An Investigation into the Molecular Mechanism of Action of the Progestins, Medroxyprogesterone Acetate and Norethisterone Acetate

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Promoter:

Professor JP Hapgood

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Declaration

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

Signature:..... Date:.....

Summary

Although the progestins medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) are widely used in reproductive therapy, the steroid receptors and their target genes involved in the actions of MPA and NET-A are not well understood. Surprisingly, it had not yet been investigated whether doses of MPA and NET-A used for contraception and HRT cause significant side effects through various target genes via the glucocorticoid receptor (GR).

In this thesis results of in vitro studies showed that, MPA, like dexamethasone (dex) and prog, significantly repressed tumour necrosis factor (TNF)-stimulated IL-6 protein production, and IL-6 and IL-8 promoter reporter constructs at the transcriptional level in L929sA cells, via interference with nuclear factor κB (NFκB) and activator protein-1 (AP-1) transcription factors. Like dex and prog, MPA did not affect NFκB DNA-binding activity. Furthermore, unlike dex and prog, MPA did not inhibit mitogen-activated protein kinase (MAPK) activity. The antagonistic effects of the GR and progesterone receptor (PR) antagonist, RU486, as well as the MPA-induced nuclear translocation of the GR, strongly suggest that the actions of MPA in these cells are mediated at least in part via the GR. Although the mechanism was not investigated as extensively as for MPA, NET-A was shown to repress IL-8 promoter reporter activity very weakly relative to dex, MPA and prog in Hek293 cells stably transfected with the rat GR. Furthermore, NET-A, like MPA, dex and prog did not interfere with the DNA-binding activity of NFκB.

Significant transactivation of a GRE-driven promoter reporter construct by MPA and dex in L929sA via endogenous GR and COS-1 cells via expressed rat GR, and by MPA, dex and prog in Hek293 cells via expressed rat GR was also observed. In contrast, NET-A, unlike MPA, dex and prog showed no transactivation in Hek293 cells.

MPA, NET-A and prog were shown to compete with dex for binding to the endogenous human GR in human lung carcinoma A549 cells. Similarly, MPA and NET-A were shown to compete with dex for binding to expressed rat GR in COS-1 cells. MPA displayed a higher relative binding affinity than NET-A for the GR in both systems, and a higher relative binding affinity than prog in A549 cells. Equilibrium dissociation constants (Ki values) for MPA (Ki = 10.8 ± 1.1 nM), NET-A (Ki = 270 ± 1.3 nM) and prog (Ki = 215 ± 1.1 nM) towards the human GR in A549 cells were also established. Furthermore, dose-response curves showed that MPA displays significantly greater GC agonist potency and efficacy than NET-A and prog for both transactivation of a synthetic GRE-reporter construct and transrepression of a synthetic IL-8 reporter construct via expressed rat GR in Hek293 cells, as NET-A showed no transactivation and very weak partial agonist activity for transrepression. Based on these observations, MPA behaves as a GR agonist whereas NET-A is proposed to be a weak antagonist. These results show that MPA and NET-A are not alike and not the same as prog in their mechanism of action via the GR, which may have serious health implications in vivo. Such insights may provide women and their clinicians with more information to facilitate the selection of contraception or reproductive therapy regimes with fewer side effects.

Samevatting

Alhoewel MPA en NET-A algemeen gebruik word in hormoontherapie, is dit nie duidelik watter steroïedreseptore en teikengene betrokke is by die werking van MPA en NET-A nie. Verrassend is dat geen studie nog gedoen is om te bepaal of die dosisse van MPA en NET-A wat gebruik word in voorbehoeding en hormoonvervangingsterapie (HVT), newe-effekte veroorsaak deur die glukokortikoïedreseptor (GR) en verskeie teikengene nie.

In hierdie tesis is in L929sA selle aangetoon dat MPA, net soos deksametasoon (dex) en prog, TNF-gestimuleerde IL-6 produksie onderdruk, en dat IL-6 en IL-8 promoter-rapporteerderkonstrukte op transkripsionele vlak onderdruk word deur middel van inmenging met NF-κB en AP-1 transkripsie-faktore. Net soos dex en prog het MPA nie die DNA-bindingsaktiwiteit van NF-κB beïnvloed nie. Anders as dex en prog het MPA egter nie MAPK aktiwiteit onderdruk nie. Die antagonistiese effekte van RU486, asook die MPA-geïnduseerde translokasie van die GR na die selkern, dui sterk daarop dat die effekte van MPA in hierdie selle ten minste gedeeltelik deur die GR geskied. Alhoewel die meganisme vir NET-A nie so breedvoerig bestudeer is as dié van MPA nie, is tog aangetoon dat, in Hek293 selle wat stabiel getransfekteer is met die rot GR, die onderdrukking van die IL-8 promoter deur NET-A baie swakker is as met dex, prog en MPA. Verder is daar ook gevind dat NET-A, net soos MPA, dex en prog, nie kon inmeng met die DNA-bindingsaktiwiteit van NF-κB nie.

Beduidende transaktivering van 'n GRE-bevattende promoter-rapporteerderkonstruk deur MPA en dex in L929sA en COS-1 selle, en deur MPA, dex en prog in Hek293 selle, is ook gevind. Daarteenoor het NET-A, anders as MPA, dex en prog, geen transaktivering in Hek293 selle getoon nie.

Verder moes die relatiewe bindingsaffiniteit (ewewigs-dissosiasiekonstantes) van MPA, NET-A en prog vir die GR, asook die relatiewe sterkte en effektiwiteit vir transaktivering en transonderdrukking van verskeie teikengene deur die GR, ook bepaal word. Daar is gevind dat MPA, NET-A en prog meeding met dex vir binding aan die endogene GR in mens longkarsinoom A549 selle. Soortgelyk hieraan is ook gevind dat MPA en NET-A meeding met dex vir binding aan rot GR wat in COS-1 selle uitgedruk is. MPA het in beide sisteme 'n hoër relatiewe bindingsaffiniteit vir die GR getoon as NET-A, asook 'n hoër relatiewe bindingsaffiniteit as prog in A549 selle. Ewewigs-dissosiasiekonstantes (Ki waardes) vir MPA (Ki = 10.8 ± 1.1 nM), NET- A (Ki = 270 ± 1.3 nM) en prog (Ki = 215 ± 1.1 nM) vir die mens GR in A549 selle is ook bereken. Dosisrespons-grafieke het ook aangedui dat MPA 'n beduidend beter GC sterkte en effektiwiteit as NET-A en prog het, vir beide transaktivering van 'n sintetiese GRE-rapporteerderkonstruk en transonderdrukking van 'n sintetiese IL-8 rapporteerderkonstruk via rot GR wat uitgedruk is in Hek293 selle. Dit kon afgelei word aangesien NET-A geen transaktivering en slegs baie swak gedeeltelike agonis-aktiwiteit vir transonderdrukking getoon het. Op grond van hierdie waarnemings tree MPA op as 'n GR agonis, terwyl dit lyk asof NET-A 'n swak antagonis is. Hierdie resultate dui aan dat MPA en NET-A nie dieselfde is nie, en ook nie dieselfde meganisme van werking deur die GR het as prog nie. Dit kan ernstige gesondheidsimplikasies inhou in vivo. Hierdie insigte kan dus meer inligting aan vroue en kliniese personeel verskaf om sodoende die keuse van voorbehoeding of voortplantingsterapie met minder newe-effekte te vergemaklik.

Format of this Thesis

The experimental work presented in this thesis is written up in manuscript format for Chapters 1, 2 and 3. The thesis is thus composed of:

(i) a literature review on the appropriate background (Chapter 1);

(ii) two manuscripts reporting and discussing the undertaken experiments and results (Chapters 2 and 3), each of which is followed by additional results not included in the manuscripts (Appendixes 1 and 2); and

(iii) a discussion of the overall results with emphasis on the implications of the study and future perspectives (Chapter 4).

The literature review presented in Chapter 1 will be submitted for review to Endocrine Reviews.

The manuscript presented in Chapter 2 has been submitted for review to Molecular and Cellular Endocrinology.

The manuscript comprising Chapter 3 will be submitted for review to Molecular Pharmacology.

Although the collective "we" and "our" is often used in this thesis, as consistent with manuscript format, the bulk of the experimental work was carried out by the candidate, with only the following exceptions. In Chapter 2, Figures 3b, 6a, 6b and 7 were performed by one of our collaborators, Wim Vanden Berghe. In Chapter 3, Figure 3a was carried out by Johann Riedemann.

Abbreviations

3βHSDII	-	type II 3β-hydroxysteroid dehydrogenase/isomerase
5-HT	-	serotonin
AF-1	-	activation function-1
AF-2	-	activation function-2
AP-1	-	activator protein-1
AR	-	androgen receptor
BMD	-	bone mineral density
C/EBP	-	CCAAT/enhancer binding protein
cAMP	-	cyclic AMP
CBG	-	corticosteroid-binding globulin
CHO	-	Chinese hamster ovary
CREB	-	cAMP-responsive element-binding protein
CRH	-	corticotropin releasing hormone
CYP3A	-	cytochrome P450 3A monooxygenase
DBD	-	DNA-binding domain
dex	-	dexamethasone
DHEA	-	dehydroepiandrosterone
DHT	-	dihydrotestosterone
EGFR	-	epidermal growth factor receptor
EMSA	-	electrophoretic mobility shift assay
ER	-	estrogen receptor
ERK	-	extracellular-regulated kinase
ES	-	human endometrial stromal
FDA	-	Food and Drug Administration

FSH	-	follicle stimulating hormone
GC	-	glucocorticoid
GdA	-	glycodelin-A
GnRH	-	hypothalamic gonadotropin-releasing hormone
GR	-	glucocorticoid receptor
GRE	-	glucocorticoid-response element
HIV	-	human immunodeficiency virus
HRT	-	hormone replacement therapy
hsp90	-	heat-shock protein 90
HSV-2	-	herpes simplex virus type 2
HUVEC	-	human umbilical venous endothelial cell
ICAM-1	-	intercellular adhesion molecule-1
IGFBP	-	insulin-like growth factor binding protein
IκB	-	inhibitory protein κB
JNK	-	c-Jun N-terminal kinase
IL-1β	-	interleukin-1 beta
IL-2	-	interleukin-2
IL-6	-	interleukin-6
IL-8	-	interleukin-8
Kd	-	equilibrium dissociation binding constant
Ki	-	equilibrium dissociation binding constant
LBD	-	ligand-binding domain
LH	-	luteinising hormone
MAPK	-	mitogen-activated protein kinase
MMTV	-	mouse mammary tumour virus
MPA	-	medroxyprogesterone acetate
MR	-	mineralocorticoid receptor

MSK-1	-	mitogen and stress activated protein kinase-1
N/C interaction	-	NTD and carboxy-terminal interaction
NEE	-	normal endometrial epithelia
NET	-	norethisterone
NET-A	-	norethisterone acetate
NET-EN	-	norethisterone enanthate
NFκB	-	nuclear factor κB
nGRE	-	negative GRE
NHLBI	-	National Heart, Lung and Blood Institute
NRF	-	National Research Foundation
NS	-	nonspecific
NTD	-	amino-terminal domain
OB-R (L)	-	long form leptin receptor
OHFL	-	hydroxyflutamide
P450c17	-	17α-hydroxylase/17, 20-lyase
P450scc	-	cholesterol side-chain cleavage enzyme
PAI-1	-	plasminogen activator inhibitor-1
PAR-1	-	proteolytically activatable thrombin receptor
PBMC	-	peripheral blood mononuclear cells
PD-ECGF	-	platelet-derived endothelial cell growth factor
PEI	-	polyethylenimine
PEPI	-	postmenopausal estrogen/progestin interventions trial
PHA	-	phytohaemagglutinin
POMC	-	pro-opiomelanocortin
PR	-	progesterone receptor
PRE	-	progesterone response element
PRL	-	prolactin

prog	-	progesterone
PTHrP	-	parathyroid hormone-related protein
PXR	-	pregnane x receptor
RANTES	-	regulated on activation, normal T cell expressed and secreted
RBA	-	relative binding affinity
RLU	-	relative light units
RU	-	RU486
SEGRA	-	selective GR agonist
SEM	-	standard error of the mean
SHBG	-	sex hormone-binding globulin
SIV	-	simian form of the HIV virus
SRC-1	-	steroid receptor co-activator 1
SRE	-	steroid response elements
STS	-	staurosporine
TAT	-	tyrosine aminotransferase
TF	-	tissue factor
TGF-β	-	transforming growth factor-beta
tk	-	thymidine kinase
TNFα	-	tumour necrosis factor alpha
VCAM-1	-	vascular cell adhesion molecule-1
VEGF	-	vascular endothelial growth factor
vs	-	versus
WHO	-	World Health Organisation
Wnt-7a	-	wingless-type mouse mammary tumour virus integration site family member 7a

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To all those I haven't mentioned; yet who have enriched my journey extraordinarily. You know who you are and I cherish our memories.

I found I had less and less to say, until finally,
I became silent, and began to listen.
I discovered in the silence, the voice of God.
SØREN KIERKEGAARD

What we are is God's gift to us;
What we become is our gift to God.
ANON

We are what we think.
All that we are arises with our thoughts.
With our thoughts,
We make the world.
BUDDHA

The miracle is not to walk on water. The miracle is to walk on the green earth,
Dwelling deeply in the present moment and feeling truly alive.
THICH NHAT HANH

I wish to dedicate this thesis to the loving memory of my dear younger brother, Rudi (17th June 1978 - 22nd September 1994), who died tragically. And to my loving father (12th December 1934 - 1st February 1997), who constantly encouraged me, and who genuinely believed in my strengths.

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Aim and Scope of this Thesis

This study is divided into two parts, which are reported in Chapters 2 and 3. The aim of the first part (Chapter 2) was to determine whether MPA, relative to dex and prog, is able to repress interleukin gene expression, with a view to gaining a better understanding of its immunosuppressive effects. There is evidence in the literature that MPA has a high affinity and is an agonist for the GR (Teulings et al., 1980; Bojar et al., 1979; Feil and Bardin, 1979)[1]. Furthermore, the affinity of the rat GR for MPA (Kd = 5.0 nM) is similar to that of dex (Kd = 4.1 nM) (Winneker and Parsons, 1981), but higher than that for its endogenous ligand, cortisol (Kontula et al., 1983). Since GCs impact on virtually every aspect of the immune and inflammatory response (Spangelo and Gorospe, 1995), we were prompted to study the GC-like repressive action of MPA, relative to dex and prog. In Chapter 2 therefore, we were interested to determine the effects of MPA on IL-6 protein production and IL-6 and IL-8 reporter construct expression and the mechanism thereof. For this purpose, mouse fibroblast (L929sA) cells were used which contain all the necessary machinery for pro-inflammatory cytokine synthesis. Furthermore, since MPA has been implicated as a promising agent for the treatment of autoimmune and/or inflammatory disease (Bamberger et al., 1999), we were also prompted to investigate its dissociative GC properties in our model system. These studies would contribute to gaining a better understanding of the molecular mechanism of action of MPA, which would provide a molecular basis for understanding its biological effects.

[1] For references, see Chapter 1.

The aim of the second part (Chapter 3) of the study was to compare the relative GC agonist properties of MPA vs. NET-A (relative to dex and prog) for transactivation of a synthetic GRE-reporter construct and transrepression of a synthetic IL-8 reporter construct via expressed rat GR in Hek293 cells. These cells were used because they express very low levels of endogenous receptors in the absence of expressed GR, and are also responsive to nanomolar concentrations of steroid. The aim was to also compare the relative binding affinity of MPA vs. NET-A (relative to dex and prog) towards the GR in human lung carcinoma (A549) cells, which contain endogenous GR. To date, no thorough comparison exists at the molecular level of the mechanism of action of MPA vs. NET or NET-A. By comparing these activities, the mechanisms by which they exert their effects should become clearer. These findings would serve to highlight the differences between these progestins, which would provide women and their clinicians with more information to facilitate the selection of method of contraception or reproductive therapy regime.

Chapter 1

A review of the mechanism of action at the molecular level of two synthetic progestins, medroxyprogesterone acetate and norethisterone acetate

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Manuscript in preparation for submission.

1. Manuscript

Summary

Medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) are two widely used progestin-only injectable contraceptives. In addition to their contraceptive uses, both MPA and NET-A are used in the treatment of endometriosis and in hormone replacement therapy (HRT), while MPA is used at higher doses in cancer therapy and as supportive therapy in anorexia/cachexia syndrome. In this review, the physiological effects of these progestins, as well as their regulation of and/or binding to serum-binding proteins and steroidogenic enzymes is discussed. In addition, their interactions with steroid receptors and their effects on various target genes is reviewed at length. Both MPA and NET have been shown to interact with a number of steroid receptors. MPA has a high affinity and is an agonist for the glucocorticoid receptor (GR), androgen receptor (AR) and progesterone receptor (PR), and has been shown to bind with low affinity to the mineralocorticoid receptor (MR). NET has been shown to bind the PR and AR, and very low affinity has been demonstrated for the GR and MR. Regarding the estrogen receptor (ER), MPA has been shown not to bind the ER, whereas for NET, evidence of estrogenic activity is unclear. It is therefore possible that MPA and NET exert therapeutic actions as well as side effects via any of these receptors. Here we review the mechanism of action, at the molecular level, of both MPA and NET via each of the above steroid receptors on various target genes.

1.1 MPA and NET-EN in contraceptive use

The injectable progestagen-only contraceptives, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) (also referred to as either norethindrone enanthate, norethisterone enantate or nuristerate), are the most widely used conventional contraceptive agents used by women in South Africa, and are both under current investigation for male contraception (Nieschlag et al., 2003). Although MPA was developed as a contraceptive during the 1960s, and approved by several countries in the late 1970s, it received FDA (Food and Drug Administration) approval for contraceptive use only in 1992. Approval was delayed due to theoretical but unproven links to breast cancer and alleged mutagenic properties (Kaunitz, 1994).

