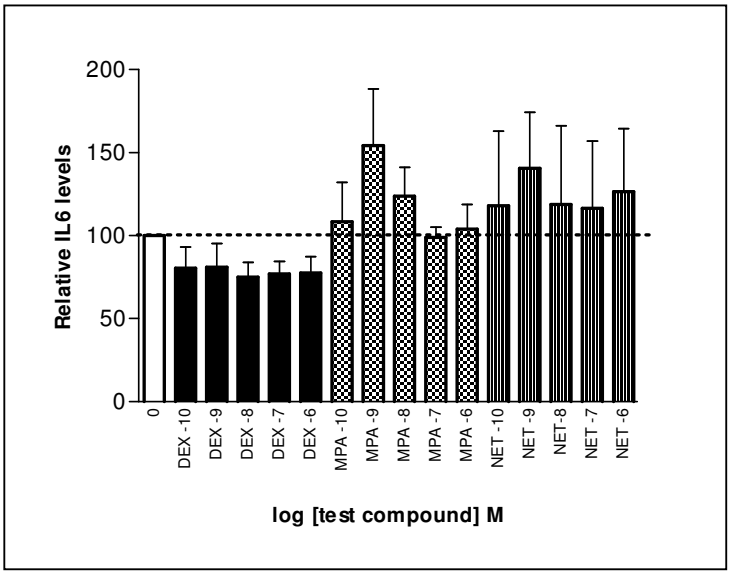


Figure D3: Agonist activity for transactivation via possible endogenous receptors, MR, AR or GR in COS-1 cells. COS-1 cells were transiently transfected with 100 ng pTAT-GRE-E1b-luc reporter plasmid plus, 10 ng pGL2basic **(A)**, pRShMR **(B)**, pSVARo **(C)** or HA-hGR-pCMV **(D)**, and 10 ng pCMV-β-galactosidase expression vectors in a 96-well plate. Subsequently, the cells were incubated with 10 µM Prog, MPA, NET-A, RU486, MIB, DHT, Ald, Dex or Cort for 24 hours. Induction is shown as fold induction relative to EtOH set as 1. Result shown is one representative figure with each condition performed in triplicate (±SEM).

D4: Effects of Dex, MPA and NET-A on IL-6 protein production in human monocytes

A



B

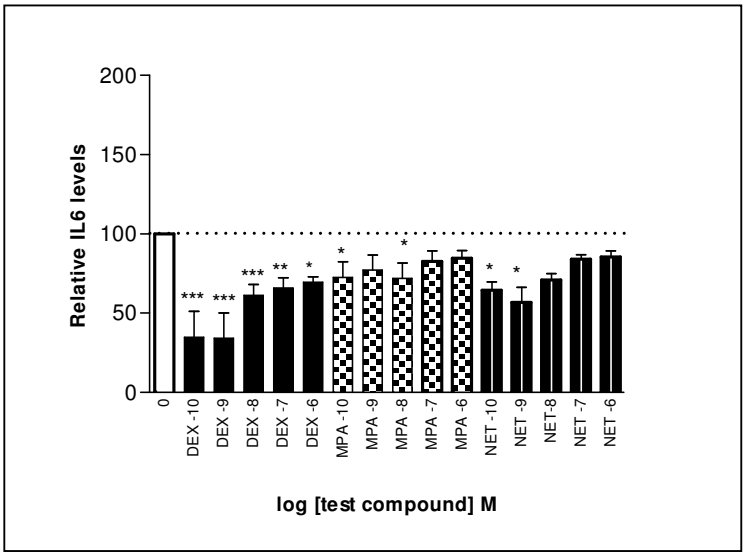


Figure D4: MPA and NET-A exert pro-inflammatory and anti-inflammatory properties. Peripheral blood was drawn from healthy volunteers. Peripheral blood mononuclear cells were isolated from peripheral blood from healthy volunteers using Ficoll-Histopaque density centrifugation. Cells were cultures in RPMI 1640 containing 10%FBS and antibiotics for one hour. Non-adherent lymphocytes were removed. Adherent monocytes were cultured for 20 hours in the absence (A) and presence (B) of 100mg/ml lipopolysaccharide (LPS), in the absence and presence of increasing concentrations of Dex, MPA or NET-A. Secretion of IL-6 was measured by ELISA. One representative experiment for each compound performed in triplicate (± S.E.M.) is shown.