When used as an injectable contraceptive in women, MPA is administered as a 150 mg aqueous suspension (referred to as Depo Provera) every three months (Mishell, 1996), whereas NET-EN is administered as a 200 mg oily suspension every two months (Garza-Flores et al., 1991). MPA, referred to as a true progestin, is a 21-carbon series steroid, containing the pregnane nucleus whereas NET-EN is a 19-nortestosterone progestin containing the androstane nucleus, which retains androgenic activity (Darney, 1995). Both formulations are administered as intramuscular injections. After injection, MPA is fairly stable and is itself the active contraceptive compound. However, NET-EN and its acetate (norethisterone acetate, NET-A) are hydrolysed to norethindrone (NET) and its metabolites, which together with NET have contraceptive action (Stanczyk and Roy, 1990). MPA elicits its contraceptive effects by abolishing peak or ovulatory gonadotropin levels and as a consequence inhibits follicular maturation and prevents ovulation (Kaunitz, 2000;

Greydanus et al., 2001). MPA also thins the endometrium and reduces glycogen secretion to low concentrations, unable to support a blastocyst entering the endometrial cavity (Mishell, 1996). In addition, MPA maintains the cervical mucus in a thick and viscous state, which interferes with sperm penetration into the uterus (Greydanus et al., 2001). NET-EN has also been shown to block ovulation, (Bhowmik and Mukherjea, 1988), but the primary contraceptive action of NET-EN involves altering the content of cervical mucus to prevent sperm penetration (Bhowmik and Mukherjea, 1987). MPA and NET-EN therefore, are highly effective contraceptive agents in women due to their multiple sites of action. Interestingly, both MPA and NET-EN, in a formulation with testosterone, are under current investigation for male contraception. Testosterone esters that had been combined with injections of MPA or NET-EN showed severe suppression of spermatogenesis, due to suppression of gonadotropin levels, which are required for normal spermatogenesis (Nieschlag et al., 2003).

Women receiving MPA by injection as a contraceptive typically have serum concentrations of approximately 1 ng/ml for the duration of contraceptive treatment (Mishell, 1996) i.e. about 2.6 nM. Levels of drug-related material have, however been shown to reach as high as 25 ng/ml a few days post injection (Kirton and Cornette, 1974). Contraceptive doses of NET-EN have been reported to result in serum concentrations of about 1.5-59 nM (Fotherby et al., 1983). As for most drugs, both MPA and NET-EN have been shown to have several side effects including irregular menses, amenorrhea, headaches, weight gain and acne (Kaunitz, 2000; Greydanus et al., 2001; Benagiano et al., 1978; Darney, 1995). For MPA, dizziness, fatigue, bloating of the abdomen or breasts, behavioural changes, reduced libido and possibly decreased bone density have also been reported (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, in particular the

shorter time lapse before return to fertility after cessation of treatment (Benagiano et al., 1978; Koetsawang, 1991).

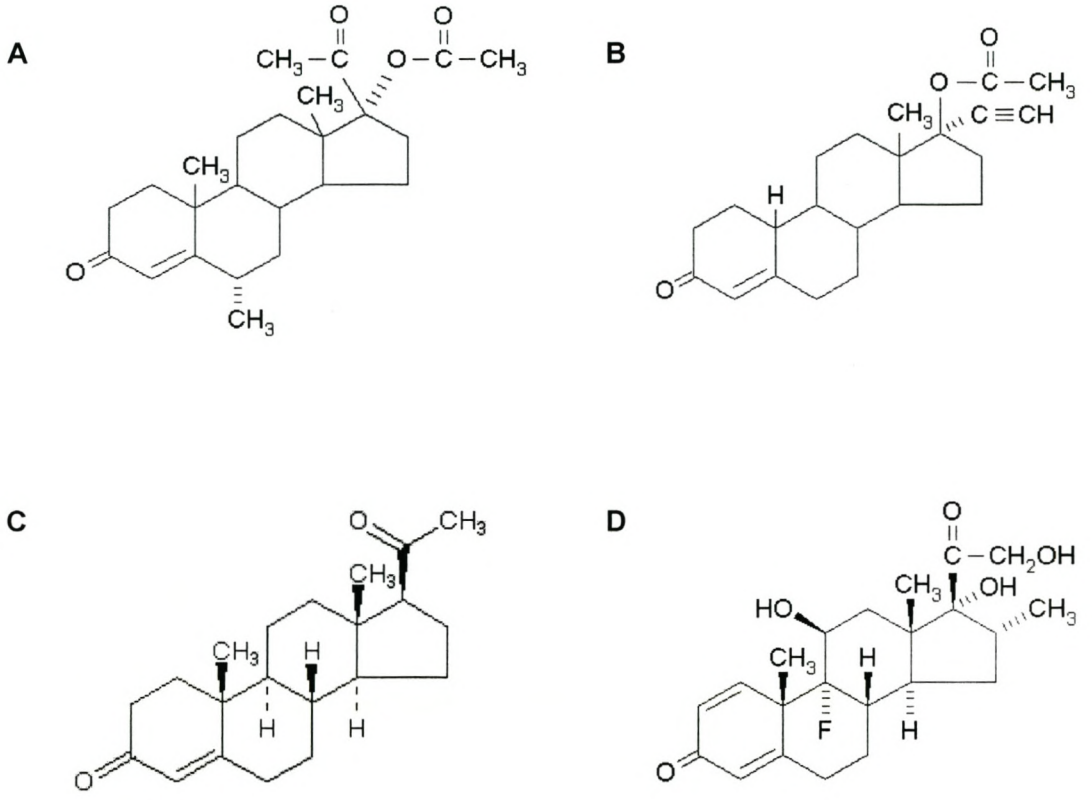

Figure 1: The chemical structures of natural and synthetic progestins, and a synthetic glucocorticoid. (A) medroxyprogesterone acetate; (B) norethisterone acetate; (C) progesterone; (D) dexamethasone.

1.2 Non-contraceptive uses of MPA and NET-A

Although MPA and NET-EN are widely employed for preventing pregnancy, these progestins have non-contraceptive uses as well. MPA is used at higher doses in cancer therapy (Etienne et al., 1992; Yamashita et al., 1996) and as supportive therapy in anorexia/cachexia syndrome (Downer et al., 1993), while both MPA and NET-A are used in the treatment of endometriosis (Irahara et al., 2001; Harrison and Barry-Kinsella, 2000; Muneyyirci-Delale and Karacan, 1998) and in hormone replacement therapy (HRT) (Brunelli et al., 1996; Taitel and Kafrissen, 1995). Doses of MPA used for cancer therapy typically range between 500 and 1500 mg orally per day for about 12 weeks (Blossey et al., 1984), for treatment of endometriosis about 50- to 100 mg/day (Harrison and Barry-Kinsella, 2000; Telimaa et al., 1989), while those used for HRT are about 10 mg/day for about 11 days (Brunelli et al., 1996). It is not clear from the literature what the typical serum concentrations of MPA are, in women on HRT. Daily doses of 5 mg NET-A and 2.5 mg NET have been reported for the treatment of endometriosis (Surrey and Hornstein, 2002; Surrey, 1999), whereas HRT doses of NET range between 0.35- to 2.1 mg orally per day (Taitel and Kafrissen, 1995). Women receiving the Activelle HRT regime (0.5 mg NET-A, estradiol 1 mg) are reported to have peak serum concentrations of NET ranging between 1.4- and 6.8 ng/ml (3.64- to 17.7 nM) (Activelle package insert reg. no. 33/21.8.2/0532, Novo Nordisk).

At high cancer therapy doses, MPA has potent glucocorticoid (GC)-like effects such as significant 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). Doses of MPA used in HRT modify

immune function in man, by affecting various immune cell subsets (Brunelli et al., 1996; Malarkey et al., 1997) and have also been shown to elicit anti-inflammatory effects (Wakatsuki et al., 2002). Furthermore, when used in the treatment of endometriosis, MPA has been shown to interfere with lipoprotein metabolism that could expose the individual to an increased risk of cardiovascular disease (Telimaa et al., 1989). Similarly, side effects of MPA and NET when used in HRT include changes in the levels of lipids, lipoproteins and vasomotion, which may increase cardiovascular risk in postmenopausal women (Sitruk-Ware, 2000). NET and MPA may also be involved in the possible increased risk of the development of breast cancer (Riis et al., 2002; Stahlberg et al., 2003). Furthermore, the National Heart, Lung and Blood Institute (NHLBI) recently terminated prematurely a major clinical trial, scheduled to run until 2005, on the risks and benefits of an HRT regime of combined estrogen and progestin (MPA) in healthy menopausal women, due to an increased risk of invasive breast cancer. A separate Women's Health Initiative study of estrogen alone continues unchanged because at this stage, the risks and benefits of estrogen are still uncertain, suggesting that MPA may be causing the increased risk. Furthermore, increases in coronary heart disease, stroke, and pulmonary embolism were observed in participants on HRT (combined estrogen and MPA) compared to women who were administered placebo pills. Although fewer cases of hip fractures and colon cancer were reported, the overall health risks were found to exceed the benefits (Rossouw et al., 2002). Recently published data by investigators working on the same trial also suggested an increase in the risk of ovarian cancer (Anderson et al., 2003).

A variety of other non-contraceptive benefits of MPA include a reduced risk of endometrial cancer, iron-deficiency anaemia, pelvic inflammatory disease, and ectopic pregnancy. With regard to endometrial cancer, a case-control study by the World Health Organisation (WHO) published in 1991 found an 80% reduction in risk

among women who had used MPA for more than 1 year prior to diagnosis. This protection was noted to be more profound than the protection associated with oral contraceptive use (Kaunitz, 2000). MPA is also used for the treatment of dysmenorrhea, menorrhagia, ovulatory pain, pain associated with ovarian disease, premenstrual dysphoria and perimenopausal symptoms (Kaunitz, 1998). Furthermore, MPA is also considered to be appropriate for mentally handicapped women who have menstrual hygiene problems. Finally, MPA has been associated with hematologic improvement in women with sickle cell disease and reduced seizure frequency in women with seizure disorders (Kaunitz, 2000). Although NET is not as widely used in reproductive therapy as MPA, NET-A is used in the treatment of acne (Zouboulis and Piquero-Martin, 2003).

1.3 Physiological effects of MPA and NET

1.3.1 Effects of MPA and NET on immune and adrenal function

On a physiological level, MPA, and to a much lesser extent NET, have been shown to exert a number of effects on immune and / or adrenal function. In the rabbit, early studies reported significant suppression of circulating antibody production due to MPA treatment (5 mg daily), to the same extent as that demonstrated with cortisol treatment (50 mg twice a week) (Hulka et al., 1965). Similarly, MPA was shown to suppress the primary humoral antibody response in rabbits and also extended survival of dogs with renal allografts as well as survival of rabbit skin allografts (Turcotte et al., 1968). 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). 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).

Surprisingly, little research appears to have been carried out in humans on the effects of MPA at contraceptive doses on immune or adrenal 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). In addition, MPA was found to compromise cell-mediated immune status in Bangladeshi women receiving these compounds as injectable contraceptives (Majumder et al., 1987). Furthermore, Jones et al., (1974) reported lowered baseline plasma cortisol levels in contraceptive users of MPA. In a later study, Aedo et al., (1981) also showed that administration of a single dose of MPA resulted in a slight but significant reduction of cortisol in normal menstruating women. However, healthy, non-lactating Thai women who received long term MPA were found to have no significant change in adrenal function (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 (Blossey et al., 1984; Papaleo et al., 1984; Lang et al., 1990; Hellman et al., 1976) and 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). Furthermore, MPA was shown to significantly reduce the proliferative response of peripheral blood mononuclear cells (PBMC) from cancer patients to phytohaemagglutinin (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). The above results are in contrast to Gronroos and Eskola, (1984) who showed no effect of MPA on

lymphocyte proliferation or on the proportion of T and B cells in endometrial cancer patients. Furthermore, MPA at 0.2 μg/ml has been shown to reduce levels of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumour necrosis factor alpha (TNFα) and serotonin (5-HT) produced in culture by PHA-stimulated PBMC from cancer patients (Mantovani et al., 1997). In addition, treatment of serotonin-induced myometrial smooth muscle cells with MPA resulted in a marked decrease in IL-1α mRNA and protein levels (Lan et al., 1999). Other studies showed that MPA at 10^{-6} M could inhibit β-chemokine production in choriodecidual cells (Kelly et al., 1997) and interleukin-8 (IL-8) production in endometrial explants and chorion cells (Kelly et al., 1994). In contrast Arici et al., (1996a) found that MPA at 10^{-7} M increased the level of IL-8 mRNA in human endometrial stromal (ES) cells.

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) infection. 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). Also, expression of CD1, a major histocompatibility class 1-like lipid antigen-presenting molecule was reduced in endometrial epithelia of BALB/c mice after treatment with MPA (Sallinen et al., 1999). In addition, significant changes were observed in the vaginal intraepithelial leukocyte population of MPA contraceptive users, which was thought to reflect altered local immune capacity (Ildgruben et al., 2003). Based on these and other studies (Stephenson, 1998), MPA may exert a negative influence on local defence mechanisms in the human female genital tract.

Collectively, the above studies raise concern, as the potential exists for MPA to exert not only local but also systemic immunosuppressive side effects in vivo. There is evidence in the literature to suggest that the use of MPA as injectable contraception has deleterious effects on human immunodeficiency virus (HIV) viral shedding, but whether MPA influences susceptibility to HIV still remains a controversial issue. 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 propose that they may be due to factors such as direct effects on the virus, effects on local genital tract physiology or effects on immune modulation of viral replication. Furthermore, progesterone implants, which could mimic hormonally based contraceptives like MPA, were shown to thin the vaginal epithelium and enhanced SIV (the simian form of the HIV virus) vaginal infection in monkeys (Cohen, 1996; Marx et al., 1996). Interestingly, female sex workers in Kenya who used MPA were found to have an increased incidence of HIV-1 infection (Martin et al., 1998). However, other studies investigating the effects of contraceptive use on the risk of HIV infection in women found no significant link (Mati et al., 1995), or the results were inconclusive (Sinei et al., 1996; Daly et al., 1994). Furthermore, a systematic review of the epidemiological evidence concerning hormonal contraception and the risk of HIV transmission to women was inconclusive as the quality of the studies reviewed was poor and inappropriate for statistical meta-analysis (Stephenson, 1998).

In contrast to what is known for MPA, very little is known about the effects of NET on immune function and to our knowledge, nothing to date on adrenal function. Early studies in animal models on the effects of 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). In the guinea pig, like MPA, NET decreased splenic macrophage Fcgamma receptor expression, which impaired 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).

1.3.2 Effects of MPA and NET on reproduction

Hypothalamic gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and binds to the GnRH receptor on the cell surface of pituitary gonadotropes. There it activates intracellular signalling transduction pathways, which stimulate the synthesis and release of luteinising hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins enter the systemic circulation to regulate gonadal function, including steroid hormone synthesis and gametogenesis (Ganong, 1991). Although the effect of MPA and NET on the regulation of GnRH expression has not yet been determined, a few studies have investigated the effects of MPA and to a lesser extent NET, on LH, FSH and steroid hormone levels. One early study measured the peripheral blood levels of FSH, LH and estradiol after intramuscular injection of a contraceptive dose of MPA in normal women. The levels of all three hormones remained in the range of the early follicular phase of the untreated cycle and ovulation was suppressed due to suppression of the LH peak. Interestingly, no suppression of basal gonadotropin levels was reported in any of the women, an effect which likely contributes to lack of menopausal-like symptoms in women receiving MPA (Jeppsson and Johansson, 1976). In another study, sixteen days after a 150 mg MPA administration, normally menstruating women showed a decline in plasma levels of estradiol, progesterone and 17α-hydroxyprogesterone to early follicular phase levels (Aedo et al., 1981). MPA administered at higher doses

(daily doses of 2000 mg every 12 hours for 30 days, 60 mg daily for 14 days or 5 mg daily for 12 days for each respective study) to post menopausal women resulted in reduced plasma LH and FSH levels (Sala et al., 1978; Saaresranta et al., 2002; Castelo-Branco et al., 1994), which increased after cessation of treatment (Castelo-Branco et al., 1994). Similarly, MPA and NET administered at contraceptive doses to post menopausal women significantly inhibited serum LH and FSH activity (Perez-Palacios et al., 1981a). Furthermore, no LH surge or ovulation was detected in patients involved in in vitro fertilisation studies who were administered 1 mg of NET over a 5-day period (Letterie, 2000).

Furthermore, MPA and to a much lesser extent NET, have been shown to influence expression of a number of genes involved in various endometrial functions. Examples of such genes include tissue factor (TF) (Krikun et al., 2000), decidual cell-expressed plasminogen activator inhibitor 1 (PAI-1) (Lockwood, 2001) and transforming growth factor-beta (TGF-β) (Arici et al., 1996b). TF, a cell membrane-bound glycoprotein, is responsible for the initiation of hemostasis and is associated with decidualisation in the uterus. MPA at 10^{-7} M was shown to significantly enhance TF gene transcription in ES cells, an effect mediated principally by Sp1 transcription factor binding to its site on the TF promoter (Krikun et al., 2000), and likely involving activation of epidermal growth factor receptors (EGFRs) (Lockwood et al., 2000). Furthermore, decidual cell-expressed PAI-1 plays a role in preventing haemorrhage during human pregnancy implantation. MPA plus estradiol, in the absence and presence of EGFR, was shown to enhance PAI-1 protein levels 8- and 65-fold respectively, in human ES cells (Lockwood, 2001). Another gene involved in endometrial functions and regulated by MPA is TGF-β, which is believed to play a role in the predecidualisation of human ES cells and in the completion of decidualisation after blastocyst implantation. In this study, the regulation of TGF-

β1 and TGF-β3 mRNA levels in human ES cells in culture was investigated. Treatment of ES cells with 1 nM MPA resulted in reduced levels of TGF-β3 mRNA, yet only a small increase in the level of TGF-β1 mRNA was observed (Arici et al., 1996b). Reis et al., (2002) however, observed increases in TGF-β3 gene and protein expression in endometrial samples from women having received higher MPA doses of 10 mg/day. Interestingly in another study, MPA did not affect TGF-β2 and TGF-β3 mRNA levels in MCF-7 human breast carcinoma cells, whereas a dramatic decrease was observed with NET (Jeng and Jordan, 1991).

1.3.3 Effects of MPA and NET on reproductive cancers

There is evidence in the literature to suggest that the use of MPA as a contraceptive does not increase breast cancer risk, and is associated with an 80% reduction of endometrial adenocarcinoma, a level of protection greater than that noted with oral contraceptives (Kaunitz, 1996). Similarly, Dillis and Schreiman, (2003) postulated a suppressive effect of MPA on breast density in women receiving contraceptive injections. Furthermore, MPA contraceptive use appears not to affect the risk of epithelial ovarian cancer or cervical neoplasia (Kaunitz, 1996). However, in contrast to the results of the above studies, recent clinical evidence suggests that MPA in HRT use increases the risk of breast (particularly) and ovarian cancers (Rossouw et al., 2002; Anderson et al., 2003), whereas no increased risk of endometrial cancer has been reported (Anderson et al., 2003). NET used in HRT has been reported to protect against the hyperplastic effects of estrogen treatment on the endometrium (Riis et al., 2002) but whether it confers an increased risk of breast cancer remains to be determined (Stahlberg et al., 2003). To our knowledge, nothing is known about the effects of contraceptive doses of NET on the development of breast or uterine cancers. Further investigation is therefore required to determine

whether NET (in contraceptive or HRT use) facilitates the development of reproductive cancer in women.

A number of studies have been undertaken in cell lines and tissue samples to determine further the effects and mechanisms of action of MPA and NET on uterine and breast cancer. Consistent with the clinical evidence suggesting no deleterious effect of MPA on the development of uterine cancers, MPA (at 10^{-8} M), unlike estradiol, did not increase the expression of platelet-derived endothelial cell growth factor (PD-ECGF), an angiogenic factor, in Ishikawa cells (Aoki et al., 2003). 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 protooncogene, c-fos is one of the putative mediators of estrogen-induced endometrial proliferation. Although NET in HRT use has been shown to have protective effects on the endometrium, its A-ring reduced derivatives (at 100 μg/100 g body weight) were able to significantly increase mRNA content of c-fos in ovariectomised rats, although this increase was lower than that found with estradiol treatment. 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 analysing the effects of NET-A on wingless-type mouse mammary tumour virus integration site family member 7a (Wnt-7a) gene expression in normal endometrial epithelia (NEE) cells showed for the first time upregulation of Wnt-7a gene expression in estrogen treated NEE cells. Upregulation of Wnt-7a is thought to be associated with the antineoplastic effects of progestins on the endometrium (Oehler et al., 2002). Although the above in

vitro studies are consistent with the clinical evidence that MPA does not increase the risk of endometrial cancer, the in vitro results for NET are inconclusive, and inconsistent with the clinical evidence.

Although a number of in vitro studies have been carried out to determine the effects of MPA and NET on breast cancer, for MPA the in vitro results do not correlate with the recently available clinical evidence suggesting an increased risk for HRT users. For NET the in vitro results are contradictory and definite conclusions cannot be drawn. Cyclin D1, which is required for cell cycle progression in the G1 phase of the cell cycle, has been implicated in the pathogenesis of breast cancer. After 24 hours of MPA (2.5×10^{-7} M) stimulation, an increased expression of cyclin D1 protein was observed, while at 72 hours the protein levels were barely detectable. Similar results were obtained at the promoter level. 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 (Thuneke et al., 2000). Similarly, endogenous RNA expression levels of calcyclin were inhibited by 10^{-7} M MPA in both human mammary carcinoma cells and hamster fibroblasts. Calcyclin is a cell cycle-related gene, which seems to play a role in the pathogenesis of some malignancies (Ghezzo et al., 1997). Furthermore, the level of parathyroid hormone-related protein (PTHrP) expressed in breast cancer tissue is closely associated with the incidence of bone metastasis. PTHrP mRNA expression was investigated in breast tissues incubated for 24 hours with 10^{-5} M MPA. MPA was shown to decrease PTHrP expression significantly, confirming its therapeutic effect in the treatment of breast cancer with bone metastases (Sugimoto et al., 1999). In a study in MCF-7 cells, MPA was shown not to affect TGF-β2 and TGF-β3 mRNA levels, whereas a dramatic decrease was observed with NET. 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. It was further suggested that NET in contraception might facilitate the development of breast cancer (Jeng and Jordan, 1991). In another study in MCF-7 cells, MPA and NET investigated in the range of 0.01 nM to 10 μM displayed significant inhibition of cell proliferation between 20- and 25% for MPA and between 23- and 41% for NET over the whole concentration range tested (Seeger et al., 2003). The above in vitro studies, in contrast to recent clinical evidence for MPA, seem to indicate that MPA may reduce the risk of breast cancer, while the effects of NET are not clear.

1.3.4 Effects of MPA and NET on skeletal function

Although the mechanism is poorly understood, a decline in bone mineral density (BMD) in women with long-term contraceptive use of MPA has been reported in a number of studies. This decrease in bone density tends to be greatest in women who start MPA use at an early age, and in those whose duration of use exceeds 15 years (Cundy et al., 2003; Cundy et al., 1991; Cromer et al., 1996; Paiva et al., 1998; Gbolade et al., 1998; Cundy et al., 1998; Scholes et al., 1999; Tang et al., 1999). Attainment of peak bone mass during adolescence is one of the primary determinants of osteoporosis risk in post-menopausal women. Concern therefore exists among clinicians that adolescents using MPA are losing bone mineralisation at a time in life when they should be experiencing a significant increase. Studies have shown that BMD in groups of adolescent women using MPA as a contraceptive was suppressed significantly (Cromer et al., 1996), especially in the femoral neck and in the lumbar spine (Busen et al., 2003). MPA acts by inhibiting pituitary gonadotropin secretion and thus reduces ovarian estrogen production (Jeppsson et al., 1982). The cause of MPA-associated bone loss is not certain, but one plausible explanation is that it is a consequence of estrogen deficiency. In the study by Cundy et al., (2003),

estrogen replacement therapy arrested MPA-related bone loss in premenopausal women with a minimum 2 year MPA use who had a below average baseline lumbar spine BMD. However, according to Westhoff, (2002), the relationship between MPA and changes in bone mineral density remains controversial despite a substantial number of studies evaluating this potential association. This author also reported that if bone density loss does occur in the presence of MPA, it is reversible and suggests further that the benefits of MPA as a contraceptive outweigh the risks involving bone mineral density (Westhoff, 2002). In addition, pre-menopausal 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, in HRT, MPA (20 mg/day) alone could not arrest spinal bone loss in postmenopausal osteoporosis. Yet, when MPA (10 mg/day) was 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 (The Writing Group for the PEPI, 1996). In addition, results from the Women's Health Initiative trial demonstrated that estrogen (0.625 mg/day) plus MPA (2.5 mg/day) increased BMD and reduced the risk of fracture in postmenopausal women (Cauley et al., 2003).

A number of studies have investigated the effects of NET oral contraceptive use on bone turnover. 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 effects of NET in HRT have also been investigated. On the one hand, 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. NET (5 mg/day) was also shown to prevent bone loss in postmenopausal osteoporosis by decreasing bone turnover (Horowitz et al., 1993). On the other hand, another study was unable to show a consistent increase in markers of bone formation in postmenopausal women treated with 5 mg NET-A for 5 weeks (Onobrakpeya et al., 2001). From the above data, there appears to be sufficient evidence to support a link between MPA contraceptive use and a reduction of bone mineral density. NET in oral contraceptive formulation together with estradiol has also been reported to have positive effects on bone mass. Whether injectable progestin only contraceptive doses of NET have similar effects on bone requires further investigation. In HRT, both MPA and NET-A in combination with estradiol appear to have positive effects on bone in postmenopausal women.

1.3.5 Effects of MPA and NET on cardiovascular function

Recent randomised clinical trials have suggested that estrogen plus progestin in HRT regimes does not confer cardiac protection and may increase the risk of coronary heart disease in postmenopausal women (Rossouw et al., 2002). In particular, the Women's Health Initiative included a randomised trial where postmenopausal participants received daily doses of estrogen (0.625 mg) and MPA (2.5 mg) and showed an elevated risk of coronary heart disease (Manson et al., 2003). The use of sex steroids in contraception or HRT has also been associated

with an increased risk of cardiovascular thromboembolic complications. Treatment of vascular smooth muscle cells with MPA, in contrast to NET, upregulated proteolytically activatable thrombin receptor (PAR-1) expression, which markedly potentiated the vascular procoagulent effects of thrombin, an effect thought to be due to the GC-like activity of MPA. In addition, long-term administration of MPA to ovariectomised rats increased PAR-1 protein level in the arterial wall, which resulted in increased responsiveness of isolated aortic rings to thrombin (Herkert et al., 2001). Furthermore, progestin addition to estradiol replacement therapy may result in a decline of beneficial effects on vasculature. MPA and NET were investigated for their effects on various markers of endothelial function. MPA and NET did not interfere negatively with estradiol-induced benefits including an increase in endothelial prostacyclin production, a decrease in the synthesis of endothelin, PAI-1 and adhesion molecule concentrations. MPA and NET also enhanced the positive effect of estradiol on the precursor of matrix metalloproteinase-1. Interestingly however, MPA unlike NET antagonised the estradiol-induced significant reduction of monocyte chemoattractant protein-1. These results indicate that progestins may differ in their effects on early stages of atherosclerosis (Mueck et al., 2002). NET-A (0.5 mg) in combination with estradiol (1 mg) in HRT has been reported to result in increases in blood pressure (Activelle package insert reg. no. 33/21.8.2/0532, Novo Nordisk). In another study, NET-A in HRT was associated with an increased risk of stroke among hypertensive but not normotensive women. The authors suggested that hypertensive postmenopausal women should avoid using HRT regimes containing NET-A (Lokkegaard et al., 2003). Furthermore, the effect of MPA and NET-A on cytokine stimulated human umbilical venous endothelial cell (HUVEC) expression of adhesion molecules was investigated. Both MPA and NET-A dose-dependently (10^{-10} to 10^{-8} M) increased expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Since monocyte adhesion to endothelial cells is an important initial event at the onset of atherosclerosis, the above results suggest

that MPA and NET may contribute unfavourably to this disease (Tatsumi et al., 2002).

1.4 Targets of MPA and NET – mechanisms of action

In reviewing their mechanism of action, MPA and NET have been shown to exert effects at various levels. Firstly, as both MPA and NET are known to 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), they could potentially alter serum concentrations and thus bioavailability of endogenous steroids to various target tissues. Secondly, MPA and NET have also been shown to influence enzymes involved in the steroidogenesis pathways (Lee et al., 1999; Anderson et al., 1990) and thirdly, MPA and NET are able to regulate an array of genes by binding to various steroid receptors (see sections 1.4.3 and 1.5). Below we briefly review what is known on the molecular level on the effects of MPA and NET on serum-binding proteins and steroidogenesis. Thereafter we focus in detail on reviewing the known effects of MPA and NET via steroid receptors.

1.4.1 Serum-binding proteins

Once steroids enter the bloodstream, they immediately interact with several serum-binding proteins, and a state of dynamic equilibrium is established that determines how they become distributed between the various protein-bound fractions and the non-protein bound or free fraction. 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 serum-binding affinity and specificity (Hammond, 2002).

CBG is mainly synthesised in the liver (Scrocchi et al., 1993) and is the major transport protein for natural GCs in all mammalian species including humans (Hammond, 1990). Human CBG also displays a relatively high affinity for progesterone and MPA (Pugeat et al., 1981; Hammond, 2002). 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 act as a buffer against potentially harmful effects of elevated GCs on tissues (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). Little is known about the effects of MPA or NET on CBG gene regulation. One study showed that MPA at 10^{-6} M 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). Furthermore, in addition to regulating CBG levels, MPA, to a greater extent than NET, has been shown to bind to CBG (Pugeat et al., 1981). This may result in the displacement of endogenous steroids and thereby increase their biological availability to tissues.

SHBG is produced by hepatocytes (Janne et al., 1998) and is a homodimeric glycoprotein that binds testosterone and estradiol with high affinity (Hammond, 2002). The primary function of SHBG is to transport biologically active androgens and estradiol in the blood, and it also plays an important role in regulating the free

fraction of these steroids (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 steroids (testosterone and estradiol) to target cells that are not in the immediate vicinity of the blood supply. MPA has been shown to influence SHBG gene regulation. In the Ishikawa human endometrial cancer cell line, low (10^{-10} M) MPA concentrations in addition to estradiol (10^{-8} M) were found to increase SHBG mRNA expression, whereas the addition of high concentrations (10^{-6} to 10^{-5} M) of MPA with or without estradiol (10^{-8} M) 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). 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). Both NET-A and NET have also been shown to influence SHBG levels in a number of studies. 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). In contrast, NET-A administration of up to 1 mg/day in combination with estradiol hemihydrate (2 mg/day) in postmenopausal women showed no variations in serum levels of SHBG (Biglia et al., 2003). Furthermore, 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 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). As discussed for CBG above, this may

result in the displacement of endogenous steroids from SHBG, which may increase their bioavailability to target tissues.

1.4.2 Effects of MPA and NET on steroidogenesis

The mechanisms by which MPA and NET inhibit human steroidogenesis are not well investigated. Steroid hormones are derived from cholesterol by a stepwise removal of carbon atoms and hydroxylation and are synthesised by cells of the adrenal cortex (in the case of GCs and mineralocorticoids) and the gonads (in the case of estrogens, progestins and androgens). For a thorough description on the synthesis of steroid hormones, refer to Zubay, (1993) and Lee et al., (1999). MPA was investigated for its ability to inhibit the first three human enzymes in steroidogenesis: the cholesterol side-chain cleavage enzyme (P450scc), the 17α-hydroxylase/17, 20-lyase (P450c17), and type II 3β-hydroxysteroid dehydrogenase/isomerase (3βHSDII). Briefly, P450scc converts cholesterol to pregnenolone. P450c17 catalyses the 17α-hydroxylase reaction using pregnenolone and progesterone as substrates to form 17-hydroxypregnenolone and 17-hydroxyprogesterone respectively. P450c17 also converts 17-hydroxypregnenolone to dehydroepiandrosterone (DHEA). 3βHSD converts pregnenolone, 17-hydroxypregnenolone and DHEA to progesterone, 17-hydroxyprogesterone and androstenedione respectively. MPA at 10 to 100 μM was shown to have no effect on P450scc activity in intact human choriocarcinoma JEG-3 cells. Similarly, concentrations of up to 100 μM MPA failed to inhibit P450c17 expressed in yeast or human adrenal microsomes. Finally, MPA (1 to 100 μM) showed substantial inhibition of 3βHSDII activity in yeast microsomes. These findings propose the mechanism of action of MPA to be inhibition of human steroidogenesis (Lee et al., 1999). NET, however, was shown not to inhibit ovarian steroidogenesis in vivo and in

vitro (Anderson et al., 1990). To our knowledge, it is unknown whether NET has an effect on adrenal steroidogenesis.

1.4.3 Steroid receptors

The steroid hormone receptor family is comprised of the glucocorticoid (GR), androgen (AR), progesterone (PR), estrogen (ER) and mineralocorticoid (MR) receptors. These are ligand-dependent transcription factors that display a high level of similarity with regards to their structure and mechanism of action. In general, the receptor members share a variable amino-terminal transactivation domain (NTD); a central and well conserved DNA-binding domain (DBD), which interacts with the DNA response elements, and a moderately conserved carboxy-terminal domain, which contains the ligand-binding domain (LBD) to which the steroid hormone binds. Furthermore, these receptors also possess transcriptional activation functions located in the NTD and LBD. It is generally accepted that in the absence of ligand, steroid receptors are coupled to heat-shock proteins and reside in the cytoplasm. Once bound by steroids these (mostly cytoplasmic) receptors are activated and translocate to the nucleus where they bind to specific DNA sequences, called response elements, and subsequently regulate gene expression (Evans, 1988; Beato, 1989).

1.4.3.1 GR mechanism of action

GCs are steroid hormones secreted from the adrenal glands in response to various environmental stimuli. They mediate development and growth, regulate glucose and mineral homeostasis and modulate stress, inflammatory and immunological responses (Jenkins et al., 2001). Due to their lipophilic nature, they diffuse freely across cell membranes and exert their effects by binding with high

affinity to the GR. In an unbound state, the GR resides in the cytoplasm and is complexed with several different protein factors including heat-shock protein 90 (hsp90) (DeRijk et al., 2002) and immunophilins (Davies et al., 2002). Upon hormone binding, receptor activation and phosphorylation occurs, resulting in substitution of one immunophilin (FKBP-51) for another (FKBP-52), as well as recruitment of the transport protein dynein. This GR-hsp90-FKBP-52-dynein complex then translocates from the cytoplasm to the nucleus where it dissociates, releasing GR to either transactivate or transrepress specific genes (Davies et al., 2002).

There are at least three mechanisms through which GCs can regulate gene transcription. The first involves activation of genes through binding of GC-activated GR homodimers to consensus sequences referred to as GC-response elements (GREs) found in the promoters of various genes (Adcock, 2000; Webster and Cidlowski, 1999; Newton, 2000). The consensus sequence for GRE binding is the palindromic 15bp sequence GGTACAnnnTGTTCT, where n is any nucleotide (Adcock, 2000). Many genes contain GREs in their promoters, including for example 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 mouse mammary tumour virus (MMTV) (Scheidereit et al., 1983).

The second mechanism of GC-mediated regulation involves repression of target genes through a direct interaction of GC-bound GR to variable negative GRE (nGRE) sequences (ATYACnnTnTGATCn) within the promoter (Adcock, 2000). Detailed footprinting revealed that the function of nGREs is to instruct GRs to bind as monomers (Radoja et al., 2000). nGREs are present in the promoters of various genes, including the ACTH precursor gene, pro-opiomelanocortin (POMC) (Drouin et al., 1993) and the corticotropin releasing hormone (CRH) gene (Malkoski and Dorin,

1999). For the POMC gene, the mechanism for GR-mediated repression may involve either protein-protein interactions with other factors (for example NUR77) or may be due to steric hindrance due to the close proximity of GR binding to the TATA box and transcription start site. This may block binding of other factors required for gene activation (Murphy and Conneely, 1997).

Thirdly, GCs may inhibit gene expression by transcriptional cross-talk where the GR mutually interferes with other signalling pathways such as those involving activator protein-1 (AP-1), nuclear factor κB (NFκB), CCAAT/enhancer binding protein (C/EBP), cyclic AMP (cAMP)-responsive element-binding protein (CREB), p53 and Smad (Webster and Cidlowski, 1999; Karin, 1998; De Bosscher et al., 2003). This may require direct or indirect binding of the GR monomer to these transcription factors. 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, which do not contain nGREs, have been reported to be repressed in this way (De Bosscher et al., 2003).

1.4.3.2 GR isoforms

An additional level of complexity of steroid receptor action involves alternative splicing of the human GR pre-mRNA, which generates two highly homologous isoforms, known as hGRα and hGRβ. hGRα is a 777 amino acid ligand-activated transcription factor which, when to bound to hormone, modulates the expression of GC-responsive genes (Hollenberg et al., 1985; Bamberger et al., 1995). In contrast, hGRβ (742 amino acids) does not bind GCs and is transcriptionally inactive. hGRβ has been shown to inhibit the effects of hormone-activated hGRα on a GC-responsive reporter gene in a concentration-dependent manner (Bamberger et al.,

1995). Furthermore, two forms of the hGRα, GR-A and GR-B, are produced by alternative translation of the same mRNA. Both receptors were shown to display similar subcellular localisation and nuclear translocation after ligand activation. Interestingly, functional analyses of these splice variants showed that the shorter hGR-B was nearly twice as effective as the longer hGR-A in transactivation, but not in transrepression (Yudt and Cidlowski, 2001).

1.4.3.3 Functional domains of the GR

As described above briefly, steroid receptors have several functional domains (see Figure 2). The NTD in the hGRα consists of amino acids 1-420, followed by the DBD, which consists of amino acids 421-488. The LBD, at the carboxyl terminus, consists of amino acids 527-777 and is separated from the DBD by a hinge region. The LBD of both the hGRα and hGRβ are identical up until amino acids 727, whereas after that the structures begin to diverge (DeRijk et al., 2002). Within this hinge region is the site of monomer interaction or the dimerisation domain. The GR is folded in such a manner that two zinc fingers protrude from the surface enabling interaction with DNA (Adcock, 2000). The two zinc fingers form a single structural domain consisting of two α-helices, one adjacent to each zinc finger (Baumann et al., 1993). Once binding to DNA has occurred, the NTD (also known as τ_1 or AF-1 for activation function-1) is involved in transactivation of genes and may also be involved in binding to other transcription factors (Adcock, 2000). In the human GR, there is another transactivating domain (τ_2 or AF-2), which also plays an important role in nuclear translocation of the receptor (Adcock, 2000).

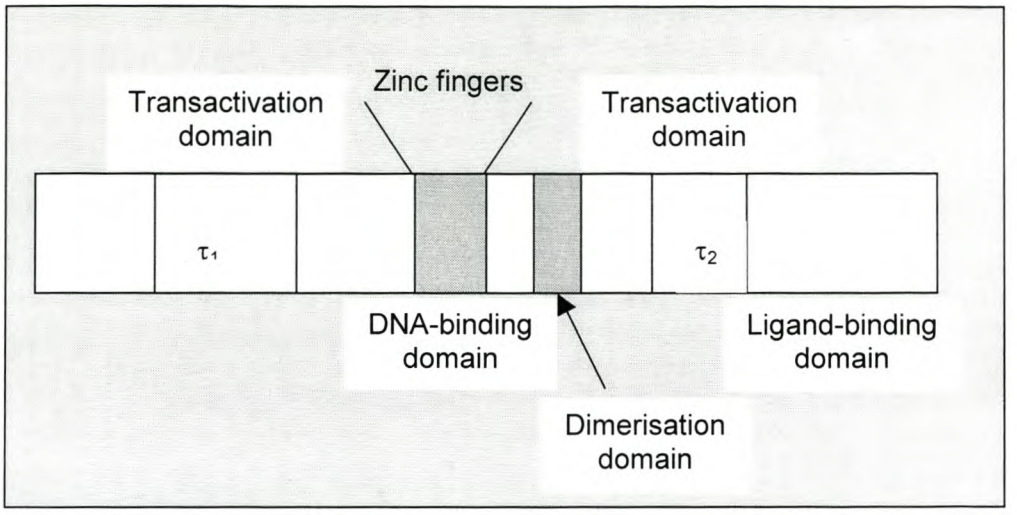

Figure 2: A schematic representation of the structural and functional domains of the glucocorticoid receptor (from Adcock, 2000).

1.4.3.4 AR, PR and MR mechanism of action

The AR, PR, MR and GR, display a high level of similarity with regards to their structure and mechanism of action. Synthetic GREs, also referred to as steroid response elements (SREs), are therefore able to mediate induction by progesterone, androgens and mineralocorticoids, in addition to GCs, via the PR, AR and MR, respectively. Furthermore, natural elements such as those found in the MMTV promoter are also able to mediate induction by the above hormones, via their respective receptors (Beato, 1989).

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 suppression of genes. This antagonism involves direct protein-protein interactions between the steroid receptors and NFκB (Palvimo et al., 1996; Kalkhoven et al., 1996).

1.5 Mechanism of action of MPA and NET on target genes via various steroid receptors

As both compounds are progestins, the contraceptive effects of MPA and NET-EN are generally assumed to be mediated via the PR. However, MPA has also been shown to bind with high affinity to the GR, AR, and PR (Teulings et al., 1980; Bentel et al., 1999; Kemppainen et al., 1999; Bojar et al., 1979; Bergink et al., 1983; Feil and Bardin, 1979) and has been shown to be an agonist for transactivation (Bamberger et al., 1999; Bentel et al., 1999; Kemppainen et al., 1999; Mueller et al., 2003; Gao et al., 2000), and an agonist for transrepression via these receptors (Bamberger et al., 1999; Zhao et al., 2002). MPA has also been shown to bind to the MR (Wambach et al., 1979). Binding and agonist activity for transactivation by NET has been demonstrated for the PR and AR (Deckers et al., 2000; Bergink et al., 1983; Bergink et al., 1985), and very low affinity has been shown for the GR (Kontula et al, 1983; Schoonen et al., 2000) and MR (Wambach et al., 1979). Regarding the ER, MPA has been reported not to have estrogenic effects (Markiewicz and Gurpide, 1994) and has also been shown not to bind to the ER (Teulings et al., 1980; Bergink et al., 1983). For NET, evidence of estrogenic activity is conflicting. On the one hand and in contrast to MPA, 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 metabolite of NET (3β, 5α-NET) has been shown to bind to the ER (Chavez et al., 1985). On the other hand, NET has been reported to

have no binding or transactivation activities via the ER (Schoonen et al., 2000; Bergink et al., 1983).

Despite their widespread use and many side effects, the target genes and precise molecular mechanisms of action of MPA and NET are not well defined. Below we review what is known on the mechanism of action at a molecular level of these two compounds via the GR, AR, PR and MR.

1.5.1 MPA and NET effects via the GR

A collection of data exists showing MPA to interact with the GR (Kontula et al., 1983; Teulings et al., 1980; Bojar et al., 1979; Selman et al., 1996; Bamberger et al., 1999; Winneker and Parsons, 1981). One study found that MPA displays considerable binding affinity towards the receptor (42%, relative to 100% binding by dexamethasone (dex), calculated from EC50 curves at the 50% competition level) in human mononuclear leukocytes. Interestingly, in the same study the relative binding affinity of the naturally occurring ligand, cortisol, to the receptor was significantly lower (25%) than that of MPA (Kontula et al., 1983). Similar results from EC50 curves for MPA binding to the GR were obtained in human renal cell carcinoma (Bojar et al., 1979) and human breast cancer cells where the molar excess of competitor (MPA) that caused 50% displacement of radioactive ligand was estimated as a measure of affinity of MPA for the GR (Teulings et al., 1980). A Kd value (31 nM), which is a more accurate measure of binding affinity, was also obtained for MPA binding to the GR in human mononuclear leukocytes, and was shown to be only 3 times higher than that of dex (Kd = 10 nM) (Kontula et al., 1983). In the beagle dog, a Ki value of 3.7 nM was calculated for MPA and was shown to be only 4-5 times higher than that of dex, also demonstrating a relatively high affinity for the GR (Selman et al., 1996). In another study, the affinity of the rat GR for MPA (Kd = 5.0 nM) was found to be

similar to that of dex (Kd = 4.1 nM) (Winneker and Parsons, 1981). In contrast to MPA, NET has been shown to bind with low affinity (0.1% relative to 100% binding by dex, calculated from EC50 curves) and was also unable to displace [^{3}H] MPA from the human GR (Kontula et al., 1983). In another study similar results were obtained where NET displayed very low binding activity (below 1%, where dex = 100%) towards the human GR in leukemic IM-9 cells (Schoonen et al., 2000). The above results indicate that in contrast to the volume of data available for MPA, very little is known about NET binding to the GR. Furthermore, no comparison of the relative binding affinities of the two progestins has been reported. Further studies are therefore required to determine precise Ki values for both MPA and NET towards the GR.

The repressive influence via the GR of MPA and to a much lesser extent NET, on in vitro functions of human lymphocytes has also been investigated. Evidence for GC-like MPA effects on lymphocyte function includes a recent study where MPA (at $2.5x10^{-7}$ M) was shown to repress transcription of a human interleukin-2 (IL-2) promoter reporter construct, stimulated with phorbol ester (TPA) and ionomycin, in normal human lymphocytes, to the same extent as dex. These observed effects of MPA are likely to be mediated by the GR, as human lymphocytes express GR but no PR, and very low levels of AR (below the detection limit of the Western blotting assay). MPA at $2.5x10^{-7}$ M was also shown to repress transcription of the IL-2 promoter construct stimulated with TPA and ionomycin, via co-transfected GR in Jurkat T-lymphoma cells. At the protein level, MPA at $2.5x10^{-7}$ M markedly suppressed TPA and ionomycin-induced IL-1, IL-2 and IL-6 protein production in normal human lymphocytes (Bamberger et al., 1999). Furthermore, 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). 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 many other target genes such as IL-6 and IL-8, in addition to those reviewed above, involved in immune function. Moreover, 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 prolactin (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 argued that orally administered MPA at high or contraceptive doses decreases bone density due to its significant GC activity. The authors proposed that bone loss associated with MPA administration 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).

Since many of the side effects of conventional GCs used to treat inflammatory or autoimmune diseases can 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. Bamberger et al., (1999) showed that in normal human lymphocytes, MPA (at 2.5×10^{-7} M) acts as a dissociated GC, since it efficiently transrepresses phorbol ester-stimulated IL-2 and IL-6 transcription, but only marginally activates transcription via GREs. Furthermore, in a cancer treatment study, MPA (over a 10 nM to 1 μM dose-range) was also shown to elevate protein expression of Nm23-H1, a metastasis suppressor, 3-fold in metastatic human breast carcinoma cells via the GR and thought to involve GREs (Ouatas et al., 2003). Although NET has been shown to display no transactivation activity towards the GR

in leukemic IM-9 cells (Schoonen et al., 2000), it has not yet been established in this or any other system whether it displays dissociative GC properties. Further work is therefore required to characterise pharmacologically in terms of potency and efficacy the effects of MPA vs. NET on GR-mediated gene regulation for both transrepression and transactivation of various target genes in the same system.

1.5.2 MPA and NET effects via the AR

A number of studies show that MPA binds to and is an agonist for transactivation via the AR (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; MacLaughlin and Richardson, 1979; Bergink et al., 1983). In the study by Hackenberg et al., (1993) the Kd of MPA for the AR was determined as 3.6 nM in MFM-223 human mammary cancer cells compared to a Kd of 0.18 nM for dihydrotestosterone (DHT), the endogenous ligand for the AR. Similarly, the Kd of MPA for the cytosolic AR from rat pituitary and hypothalamus was determined as 1.7 nM and 2.9 nM respectively (Perez-Palacios et al., 1983).

The mechanism of AR activation by MPA has recently been investigated. A two-hybrid protein transfection assay in Chinese hamster ovary (CHO) cells showed that MPA (up to 1 μM) failed to promote an NH_2- and carboxy-terminal (N/C) interaction of the AR, which is usually induced by high-affinity agonists and believed to be inhibited by antagonists. MPA was therefore proposed to activate the AR through a mechanism that does not involve the N/C interaction. Although not an antagonist, MPA was also shown to inhibit the DHT-induced AR N/C interaction at low concentrations (as low as 1 nM) in the same cells. Furthermore, MPA (at 0.1 nM) was shown to be able to induce transcriptional activation of a MMTV-luciferase

reporter construct in CV-1 monkey kidney cells via the AR. Agonist potency tended to parallel the ligand-induced N/C interaction as lack of ligand-induced N/C interaction by MPA was associated with 100-fold higher MPA concentrations relative to DHT necessary for transactivation (Kemppainen et al., 1999). In another study, MPA (at 2.5×10^{-7} M) was reported to transactivate a GRE-driven reporter construct via co-transfected AR in Jurkat cells. In addition, MPA (at 2.5×10^{-7} M) was reported to mediate transrepression of an IL-2 promoter reporter construct stimulated with TPA and ionomycin, via co-transfected AR in the same cells (Bamberger et al., 1999).

Consistent with the presence of an androstane nucleus, which retains androgenic activity (Darney, 1995), NET has also been shown to bind to and have agonist activity for transactivation via the AR (Perez-Palacios et al., 1981a; Schoonen et al., 2000; Deckers et al., 2000; Chavez et al., 1985; Bergink et al., 1983; Bergink et al., 1985). In the studies by Schoonen et al., (2000) and Deckers et al., (2000), the relative binding and relative agonist activity values for transactivation of NET for the AR were relatively low: 3.2 and 1.1% relative to that of DHT, respectively. Similarly, the Kd of NET for the AR was determined as 1.9×10^{-8} M (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 AR in 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 Kd values or from EC50 curves. To our knowledge, the transrepressive effects of NET via the AR on target genes have not yet been investigated. In addition, very little is known about the relative transactivation and transrepressive effects of MPA vs. NET via the AR. In an early study, administration of either NET-EN or MPA, in contrast to progesterone,

resulted in a significant inhibition of serum gonadotropin levels in postmenopausal women. Although the mechanism is unclear, this effect was suggested to be mediated via the AR (Perez-Palacios et al., 1981a). Further pharmacological investigation into the relative binding affinity and relative agonist potency for transactivation and transrepression towards the AR of MPA vs. NET is required.

1.5.3 MPA and NET effects via the PR

Consistent with MPA being a synthetic progestin, it has been shown in a number of studies to bind to the PR (MacLaughlin and Richardson, 1979; Bergink et al., 1983; Feil and Bardin, 1979; Zhang et al., 2000; Selman et al., 1996). In the beagle dog, competition experiments for binding to the PR demonstrated that MPA (Ki = 1.5 nM) had an affinity equal to that of Org 2058 (Ki = 1.6 nM) (16α-ethyl-21-hydroxy-19-nor-4-pregnen-3, 20-dione), a synthetic high affinity progestin (Selman et al., 1996).

MPA has also been shown to be a PR agonist for transactivation via synthetic SRE sequences as well as via natural promoter-reporter constructs for several target genes involved in different physiological processes such as tumour development. In Jurkat cells, MPA at 2.5 x 10^{-7} M was able to transactivate a GRE-driven reporter construct via co-transfected PR (Bamberger et al., 1999). MPA (at 100 nM) was also shown to increase vascular endothelial growth factor (VEGF) promoter construct activity in endometrial adenocarcinoma cells co-transfected with either hPRA or hPRB (different isoforms of the human PR) via three functional progesterone response elements (PREs) identified in the VEGF promoter (Mueller et al., 2003). Similarly, in another study, MPA treatment (10^{-8} M) also increased VEGF levels in the media of T47-D human breast cancer cells via the PR. 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).

Furthermore, MPA has been shown in many studies to play a significant role in various endometrial functions via the PR. Insulin-like growth factor binding proteins (IGFBPs) regulate insulin-like growth factor action, which is believed to play a role in endometrial differentiation. MPA was shown to increase the promoter activity of IGFBP-1 in ES cells co-transfected with either hPRA or hPRB. The induced activity of IGFBP-1 was mediated through the progesterone response elements, PRE1 and PRE2 (Gao et al., 2000). MPA (at 0.1 μM) has also been shown to increase the activity of a deleted glycodelin-A (GdA) promoter reporter construct 3-fold in a human endometrial adenocarcinoma cell line via the PR. GdA expression is highly regulated and the protein is abundantly produced in the glandular cells of human endometrium. Since GdA potentially inhibits fertilisation but enhances implantation, the timing of GdA expression is critical for fertility regulation (Gao et al., 2001). This increase in GdA expression by MPA may possibly play a role in its contraceptive mechanism of action. Furthermore, MPA (at 10^{-6} M) was also shown to enhance decidual PRL gene activation in the presence of elevated intracellular cAMP levels in human ES cells (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. Similar results were obtained in the human endometrium in vivo, where PRL expression (mRNA and protein level) was increased significantly after treatment with MPA at 10 mg/day (Reis et al., 1999). This synergy between MPA and cAMP on decidual PRL expression was postulated to be dependent upon cellular levels of PR. Transient expression of steroid receptor co-activator 1e (SRC-1e) but not SRC-1a facilitated the synergy between cAMP and MPA (Brosens et al., 1999). Recent evidence revealed an increase in STAT5 in response to cAMP/MPA treatment, which served to enhance PRL expression in human ES cells (Mak et al.,

2002). Furthermore, MPA (at 10^{-8} and 10^{-7} M) suppressed mRNA expression of the long form leptin receptor (OB-R (L)) in organ-cultured proliferative endometrial specimens by 50%. This progestin-induced suppression was blocked by the addition of the antiprogestin RU486, indicating that in the human endometrium, MPA acts via the PR to suppress functional OB-R (L) mRNA expression. 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 (Koshiba et al., 2001).

NET has also been shown to bind to the PR (Bergink et al., 1983; Deckers et al., 2000; Chavez et al., 1985; Kontula et al., 1975). The relative binding affinity of NET for the PR was determined as 22%, relative to 100% binding by Org 2058 (Deckers et al., 2000; Schoonen et al., 2000). Furthermore, when comparing MPA and NET, both progestins have been shown to bind to the PR with almost equal affinity (Kontula et al., 1975). Similarly, in cytosol fractions of MCF-7 cells, MPA and NET both showed similar binding affinities for the PR (44- and 38% respectively, relative to 100% binding by Org 2058). However, in intact MCF-7 cells, NET showed lower binding affinity (41%) than MPA (65%) for the PR, relative to 100% binding by Org 2058 (Bergink et al., 1983). It is unclear from the above data whether the relative binding activity was determined from Kd values or from EC50 curves. Furthermore, 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 CV-1 cells stably transfected with the rabbit PR, and this activity was inhibited by RU486 (Pasapera et al., 2001). In CHO cells 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). In another study, NET at 10^{-8} M (like MPA) was also shown to increase VEGF in the media of cultured T47-D cells (Hyder et al., 1998), an effect likely mediated via the three

functional PRE elements located in the promoter (Mueller et al., 2003). Furthermore, 0.1 μM 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).

Several genes regulated by MPA and/or NET via the PR are involved in a range of physiological functions. Both MPA and NET-A were shown to dose-dependently (10^{-10} to 10^{-8} M) increase expression of ICAM-1 and VCAM-1. This increase was blocked by the addition of RU486, but not by hydroxyflutamide, an AR antagonist, indicating that this stimulation was mediated predominantly via the PR (Tatsumi et al., 2002). Prolonged MPA exposure (10^{-7} M for 8 days) was shown to repress RANTES (regulated on activation, normal T cell expressed and secreted) transcriptional activity by 36% and protein production by 50% via the PR in human ES cells. RANTES is a critical chemokine in the pathogenesis of endometriosis and has been suggested to play an early role in the inflammatory response (Zhao et al., 2002). 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 and relative agonist potency for both transactivation and transrepression of MPA vs. NET via the PR is required.

1.5.4 MPA and NET effects via the MR

MPA, NET and NET-A have been investigated for their ability to bind to the MR. Both synthetic progestins were less active relative to progesterone for competing with [^{3}H] aldosterone for cytoplasmic receptor binding in rat kidney. The relative binding affinities were obtained from EC50 curves and determined as 4% for MPA and <1% for both NET and NET-A, compared to 100% binding by progesterone, an antagonist for the MR (Wambach et al., 1979). Further

investigations are required to determine precise Ki values for the relative binding of both MPA and NET to the MR. Furthermore, no comparison exists on the relative agonist potency of MPA and NET for both transactivation and transrepression of target genes via the MR. These findings may result in an improved understanding of the relative effects of MPA and NET on blood pressure and in cardiovascular disease.

1.6 Conclusion

The synthetic progestins, MPA, NET-EN and NET-A are widely used by millions of women in reproductive therapy. Although both progestins have been shown to exert a range of side effects when used in vivo, very little is known about their mechanism of action. The importance of investigating at the molecular level the mechanism of action of synthetic progestins is highlighted by recent clinical evidence showing that MPA increases the risk of the development of breast cancer in HRT users (Rossouw et al., 2002). In addition, 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. Clearly more research is required to investigate the relative effects of synthetic progestins on immune function in the reproductive tract. MPA, unlike prog, may also have damaging effects on neurological processes (Nilsen and Brinton, 2003). Interestingly to date, 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 has not yet been determined. Furthermore, their interactions with serum-binding proteins as well as their effects on steroidogenesis requires further investigation. These studies would be important in defining differences in the mechanism of action of various synthetic progestins, and hence facilitate an informed choice of progestins for reproductive intervention and therapy.

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Chapter 2

Medroxyprogesterone acetate downregulates cytokine gene expression in mouse fibroblast cells

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2.1 Manuscript

Summary

Although medroxyprogesterone acetate (MPA) is used as an injectable contraceptive, in hormone replacement therapy and in treatment of certain cancers, the steroid receptors and their target genes involved in the actions of MPA are not well understood. We show that MPA, like dexamethasone (dex), significantly represses TNF-stimulated interleukin-6 (IL-6) protein production in mouse fibroblast (L929sA) cells. In addition, MPA repressed IL-6 and interleukin-8 (IL-8) promoter reporter constructs at the transcriptional level, likely via interference with nuclear factor κB (NFκB) and activator protein-1 (AP-1). Furthermore, like dex, MPA does not affect NFκB DNA-binding activity. We also observed significant transactivation by MPA of a glucocorticoid response element (GRE)-driven promoter reporter construct in both L929sA and COS-1 cells. The MPA-induced nuclear translocation of the glucocorticoid receptor (GR), as well as the antagonistic effects of RU486, strongly suggest that the actions of MPA in these cells are mediated at least in part via the GR.

Introduction

Medroxyprogesterone acetate (MPA), a 17α-acetoxy-6α-methyl synthetic analog of the naturally occurring 17α-hydroxyprogesterone, is one of the major conventional contraceptive agents used in Southern Africa. It is administered as Depo Provera, by intramuscular injection of an aqueous suspension of 150 mg every 3 months. MPA is also used at higher doses for hormone replacement therapy (HRT) (Brunelli et al., 1996), cancer therapy (Blossey et al., 1984; Etienne et al., 1992; van Veelen et al., 1986; Yamashita et al., 1996) and for the treatment of endometriosis (Irahara et al., 2001; Harrison and Barry-Kinsella, 2000; Telimaa et al., 1989). Doses for cancer therapy typically range between 500 and 1500 mg orally per day for about 12 weeks (Blossey et al., 1984), while those used for HRT are about 10 mg/day for about 11 days (Brunelli et al., 1996). As for most drugs, Depo Provera has been shown to have several side effects (Kaunitz, 2000; Greydanus et al., 2001). Furthermore, at high doses such as those used in cancer therapy, MPA has been shown to have potent glucocorticoid (GC)-like effects such as complete inhibition of adrenal function (Blossey et al., 1984; Papaleo et al., 1984) and immunosuppression (Yamashita et al., 1996; Mallmann et al., 1990; Scambia et al., 1988). Doses of MPA used in HRT have been shown to modulate immune function in humans, selectively affecting various immune cell subsets (Brunelli et al., 1996; Malarkey et al., 1997). Surprisingly, little research appears to have been carried out in humans on the effects of contraceptive doses of MPA on immune or adrenal function, although some early findings showed that, even at these low doses, MPA may have significant effects (Gerretsen et al., 1979; Gerretsen et al., 1980; Jones et al., 1974; Aedo et al., 1981). A better understanding of the molecular mechanism of

action and relative potency of MPA for the steroid receptors would provide a molecular basis for understanding its biological effects.

The receptors mediating the effects of MPA as well as their target genes are not well defined. The actions of MPA are generally assumed to be mediated via the progesterone receptor (PR). However, MPA has a high affinity for the glucocorticoid receptor (GR), androgen receptor (AR) and PR (Teulings et al., 1980; Bentel et al., 1999; Kemppainen et al., 1999; Bojar et al., 1979; Bergink et al., 1983; Feil and Bardin, 1979). Furthermore, the affinity of the rat GR for MPA (Kd = 5.0 nM) is similar to that of dexamethasone (dex) (Kd = 4.1 nM) (Winneker and Parsons, 1981), but higher than that for the endogenous ligand, cortisol (Kontula et al., 1983). It is thus possible that MPA may exert many of its therapeutic actions as well as side effects via any of these receptors. For example, in tumour regression, MPA may act partly via the PR (Blossey et al., 1984; Braunsberg et al., 1987), but predominantly via the GR (Bojar et al., 1979) or the AR (Teulings et al., 1980).

Interleukin-6 (IL-6) is a multi-functional cytokine that is involved in immune and inflammatory responses including acute phase reactions, and hematopoietic activities. IL-6 is produced by a number of cells including fibroblasts, T cells and mononuclear phagocytes. In immune responses, IL-6 is a growth factor for mature B cells and induces their final maturation into antibody-producing plasma cells. IL-6 is also involved in activation and differentiation of T cells (Feghali and Wright, 1997; Ershler and Keller, 2000). Interleukin-8 (IL-8) belongs to a chemotactic cytokine family and is responsible for the chemotactic migration and activation of neutrophils, lymphocytes, monocytes and other cell types at sites of inflammation (Mukaida et al., 1994; Feghali and Wright,

1997). The promoters of the interleukin-6 (IL-6) and interleukin-8 (IL-8) genes both contain a variety of binding sites for known transcription factors, including activator protein-1 (AP-1) and nuclear factor κB (NFκB) (Ershler and Keller, 2000; Vanden Berghe et al., 2000). For IL-6 gene activation in response to tumour necrosis factor (TNF), the main transcription factor involved is NFκB (Vanden Berghe et al., 1998). Furthermore, GC-mediated repression of IL-6 and IL-8 genes has been shown to involve the GR (De Bosscher et al., 2003). Although the precise molecular mechanism of gene repression by GCs is still controversial, nuclear GR is believed to repress IL-6 gene expression by interference with the activity of transcription factors NFκB and AP-1 at the promoter level (Ershler and Keller, 2000; De Bosscher et al., 2000a). Recently, studies have been initiated to investigate the effects of MPA on the immune system. Bamberger et al., (1999) reported that MPA represses transcription of a human interleukin-2 (IL-2) promoter reporter construct in normal human lymphocytes, to the same extent as dex. Furthermore, Mantovani et al., (1997) showed that MPA reduced levels of interleukin-1β (IL-1β), IL-6, TNFα and serotonin (5-HT), produced in culture by activated mononuclear leukocytes from cancer patients. Other studies showed that MPA can inhibit lymphocyte proliferation in vitro (Kontula et al., 1983; Mantovani et al., 1997), β-chemokine production in choriodecidual cells (Kelly et al., 1997) and IL-8 production in endometrial explants and chorion cells (Kelly et al., 1994). In contrast Arici et al., (1996) found that MPA increased the level of IL-8 mRNA in human endometrial stromal cells. In the current study we investigated the effects of MPA on TNF-induced IL-6 and IL-8 reporter gene expression in a mouse fibrosarcoma cell line, L929sA, as well as the mechanism thereof.

Materials and methods

Cell culture, cytokines and inducing compounds

Murine fibrosarcoma L929sA and HeLa cells were maintained as previously described (Vanden Berghe et al., 1998). COS-1 cells were cultured in high glucose (1 g/ml) DMEM supplemented with 10% fetal calf serum (Highveld Biologicals, South Africa), 50 IU/ml penicillin and 50 μg/ml streptomycin (GibcoBRL). The origin and activity of TNF has been described previously (Vanden Berghe et al., 1998). Staurosporine (Calbiochem-Novabiochem International (San Diego, CA)) was stored as a 2 mM solution in DMSO at -20°C. Dex, MPA, progesterone (prog), R5020 and RU486 were purchased from Sigma Aldrich. R1881 was purchased from NEN™ Life Science (Boston, MA). Hydroxyflutamide was obtained from Dr C. Tendler (Schering Plough Research Institute, Kenilworth, NJ). Stock solutions were routinely dissolved in ethanol or DMSO and stored at -20°C. RU486 and hydroxyflutamide were used at 2×10^{-6} M.

Plasmids

The plasmids p1168hIL-6luc+, p(IL-6κB)$_3$50hIL-6luc+, p1168(κBmut)IL-6luc+, p(GRE)$_2$50hluc+ and pAP-1luc have been described previously (Vanden Berghe et al., 1998; Vanden Berghe et al., 1999a; Plaisance et al., 1997; De Bosscher et al., 2000b). The deleted IL-8 promoter variants, p546hIL-8luc+, p133hIL-8luc+, p98hIL-8luc+ were kindly provided by Dr N. Mukaida (Cancer Research Institue, Kanazawa, Japan) (Mukaida et al., 1990). The rat GR cDNA (pSVGR1) (Delauney et al., 1996), the human PRB cDNA (PMT human PRB) and 2xGREtkluc (Delauney et al., 1996) were obtained from Prof S. Okret (Karolinska Institute, Sweden). The pPGKβGeopbA (Soriano et al.,

1991) constitutively expressing a neomycin-resistant/β-galactosidase fusion protein under the control of the pPGK promoter from the mouse housekeeping enzyme, 3-phosphoglycerate kinase, was a kind gift from Dr P. Soriano (Fred Hutchinson Cancer Research Centre, Seatle, WA).

Inductions and transfections

Stable transfection of L929sA cells has been described previously (Vanden Berghe et al., 1998). L929sA cells were stably transfected with a plasmid of interest together with the β-galactosidase expression plasmid, pPGKβGeopbA (Soriano et al., 1991). For inductions, 1.5 x 10^5 cells per well were seeded in 24-well dishes twenty-four hours before induction, such that they were 80% confluent at the time of the experiment. The medium was removed and replaced by antibiotic-supplemented phenol red-free medium (GibcoBRL) without serum, plus various test compounds. After induction, cell culture supernatant was collected for quantification of secreted IL-6 (Landegren, 1984). Cells were washed with DMEM without serum and lysed according to reporter gene assay instructions. Luciferase and β-galactosidase reporter gene assays were performed as described previously (Vanden Berghe et al., 1998). Transient transfection of COS-1 cells was performed either with FuGENE 6 (Roche Biochemicals) (according to manufacturer's instructions) or by the calcium phosphate precipitation procedure according to standard protocols. Briefly, cells were seeded 4 x 10^4 cells per well in 12-well plates and transfected the following day with 5 μg 2xGREtkluc and 2.5 μg pSVGR1 and pPGKβGeopbA respectively. Cells were then incubated with DNA precipitates for 24 hours. Thereafter, cells were washed twice with PBS and medium without serum was added. Cells were incubated with test compounds for a further 24 hours followed by harvesting with Reporter Lysis Buffer (Promega Luciferase Assay System). The β-

galactosidase (Galacto-*Star*™ assay system) and luciferase assays (Promega Luciferase Assay System) were performed in a Labsystems Luminoskan RS luminometer. Luciferase activity was normalised to co-expressed β-galactosidase activity to correct for differences in transfection efficiencies. Where indicated, the Bradford assay was used to determine total protein concentration.

Electrophoretic mobility shift assay (EMSA)

L929sA and HeLa cells seeded 3 x 10^5 per well in 6-well plates, were left untreated or were induced with 2000 IU/ml TNF for 30 minutes, in the absence or presence of 1 μM dex, MPA or prog in phenol-red free medium (GibcoBRL) for two hours. Total cell extracts were prepared using TOTEX buffer (20 mM Hepes/KOH pH 7.9, 350 mM NaCl, 20% glycine, 1% NP 40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 2 mM Pefablock and 5 mM DTT). Equal amounts of protein were incubated for 30 minutes with an NFκB-specific ^{32}P-labeled oligonucleotide (Vanden Berghe et al., 1999b), for EMSA analysis as described in Plaisance et al., (1997). Binding complexes were analysed using Phosphoimager technology.

Immunofluorescence

The procedure was performed as described previously (Vanden Berghe et al., 1999a). Essentially, L929sA cells grown on coverslips were stimulated with test compounds at the concentrations indicated for 30 min, followed by fixing and washing of cells. Incubations with rabbit polyclonal anti-GR (H-300) antibody (SantaCruz) were performed overnight at 4 °C, followed by washing and incubation with secondary antibody (goat anti-rabbit IgG biotin-conjugated antibody) (SantaCruz) at room temperature for an hour. After washing, cells were incubated with an IgG streptavidin-

Alexafluor marker (Molecular Probes) for one hour. Slides were then examined and images recorded using a Zeiss Axiophot fluorescence microscope with CCD video camera.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism software, using one-way analysis of variance with either Bonferroni or Dunnett post tests. In most figures, the letters a,b,c etc. are used to denote statistically significant differences, where all those values which differ significantly ($P<0,05$) from others, are assigned a different letter. In remaining figures, statistical significance of differences is denoted by *, ** or ***, to indicate $P<0.05$, $P<0.01$ or $P<0.001$, respectively.

Results

MPA inhibits TNF-induced endogenous IL-6 protein production in L929sA cells.

The mechanism of GR-mediated repression of IL-6 has been extensively investigated in L929sA mouse fibrosarcoma cells (De Bosscher et al., 2000a; Vanden Berghe et al., 2000). We therefore carried out experiments using these cells as a model cell line. Our aim was to determine the effects and mechanisms of action of MPA on interleukin gene expression relative to the reference ligand, dex. Figure 1 shows that MPA and prog, like dex, repress TNF-induced IL-6 protein secretion in L929sA cells, although the inhibition by MPA and prog at 0.1 μM is less pronounced than that observed for dex.

MPA represses TNF-induced expression of IL-6 and IL-8 promoter reporter constructs, most likely via interference with the transcription factor NFκB.

To determine whether MPA can repress the IL-6 gene at a transcriptional level, we investigated the effect of MPA on an IL-6 promoter-reporter construct, p1168hIL-6luc+. Figure 2a shows that TNF-mediated induction of p1168hIL-6luc+, stably integrated into L929sA cells, can be inhibited significantly by dex, MPA and prog. In parallel to IL-6 promoter regulation, we also investigated the effect of MPA on the IL-8 promoter reporter construct, p546hIL-8luc+. Figure 2b shows clearly that MPA at 0.1 μM, 1 μM and 10 μM concentrations can inhibit TNF-induced IL-8 promoter activity significantly. MPA inhibits the promoter to a significantly greater extent than prog, yet less efficiently than dex. This is particularly apparent at the 1 μM concentration point for

each test compound. Similar results were obtained with shorter IL-8 promoter variants i.e., p133hIL-8luc+ and p98hIL-8luc+ (data not shown).

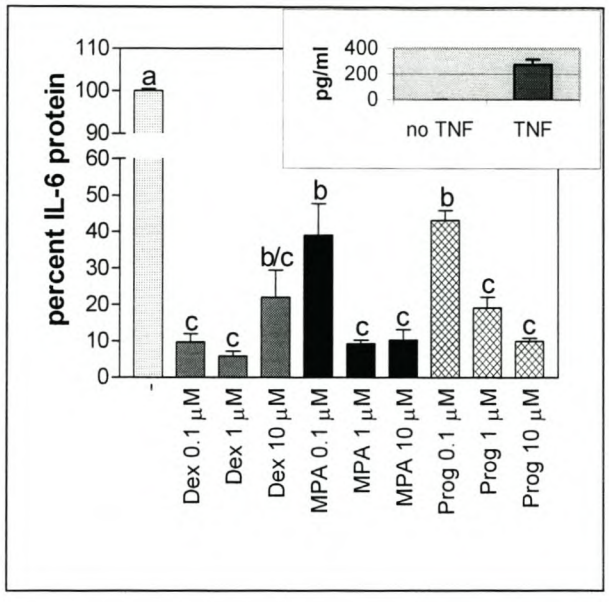


Figure 1: Effect of dex, MPA and prog on TNF-induced IL-6 protein production in mouse L929sA cells.

Cells were untreated or pre-treated with dex, MPA or prog in the presence of 2000 IU/ml TNF. Steroid hormone was added two hours before TNF, for a total period of eight hours. After induction, cell culture supernatant was collected for quantification of secreted IL-6. The combined results of two independent experiments are shown, where each condition was performed in triplicate. IL-6 protein for each test compound condition was normalised relative to protein in the presence of TNF in the absence of test compounds, which was taken as 100%. The letters a,b,c etc. are used to denote statistically significant differences, where all those values which differ significantly ($P<0,05$) from others, are assigned a different letter.

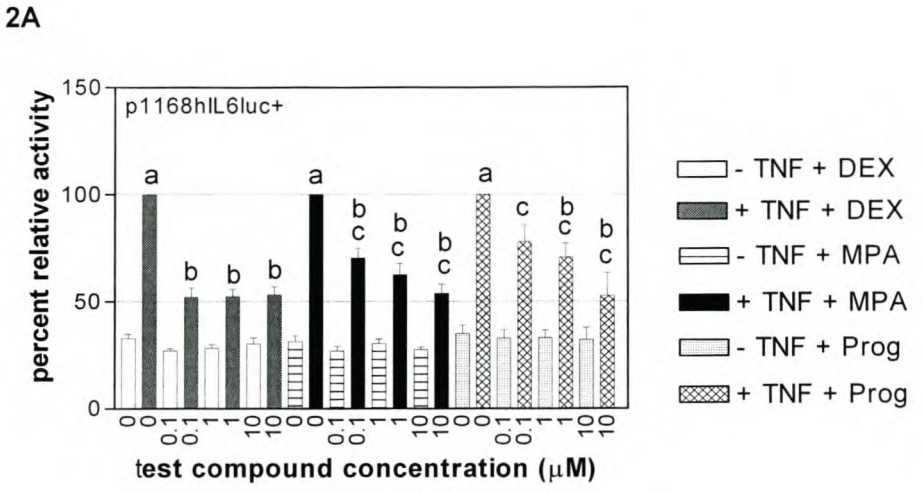
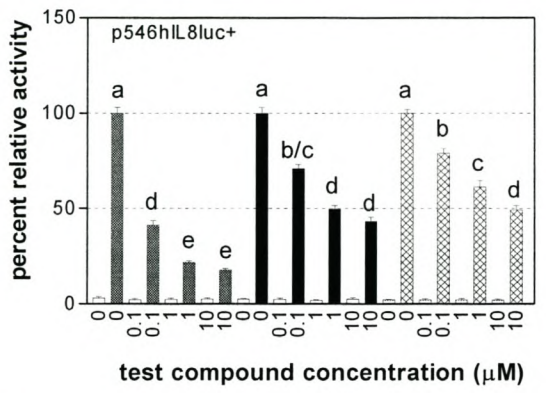


Figure 2: Effect of dex, MPA or prog on TNF-induced L929sA cells stably transfected with either p1168hIL-6luc+ (A), p546hIL-8luc+ (B, C), or p(IL-6κB)$_3$50IL-6luc+ (D) promoter reporter constructs, together with the β-galactosidase construct, pPGKβGeopbA.

Dose-response curves for each dex-, MPA- and prog-treated L929sA cells, stably transfected with the promoter construct p546hIL-8luc+ are shown in (C). Confluent L929sA cell monolayers were untreated or pre-treated with dex, MPA or prog (the key in (A) also applies to (B) and (D)) in the absence or presence of 2000 IU/ml TNF. Steroid hormone was added two hours before TNF, for a total period of eight hours, after which cells were lysed and assayed for luciferase and β-galactosidase expression. Luciferase/β-galactosidase activity for each test compound condition was normalised relative to the activity of the reporter in the presence of TNF and in the absence of test compounds, which was taken as 100%. For (A), (B) and (D), the combined results of two independent experiments are shown, where each condition was performed in triplicate. For (C) the results shown are representative of four independent experiments, where each condition was performed in triplicate, and where a representative experiment is shown. The letters a,b,c etc. are as for Figure 1.

2B



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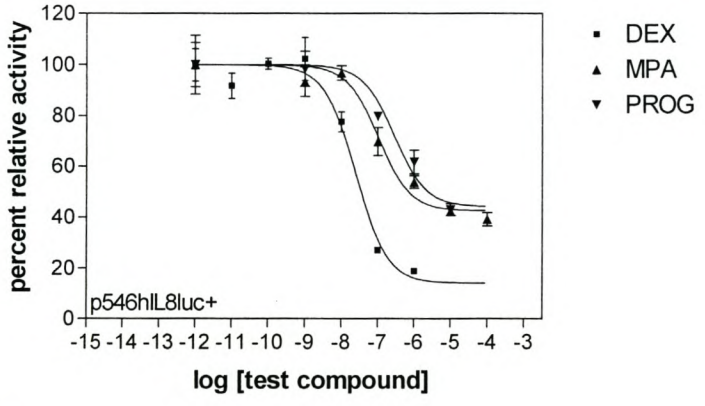

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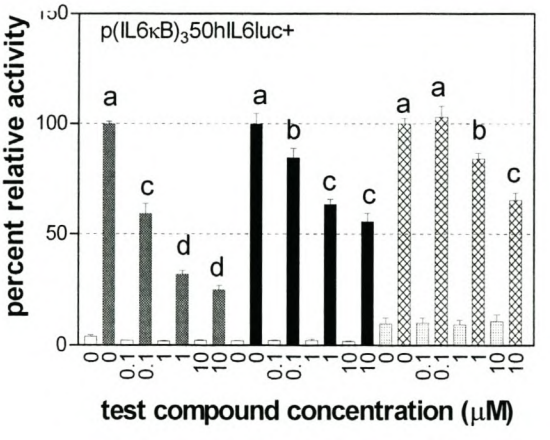


Ligand dose-response curves for transrepression were determined for the p546hIL-8luc+ reporter construct stably integrated into L929sAs. Figure 2c shows a representative dose-response curve for MPA, dex, and prog, and Table 1 shows the EC50 (defined as potency) and maximal repression (defined as efficacy) values extrapolated from four experiments. Results confirm that MPA represses the IL-8 promoter significantly more potently than prog ($p<0.05$), but less potently than dex ($p<0.05$). The maximal repression values for MPA and prog are similar (about 60%), but less than that for dex (about 80%), showing that both these compounds act here as partial agonists for transrepression.

As NFκB is a crucial transcription factor involved in TNF-mediated IL-6 and IL-8 gene induction (Mukaida et al., 1994; Vanden Berghe et al., 1998), we further investigated whether our results could be mimicked with the synthetic promoter reporter construct p(IL-6κB)$_3$50hIL-6luc+, containing three NFκB sites in front of a minimal IL-6 promoter, stably transfected in L929sA cells. Figure 2d shows that MPA at 0.1 μM, 1 μM and 10 μM can significantly inhibit the TNF-induced activation of the p(IL-6κB)$_3$50hIL-6luc+ construct. Furthermore, MPA represses the promoter significantly more efficiently than prog, but less effectively than dex, similar to our results obtained with IL-6 and IL-8 reporter promoter constructs.

Potency (EC50) and efficacy (MAX) for transrepression (A) and transactivation (B)

A

	EC50 (+/- SEM)	MAX (+/- SEM)
DEX	$2.2X10^{-8}$ (1.2)	19.7 (0.7)
MPA	$9.0x10^{-8}$ (1.4)	40.6 (1.5)
PROG	$4.7x10^{-7}$ (1.4)	39.5 (1.8)

B

	EC50 (+/- SEM)	MAX (+/- SEM)
DEX	$5.6x10^{-8}$ (1.3)	11.9 (0.9)
MPA	$9.2x10^{-8}$ (1.3)	5.7 (0.2)
PROG	$9.3x10^{-7}$ (1.6)	1.9 (0.09)

Table 1: A) EC50 (M) and maximal values for transrepression (% activity relative to no test compound, taken as 100% activity) were extrapolated from dose-response curves for each dex-, MPA- and prog-treated L929sA cells stably transfected with the promoter reporter construct p546hIL-8luc+ as shown in Figure 2C. MPA vs DEX $p<0.05$; MPA vs PROG $p<0.05$.

B) EC50 (M) and maximal values for transactivation (relative arbitrary units) extrapolated from dose-response curves for each dex-, MPA- and prog-treated L929sA cells stably transfected with the promoter reporter construct $p(GRE)_2$50hluc+ as shown in Figure 5A. MPA vs DEX $p>0.05$; MPA vs PROG $p<0.05$.

MPA inhibits a staurosporine (STS)-induced AP-1-driven minimal IL-6 promoter.

To determine whether the inhibitory action of MPA also affects AP-1 activity, we transfected L929sA cells with an IL-6 promoter reporter gene variant with a mutated κB site. STS was used to induce this promoter, which has lost its TNF inducibility but still responds to STS via AP-1 dependent gene expression (Vanden Berghe et al., 1999a). Figure 3a shows that MPA at 0.1 μM, 1 μM and 10 μM can significantly repress STS-induced promoter activity, while prog is significantly less potent than MPA. These results were further strengthened with the synthetic reporter construct pAP-1luc, containing three AP-1 sites in front of a minimal promoter, which mimics the response obtained with the NFκB mutated IL-6 promoter (Figure 3b).

MPA does not interfere with the DNA-binding activity of NFκB.

Different mechanisms have been proposed to account for inhibition of NFκB activity in response to anti-inflammatory drugs, which could interfere with cytoplasmic (inhibitory protein κB (IκB) turnover) or nuclear (NFκB DNA-binding) events (Vanden Berghe et al., 2000). Previous studies have demonstrated that the GR represses NFκB-driven genes without affecting DNA-binding activity of NFκB (De Bosscher et al., 1997; Vanden Berghe et al., 1999b). Since MPA has a high affinity for the GR, we were interested to compare its effects relative to dex at the NFκB DNA-binding level. Figure 4 shows the results of a gel shift experiment in L929sA cells where 1 μM MPA and prog, like dex, had no effect on the DNA-binding activity of NFκB. Similar results were obtained for HeLa cells (data not shown).

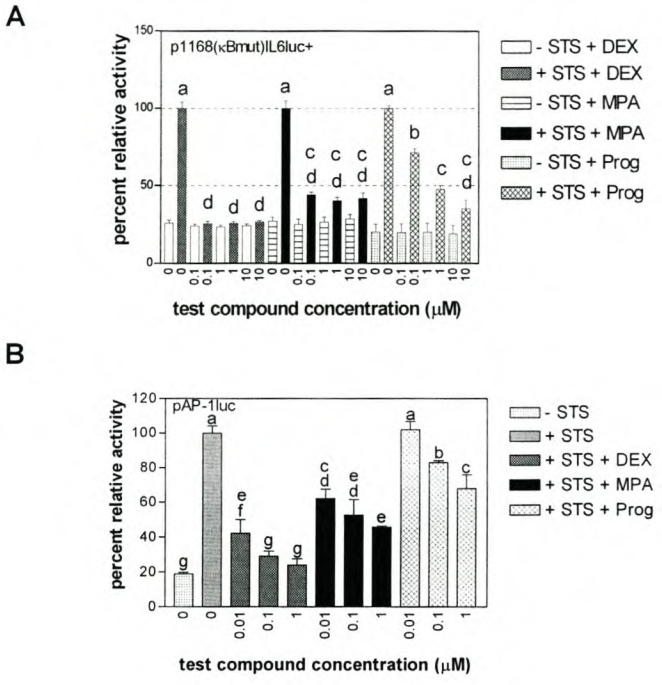


Figure 3: Effect of dex, MPA or prog on STS-induced L929sA cells stably transfected with the promoter construct p1168(kBmut)IL6luc (A) and pAP-1luc (B) together with the β-galactosidase expression vector pPGKβGeopbA. Confluent L929sA cell monolayers were untreated or pre-treated with dex, MPA or prog in the absence or presence of 60 nM STS. Steroid hormone was added two hours before STS, for a total period of eight hours, after which cells were lysed and assayed for luciferase and β-galactosidase expression. Luciferase/β-galactosidase activity for each test compound condition was normalised as for Figure 2. For (A) the combined results of two independent experiments are shown, where each condition was performed in triplicate. For (B) the results shown are representative of three independent experiments, where each condition was performed in duplicate, and where a representative experiment is shown. The letters a,b,c etc. are as for Figure 1.

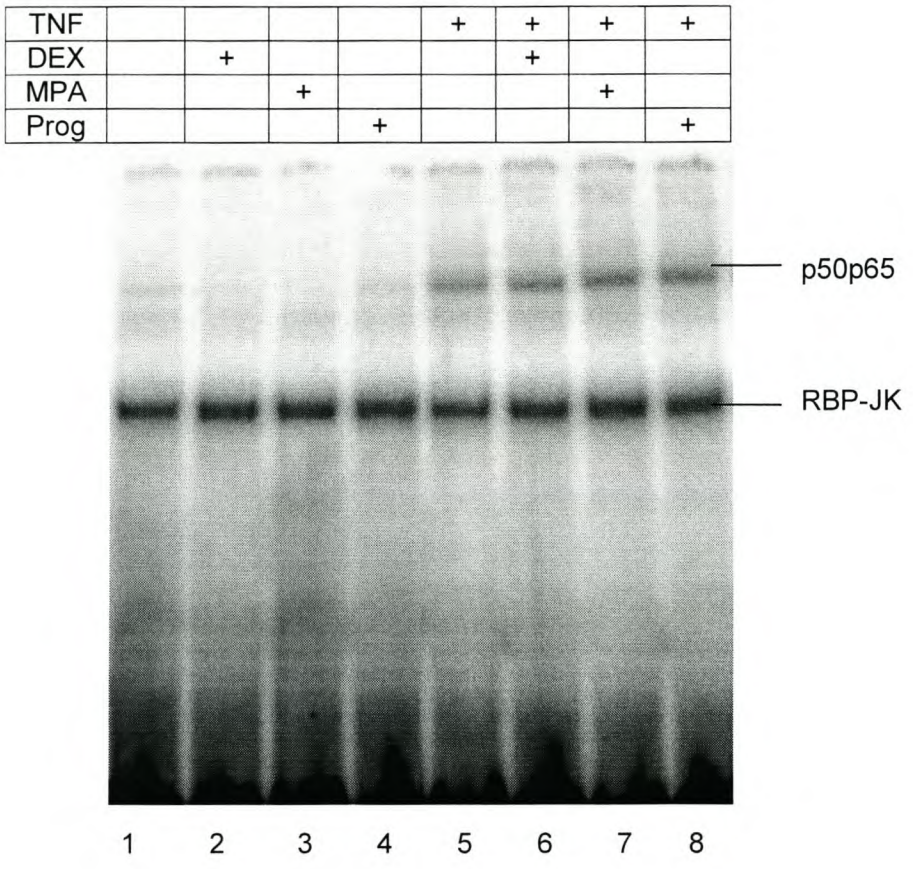


Figure 4: Effect of dex, MPA and prog on NFκB-DNA binding.

L929sA cells were untreated or pre-treated with 1 μM dex, MPA or prog for two hours in the absence or presence of 2000 IU/ml TNF for 30 minutes. Total cell extract was incubated with an NFκB-specific oligonucleotide and protein-DNA complexes were analysed in an electrophoretic mobility shift assay (EMSA). The result shown is typical of two independent experiments. RBP-JK is the constitutively expressed recombination binding protein.

MPA exhibits transactivation potential on a GRE-dependent reporter gene.

To determine whether MPA acts as a dissociated GC, we performed transactivation studies using L929sA cells stably transfected with the promoter reporter construct $p(GRE)_2$50hluc+, consisting of two copies of a GRE in front of a minimal IL-6 promoter (Vanden Berghe et al., 1999b). Figure 5a shows dose-response curves for MPA, dex, and prog, and Table 1 shows the EC50 (potency) and maximal (efficacy) values extrapolated from each curve. Although MPA and dex achieve different levels of maximal induction, with dex exhibiting a greater efficacy, their EC50 values are not significantly different (MPA vs dex, $P>0.05$). However, MPA, a partial agonist here for transactivation, is significantly more potent and has a greater efficacy than prog (MPA vs prog, $p<0.05$). Transactivation studies were also performed on COS-1 cells transiently transfected with a 2xGREtkluc reporter construct (containing two copies of a GRE upstream of the tk promoter) with and without rat GR cDNA, in the presence of steroid hormone. Results show that MPA, like dex, is able to transactivate the reporter at nanomolar ligand concentrations, which are typically observed in the serum of women using MPA as a contraceptive (Mishell, 1996) (Figure 5b). Furthermore, the latter effect was clearly dependent on the presence of the GR (Figure 5b).

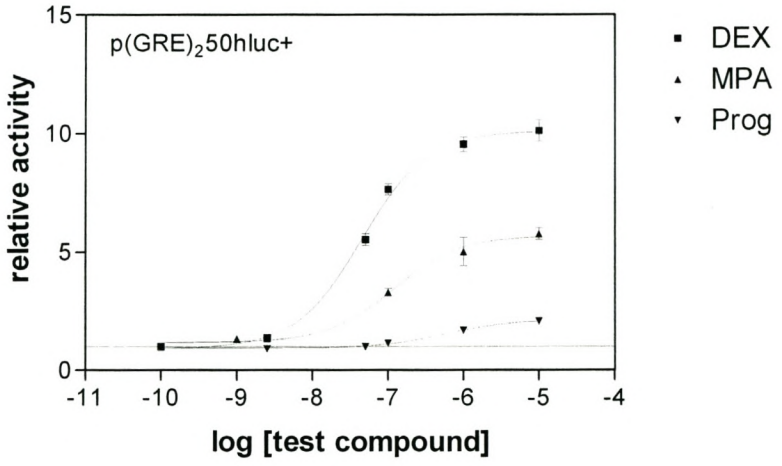


Figure 5A: Effect of dex, MPA or prog on L929sA cells stably transfected with the promoter construct p(GRE)$_2$50hluc+ together with the β-galactosidase construct, pPGKβGeopbA. Confluent L929sA cell monolayers were untreated or treated with dex, MPA or prog for 24 hours, after which cell lysate was harvested. The combined results of two independent experiments are shown, where each condition was performed in quadruplicate.

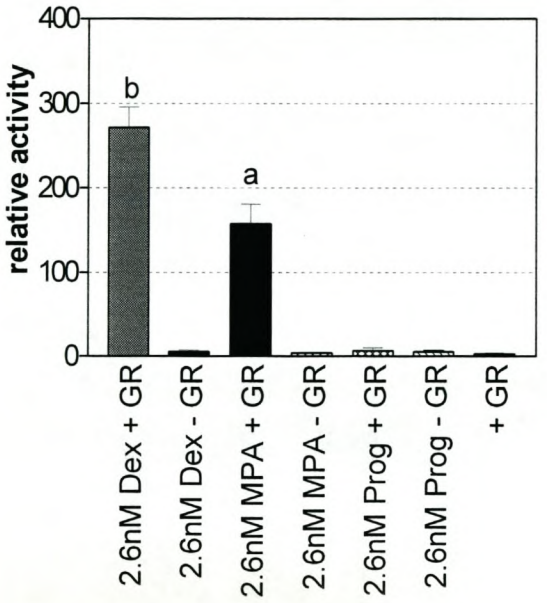


Figure 5B: Effect of dex, MPA and prog on COS-1 cells transiently transfected with 5μg 2xGREtkluc and pPGKβGeopbA, with or without 2.5μg pSVGR1. Cells were incubated the following day for 24 hours with or without 2.6 nM test compounds and lysate was harvested. The results shown are typical of three independent experiments. The letters a,b,c etc. are as for Figure 1. Luciferase/β-galactosidase activity for each test compound condition was normalised relative to the activity of the reporter in the absence of test compounds. For A, this value was taken as 1.

The effects of MPA in L929sA cells are mediated at least in part via the GR.

It is generally known that the GR, PR and AR are able to transactivate a GRE-driven promoter in a ligand-dependent fashion. Thus receptor-specific antagonists were used to investigate which receptor mediates the transactivation effects of MPA in L929sA cells stably transfected with the $(GRE)_2$50hluc+ promoter reporter construct. Hydroxyflutamide, an AR-specific antagonist, was unable to reverse the transactivation observed with dex and could only slightly (but not significantly) antagonise the effects seen with MPA (Figure 6a). As a control, we found that R1881, an AR agonist, significantly transactivates the reporter, while hydroxyflutamide significantly reverses this transactivation (data not shown). Thus although the AR is indeed present in L929sA cells, it does not appear to mediate transactivation by MPA. However, Figure 6b shows that RU486, a PR and GR-specific antagonist, is able to significantly reverse transactivation by dex and MPA, and marginally by prog, indicating that either or both of these receptors are involved.

Since dex is specific for the GR, the observed effects of dex can be assumed to be due to the presence of the GR. To determine whether PR is present in L929sA cells, cells were treated with test compounds in the presence of the prog-specific agonist R5020. We found that R5020 is unable to transactivate the reporter, indicating that PR is either present at very low levels undetectable by this assay, present but inactive for transactivation, or is altogether absent in L929sA cells (data not shown). In contrast, in a control experiment in COS-1 cells, R5020 clearly stimulates transactivation of a transiently transfected GRE-driven promoter in presence of co-transfected PR, demonstrating that the PR ligand is active. Reciprocally, no transactivation was observed in the absence of co-transfected PR (data not shown).

6A

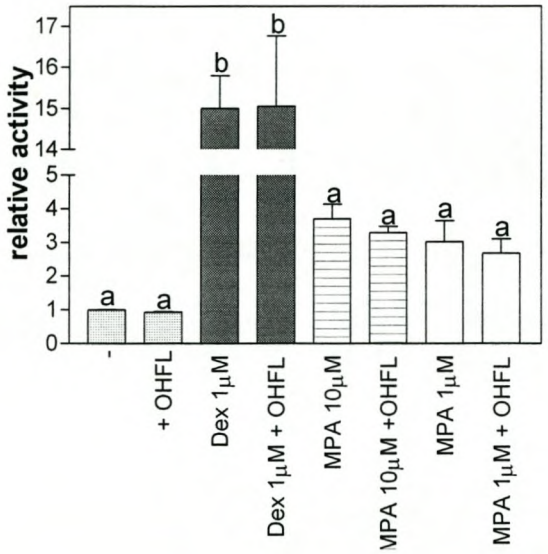
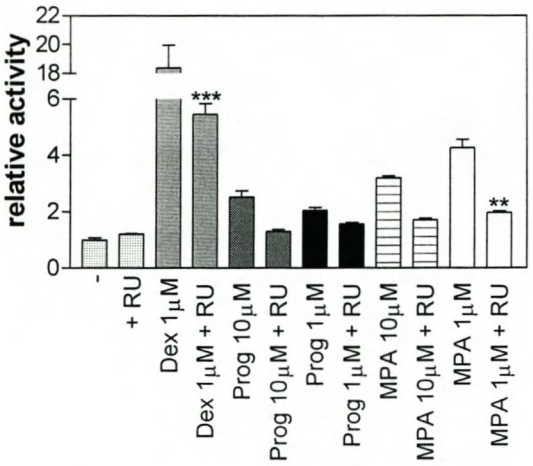


Figure 6: (A) Effect of dex or MPA, alone or in combination with hydroxyflutamide (OHFL) on L929sA cells stably transfected with the promoter construct p(GRE)$_2$50hluc+. (B) Effect of dex, prog or MPA, alone or in combination with RU486 (RU) on L929sA cells stably transfected with the promoter construct p(GRE)$_2$50hluc+. Cells were incubated with test compounds for 24 hours, followed by lysis and determination of luciferase levels. Results are expressed as relative activity, where the activity in the absence of test compounds was taken as 1. The results shown are representative of two (A) or three (B) independent experiments, where each condition was performed in quadruplicate, and where a representative experiment is shown. For (A), the letters a,b,c etc. are used, as for Figure 1. For (B), statistical significance of differences is denoted by *, ** or ***.

6B



To confirm the involvement of the GR in our observed effects of MPA in L929sA cells, we performed GR-localisation studies assessed by immunofluorescence assays. In the absence of test compounds, the GR is localized mainly in the cytoplasm, whereas some GR can also be observed in the nucleus, possibly due to phenol red effects or trace amounts of steroid present in the serum. However, upon incubation with MPA, a substantial increase in nuclear GR can be observed, with a concomitant decrease in cytoplasmic GR (Figure 7). These effects can clearly be reversed by RU486 (data not shown). Similar effects were recorded for dex (data not shown). Taken together, these results strongly suggest that MPA exerts its effects in these cells at least in part via the GR.

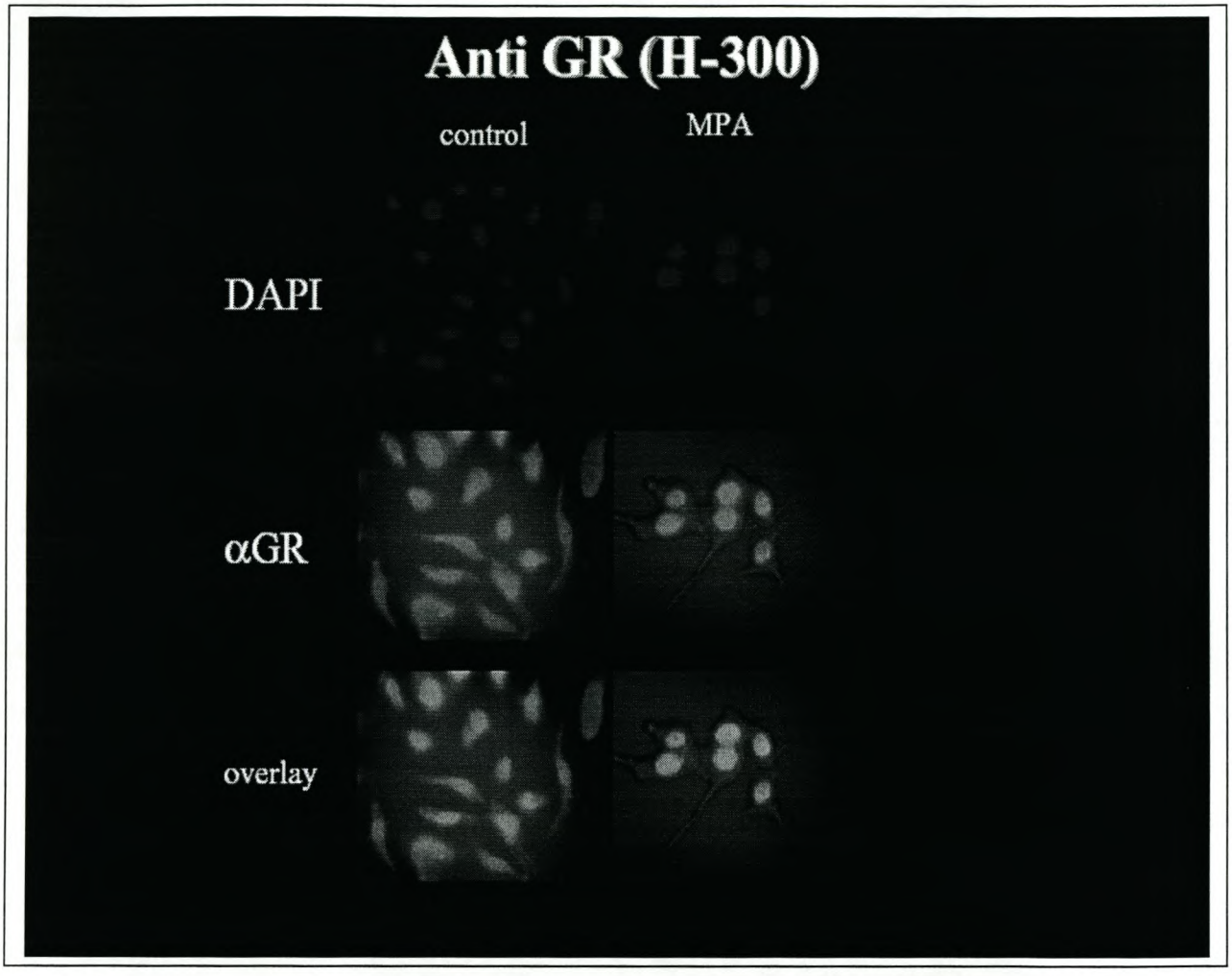


Figure 7: Effect of MPA on GR localisation in L929sA as revealed by immunofluorescence assay. Cells were stimulated with MPA for 30min followed by fixing and washing of cells. Incubations with polyclonal anti-GR (H-300) antibody were performed overnight at 4°C followed by incubation with secondary antibody (goat anti-rabbit IgG biotin-conjugated antibody). IgG Streptavidin-Alexafluor marker was used for visualization.

Discussion

Our results show that MPA represses both TNF-induced IL-6 protein and promoter reporter activity in our model cell line, L929sA (Figures 1 and 2a). At the protein level, we observed repression down to approximately 5-8% by both dex and MPA, whereas activation of the corresponding promoter reporter construct was repressed to only 50%. The greater degree of repression at the protein level could be due to post-transcriptional effects. We also showed that MPA represses synthetic promoter reporter constructs, consisting of either three NFκB responsive elements, or three AP-1 elements coupled to a minimal promoter (Figure 2d and Figure 3b, respectively), consistent with a mechanism involving interference with NFκB and AP-1 activity. In analogy with IL-6, MPA can repress IL-8 reporters, to a lesser extent than dex, but more efficiently than prog (Figure 2b). Consistent with this trend were the EC50 and maximal repression values extrapolated from ligand dose-response curves established for the p546hIL-8luc+ reporter construct stably integrated into L929sA cells (Table 1, Figure 2c). To our knowledge, this is the first report showing repression by MPA of IL-8 promoter activity. Towards further investigating the mechanism of repression of the IL-6 and IL-8 promoters by MPA, we tested whether MPA has any effect on NFκB DNA-binding. MPA treatment of TNF-induced L929sA cells (Figure 4) and HeLa cells (data not shown) could not disrupt DNA-binding by NFκB. This result is consistent with previous reports showing that the GR does not disrupt DNA-binding by NFκB in vitro (De Bosscher et al., 1997; Vanden Berghe et al., 1999b; Nissen and Yamamoto, 2000) and in vivo (Nissen and Yamamoto, 2000).

When assessing the dissociative properties of MPA as compared to dex in L929sA cells, the fold differences relative to dex for transrepression compared with transactivation were found not to differ substantially. MPA and dex display a similar transactivation potential (1.6-fold difference in EC50 values) and only a four-fold difference with regards to their relative transrepression potency (Table 1). With respect to maximal effects, MPA displayed about 26% lower efficacy relative to dex for transrepression (Figure 2c, Table 1), and approximately 50% lower efficacy relative to dex for transactivation (Figure 5a), illustrating that MPA is a partial agonist for both transactivation and transrepression in these cells. Hence, MPA did not exhibit dissociated GC properties in our experimental model system. However, in the studies by Bamberger et al., (1999) and Bamberger and Schulte, (2000), MPA behaved as a dissociated GC, as it only marginally transactivated a GRE-reporter construct in HeLa cells and in normal human lymphocytes. One explanation for these differences may be that cell type-dependent variations in the efficacy of MPA may, as has been shown for GCs, be due to differences in receptor or cofactor expression levels, presence of different receptor isoforms, and different components of the intracellular signalling machinery (Wallace and Cidlowski, 2001; Schneikert et al., 2000; Bamberger et al., 1996).

Our data strongly suggest that the effects of MPA on transactivation of a GRE reporter in L929sA cells are largely mediated via the GR. Hydroxyflutamide, which acts as an antagonist of the endogenous AR in L929sA cells, has no effect on MPA-mediated transactivation in these cells (Figure 6a), suggesting that the AR is not involved. In contrast, we show in Figure 6b that the transactivation effects of MPA via endogenous receptors in L929sA cells can be fully reversed by the GR- and PR- specific

antagonist, RU486. The absence of PR activity for transactivation using a strong PR agonist, together with the RU486 results, suggest that only the GR, and not the PR is involved. In addition, our immunofluorescence results show that MPA causes nuclear translocation of the GR, strongly supporting a role for MPA in ligand-mediated activation of the GR. In the case of transrepression, we are unable to exclude the involvement of the PR. Although our results suggest these cells do not contain transactivation competent PR, and although there are reports in the literature that PR is undetectable in binding assays in these cells (Jung-Testas et al., 1976), we were able to detect PR mRNA and protein in our cells by RT-PCR and Western blotting, respectively (data not shown). It is thus possible that the PR-A isoform, a poor activator of transactivation but a potent activator of transrepression (Kalkhoven et al., 1996), is expressed and mediates some of the transrepressive activity of MPA. Since it is well established that MPA does not bind to the estrogen receptor (ER), we can exclude a role for the ER in transrepression (Teulings et al., 1980).

Interestingly, the transactivation activity of prog is also reversed by RU486, although not significantly (Figure 6b). Given that transactivation agonist activity of the PR could not be detected in these cells with R5020, prog appears to act as a very weak partial agonist for transactivation via the GR, with about a ten-fold lower potency than MPA (Table 1), and a much lower efficacy (Figure 5a, Table 1) (16% of the maximal activity of dex, versus 50% for MPA). However, prog is a relatively stronger agonist for transrepression, showing approximately five-fold lower potency (Table 1) and similar efficacy (Figure 2c, Table 1) to that of MPA (both about 25% less repression than dex). It needs to be further investigated whether selective effects of PR isoforms could

perhaps explain these differences in the relative efficacy and potency of prog for transactivation versus transrepression.

In summary, our data show that MPA acts like a partial to full GC agonist in repressing both IL-6 and IL-8 promoters, with a potency of about four-fold less than that of dex (Table 1) and a similar (Figures 2a, 3a) to lower (Figures 2b, 2c, 2d, 3b) efficacy than dex. Given the multiple functions of the IL-6 and IL-8 cytokines, our results suggest that MPA may exert significant side effects via repression of these genes on various biological processes including those involved in the immune response, such as B cell maturation and T cell differentiation and activation, and bone metabolism and differentiation (Feghali and Wright, 1997; Ershler and Keller, 2000; Mukaida et al., 1994; Ishida and Heersche, 2002). Moreover, our results suggest that GC-like actions of MPA via the GR, resulting in transrepression via NFκB or AP-1 transcription factors, or transactivation via GREs, may be involved in regulating expression of several genes involved in many physiological processes.

Acknowledgments

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Appendix 1

Data not included in manuscript

TNF-activated p38 and extracellular-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathways have been shown to contribute to transcriptional activation of the IL-6 promoter (Vanden Berghe et al., 1998). In addition, inhibition of these pathways have been reported to repress TNF-mediated IL-6 gene expression in L929sA cells (Vanden Berghe et al., 1998; Beyaert et al., 1996)[1]. Furthermore, De Bosscher et al., (2001) showed that dex is able to inhibit the amount of phosphorylated c-Jun N-terminal kinase (JNK), but not of phoshorylated p38 or ERK in L929sA cells. To therefore determine whether the mechanism of MPA repression involves interference with MAPK pathways, a range of Western blotting experiments of L929sA cell lysates was carried out after treatment with or without TNF, in the presence of different steroid ligands as indicated. Results showed that MPA, in contrast to dex and prog, was unable to inhibit the amount of phosphorylated JNK p46 and p54 isoforms. Furthermore, the amount of phosphorylated p38 and ERK MAPK was not affected by dex, MPA or prog. The results of these experiments are discussed further in Chapter 4.

[1] For References, see Manuscript.

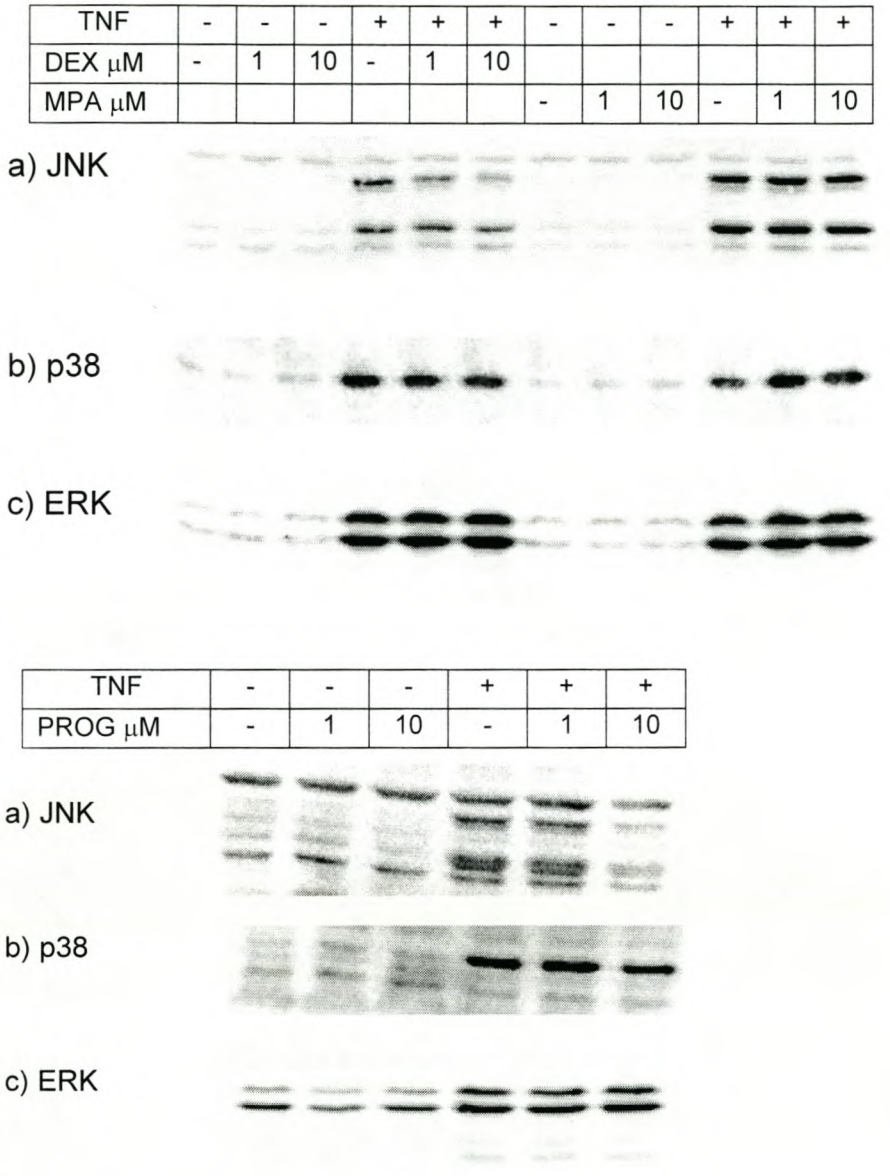

Figure 8: MPA does not inhibit MAPK activity.

Effect of dex, MPA and prog on JNK, p38 and ERK phosphorylation levels in L929sA cells. Cells were untreated or pre-treated with 1 or 10 μM dex, MPA or prog for two hours, followed by incubation with or without TNF (2000 IU/ml) for 10 minutes. Cell lysates were made and activated a) JNK, b) p38, and c) ERK were detected using the corresponding phospho-specific MAPK antibodies. The result shown is typical of three independent experiments. Non-phospho-JNK was used to check for equal protein loading (data not shown).

Pharmacological definitions

To characterise the way in which a ligand binds to its receptor and what effects it will elicit in terms of transactivation and transrepression of genes, certain parameters need to be determined. A full agonist is a drug or ligand that produces a maximal response, whereas a partial agonist is a ligand or drug that provokes a response less than the maximal response of that of a full agonist. An antagonist is a drug that does not elicit a response itself, but blocks agonist-mediated responses. The efficacy of a ligand is its ability to cause a response once bound to its receptor, in other words, the maximal effect it can elicit in a given tissue under particular experimental conditions. The potency of a ligand or drug is commonly quantified as the EC50, the concentration that leads to half the maximal response and reflects both the affinity of the ligand as well as its efficacy (Jenkinson et al., 1995; GraphPad Prism software). The equilibrium dissociation constant (Kd or Ki) is a measure of the affinity of a ligand for its receptor, and is equivalent to the concentration of ligand required to bind to half the available receptors at equilibrium. Kd and Ki can be interpreted the same way, and the subscript i is used to indicate that the competitor inhibits radioligand binding. When the concentration of ligand equals the Kd or Ki, half of the receptors will be occupied at equilibrium. When the receptors have a high affinity for the ligand, the Kd or Ki will be low, as it will take a low concentration of ligand to bind half the available receptors. The relative binding affinity (RBA), also referred to as the EC50, is a measure of the concentration of competitor that competes for half the specific binding. If the affinity (Kd or Ki) of a ligand for its receptor is high, the EC50 will be low. Kd or Ki values however, represent a more accurate measure of relative binding affinity of a ligand for its receptor than EC50 values. The Kd or Ki is a constant value for a particular receptor, not influenced by

experimental variations, whereas the EC50 is a relative term, which is influenced by different experimental conditions, including receptor number or radiolabelled ligand concentrations. The EC50 obtained from competitive binding curves is thus not the same as the Kd or Ki. Different EC50s can be obtained under different experimental conditions, for the same receptor in the same tissue/cell sample. The relative EC50 values can also differ for two different competitors, for the same receptor in the same tissue/cell sample, depending on the experimental conditions of the binding assay (GraphPad Prism software).

Analysis of radioligand binding experiments is based on the law of mass action. This model 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 rate constant or on-rate constant. Once binding has occurred, the ligand-receptor complex remains intact for a random amount of time. The probability of dissociation is the same 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 rate constant 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. At equilibrium therefore,

[Ligand].[Receptor].K_{on} = [Ligand.Receptor].K_{off}. If this equation is rearranged, the Kd is defined as K_{off} / K_{on} which is also equal to [Ligand].[Receptor] / [Ligand.Receptor] (GraphPad Prism software).

Calculations of parameters

The conventional method used for calculating Kds is by saturation binding experiments, where the concentration of radioligand is varied. An alternative method is by homologous/heterologous competitive binding experiments, where the radioligand concentration remains constant, and competes with varying concentrations of unlabelled competitor ligand for the receptor. In the current study, Kd and Ki values were determined by homologous and heterologous binding experiments respectively (Chapter 3), since [^{3}H] MPA and [^{3}H] NET-A are not commercially available. Kd and Ki values were obtained from pooled data of four independent experiments, and were also represented relative to 100% binding by dex to the GR (Chapter 3). Once it was determined that binding had reached equilibrium, and that the EC50 was two to ten times larger than the concentration of [^{3}H] dex used, Kd was calculated from homologous binding experiments by GraphPad Prism software according to the following equation: Total binding = (B_{max}.[Hot] / [Hot] + [Cold] + Kd) + NS, where NS is nonspecific binding. Once Kd for dex was determined, heterologous binding experiments were performed where Ki values for MPA, NET-A and prog were determined according to the Cheng-Prussoff equation: Ki = EC50/ 1 + ([ligand]/Kd), where the EC50 value is that determined for the competing ligand, [ligand] is the concentration of radioligand used and Kd is determined from homologous binding experiments. Binding data was analysed using GraphPad Prism software. Briefly, the following parameters were chosen: Nonlinear regression (curve fit), homologous competitive binding curve, one class of binding sites, with weighting $1/y^2$ (which minimises relative distances squared), and the mean y value of each point was considered. A stricter (slower) criterion for convergence was used. Furthermore, runs test and residuals test were used to determine whether the curve deviated systematically from the data.

Another important parameter that was controlled for in this study was ligand depletion. If a large fraction of the ligand binds to the receptors or binds non-specifically, then the concentration of ligand free in the solution does not approximate to the concentration added. Considering that this discrepancy is not the same in each well, as a rule of thumb, less than 10% ligand depletion is considered satisfactory. Ligand depletion in our experiments was always below 2%.

A comparison of the glucocorticoid agonist properties of the synthetic progestins, medroxyprogesterone acetate and norethisterone acetate

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Manuscript in preparation for submission.

3.1 Manuscript

Summary

The progestins, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN), are widely used as female contraceptive agents. In addition, MPA and norethisterone acetate (NET-A) are popular compounds in hormone replacement therapy (HRT). Surprisingly, it remains unknown whether doses of these progestins used for contraception and HRT cause significant side effects through various target genes via the glucocorticoid receptor (GR), and no thorough comparison exists at the molecular level of their mechanism of action. By competitive binding in whole cells, we show that both MPA and NET-A compete with dexamethasone (dex) for binding to the endogenous human GR in lung carcinoma A549 cells and transiently expressed rat GR in COS-1 cells. Equilibrium dissociation binding constants (Kds) revealed that MPA displays a higher relative binding affinity than NET-A and progesterone (prog) for the human GR (Kds of 4.2, 10.8, 270 and 215 nM for dex, MPA, NET-A and prog, respectively). By performing dose response analysis for transactivation of a synthetic promoter-reporter construct containing glucocorticoid (GC) response elements, we show that MPA displays greater GC transactivation agonist potency than NET-A (EC50s of 1.1, 7.2, >1000 and 280 nM for dex, MPA, NET-A and prog, respectively). Similarly, using an interleukin-8 (IL-8) promoter reporter construct, we show that MPA displays greater GC transrepression agonist potency than NET-A (EC50s of 0.21, 2.7, >100 and 26 nM for dex, MPA, NET-A and prog, respectively). These results show that binding is a poor predictor of

agonist potency. They also clearly demonstrate that these two synthetic progestins exhibit very different GC-like properties and hence are likely to exhibit different side effects via the GR, which may be particularly relevant to immune function and effects on bone density.

Introduction

The injectable progestagen-only contraceptives, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN), are the most widely used female conventional contraceptives in South Africa. Although widely employed for preventing pregnancy, these progestins have non-contraceptive uses as well. MPA is used at higher doses in cancer therapy (Etienne et al., 1992; Yamashita et al., 1996), while both MPA and norethisterone acetate (NET-A) are used in the treatment of endometriosis (Irahara et al., 2001; Harrison and Barry-Kinsella, 2000; Muneyyirci-Delale and Karacan, 1998; Surrey and Hornstein, 2002) and in hormone replacement therapy (HRT) (Brunelli et al., 1996; Taitel and Kafrissen, 1995). In contraceptive use, MPA is administered as a 150 mg aqueous suspension (referred to as Depo Provera) every three months, whereas NET-EN is administered as a 200 mg oily suspension every two months, both as intramuscular injections. After injection, MPA is fairly stable and is itself the active contraceptive compound. However, NET-EN and its acetate (NET-A) are hydrolysed to norethisterone (NET) and its metabolites, which together with NET have contraceptive action (Stanczyk and Roy, 1990). Both MPA and NET-EN are highly effective contraceptive agents in women due to their multiple sites of action. MPA and NET-EN have both been shown to block ovulation (Kaunitz, 2000; Greydanus et al., 2001; Bhowmik and Mukherjea, 1988) and alter the content of cervical mucous to prevent sperm penetration (Greydanus et al., 2001; Bhowmik and Mukherjea, 1987). Interestingly, both MPA and NET-EN in a formulation with testosterone, are currently being tested as potential male contraceptives. Since both LH and FSH are required for normal spermatogenesis, both gonadotropins need to be suppressed, an effect which can be achieved with MPA or NET-EN (Nieschlag et al., 2003).

Both MPA and NET-EN have been shown to have several side effects including irregular menses, amenorrhea, and headaches (Kaunitz, 2000; Greydanus et al., 2001; Benagiano et al., 1978) when used as contraceptives in women. For MPA, fatigue, bloating of the abdomen or breasts, behavioural changes, reduced libido, weight gain, dizziness and possibly decreased bone density have also been reported (Kaunitz, 2000; Greydanus et al., 2001). The side effect profile of NET-EN is assumed to be similar to but less severe than that of MPA. In particular, the return to ovulation following the use of NET-EN occurs earlier (2.6 months) as compared with MPA (5.5 months) (Benagiano et al., 1978; Koetsawang, 1991; Garza-Flores et al., 1985). At high doses such as those used in cancer therapy, MPA has potent glucocorticoid (GC)-like effects such as complete inhibition of adrenal function (Blossey et al., 1984; Papaleo et al., 1984) and immunosuppression (Yamashita et al., 1996; Mallmann et al., 1990; Scambia et al., 1988). Furthermore, when used in the treatment of endometriosis, MPA might be linked to an increased risk of cardiovascular disease (Telimaa et al., 1989). Doses of MPA used in HRT also modulate immune function in women, selectively affecting various immune cell subsets (Brunelli et al., 1996; Malarkey et al., 1997). Other side effects of MPA and NET used in HRT include changes in the levels of lipids, lipoproteins and vasomotion, which may increase cardiovascular risk (Sitruk-Ware, 2000) as well as increase the risk of breast cancer in post-menopausal women (Riis et al., 2002; Stahlberg et al., 2003). Furthermore, a large clinical trial on the risks and benefits of an HRT regime (MPA combined with estrogen) in healthy menopausal women, scheduled to run until 2005, was terminated early due to an increased risk of invasive breast cancer. Increases in coronary heart disease, stroke, and pulmonary embolism were also observed in participants on HRT. Although fewer cases of hip fractures and colon cancer were reported, the overall health risks were found to be greater than the benefits (Rossouw et al., 2002). Recently published investigations also suggested an increase in the risk of ovarian cancer (Anderson et al., 2003).

Given these established side effects, and the apparent arbitrary choice between the use of MPA vs. NET as synthetic progestin in various reproductive intervention treatments, it is surprising that very little information is available about the molecular mechanism of action of MPA and NET, in particular their relative properties. As both compounds are progestins, the physiological effects of MPA and NET-EN are generally assumed to be mediated via the progesterone receptor (PR). However, MPA has also been shown to have a high affinity and to be an agonist for the glucocorticoid receptor (GR) (Teulings et al., 1980; Bojar et al., 1979; Feil and Bardin, 1979; Bamberger et al., 1999). Furthermore, the affinity of the rat GR for MPA (Kd = 5.0 nM) is similar to that of dexamethasone (dex) (Kd = 4.1 nM) (Winneker and Parsons, 1981), but higher than that for the endogenous ligand, cortisol (Kontula et al., 1983). In the beagle dog, the Ki value (3.7 nM) was shown to be only 4-5 times higher than that of dex, demonstrating a relatively high affinity for the GR (Selman et al., 1996). In human mononuclear leukocytes, MPA was shown to bind to the endogenous GR with a Kd of 31 nM, which was shown to be only 3 times higher than that of dex (Kd = 10 nM) (Kontula et al., 1983). For NET little is known regarding its interaction with the GR. One study established relative binding affinity from EC50 curves for NET, which displayed a very low binding activity (below 1%, where dex = 100%) for the GR (Schoonen et al., 2000). Similarly, in another study, NET showed very low binding affinity (0.1% determined from EC50 curves, relative to dex) towards the GR in human mononuclear leukocytes (Kontula et al., 1983). Clearly, accurate Kds of MPA and NET for the GR (as related to the serum concentrations found in patients on various treatments) would be useful information in assessing their potential side effects via the GR.

Women receiving MPA by injection as a contraceptive in general have been shown to have serum concentrations of about 1 ng/ml for the duration of

contraceptive treatment (Mishell, 1996) i.e. about 2.6 nM. However, levels of drug-related material have been reported to reach 25 ng/ml (65 nM) a few days post injection (Kirton and Cornette, 1974). Contraceptive doses of NET-EN have been reported to result in serum concentrations of about 1.5-59 nM (Fotherby et al., 1983). Doses of MPA used for cancer therapy typically range between 500 and 1500 mg orally per day for about 12 weeks (Blossey et al., 1984), for treatment of endometriosis about 50- to 100 mg/day (Harrison and Barry-Kinsella, 2000; Telimaa et al., 1989), while those used for HRT are about 10 mg/day for about 11 days (Brunelli et al., 1996). From the literature, it is not clear what serum concentrations of MPA women typically have during HRT use. Doses of NET used in HRT range from about 0.35- to 2.1 mg orally per day (Taitel and Kafrissen, 1995). Women receiving the Activelle HRT regime (0.5 mg NET-A, estradiol 1 mg daily) are reported to have peak serum concentrations of NET ranging between 1.4- and 6.8 ng/ml (3.64- to 17.7 nM) after single dose administration (Activelle package insert reg. no. 33/21.8.2/0532, Novo Nordisk). For the treatment of endometriosis a daily dose of 5 mg of NET-A is used (Surrey and Hornstein, 2002). The extent to which these various doses of progestins exert effects on various target genes in vivo via the GR is not known.

The GR is a ligand-dependent transcription factor that belongs to the steroid hormone receptor family. It is composed of an amino-terminal transactivation (activation function-1, AF-1) domain (NTD), a central DNA-binding domain (DBD), which interacts with the DNA response elements, and a carboxy-terminal domain, which contains the ligand-binding (AF-2) domain (LBD) to which the steroid hormone binds (Evans, 1988; Beato, 1989; Bledsoe et al., 2002). In an unbound state, the GR resides in the cytoplasm and is complexed with several different protein factors including heat-shock protein 90 (hsp90) (DeRijk et al., 2002) and immunophilins (Davies et al., 2002). Upon hormone binding, receptor activation and phosphorylation

occurs, resulting in translocation of the GR complex from the cytoplasm to the nucleus where it dissociates, releasing GR to either transactivate or transrepress specific genes (Davies et al., 2002). There are at least three mechanisms through which GCs can regulate gene transcription. The first involves activation of genes through binding of GC-activated GR homodimers to consensus sequences referred to as GC-response elements (GREs) found in the promoters of various genes (Adcock, 2000; Webster and Cidlowski, 1999; Newton, 2000). The second mechanism of GC-mediated regulation involves repression of target genes through a direct interaction of GC-bound GR to variable negative GRE (nGRE) sequences within the promoter (Adcock, 2000). Thirdly, GCs may inhibit other genes such as the interleukin-8 (IL-8) gene by transcriptional cross-talk where the GR mutually interferes with other signalling pathways such as those involving activator protein-1 (AP-1) and nuclear factor κB (NFκB) (Webster and Cidlowski, 1999; Karin, 1998; De Bosscher et al., 2003; Mukaida et al., 1994).

In the current study, we performed whole cell binding assays and determined the Kds of MPA and NET-A for the GR. In addition, we determined the relative potencies and efficacies of these compounds for transactivation of synthetic GREs and transrepression of an IL-8 promoter reporter gene via the GR.

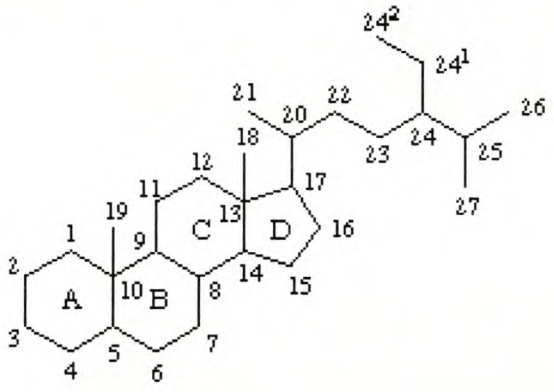


Figure 1: The steroid hormone carbon numbering system.

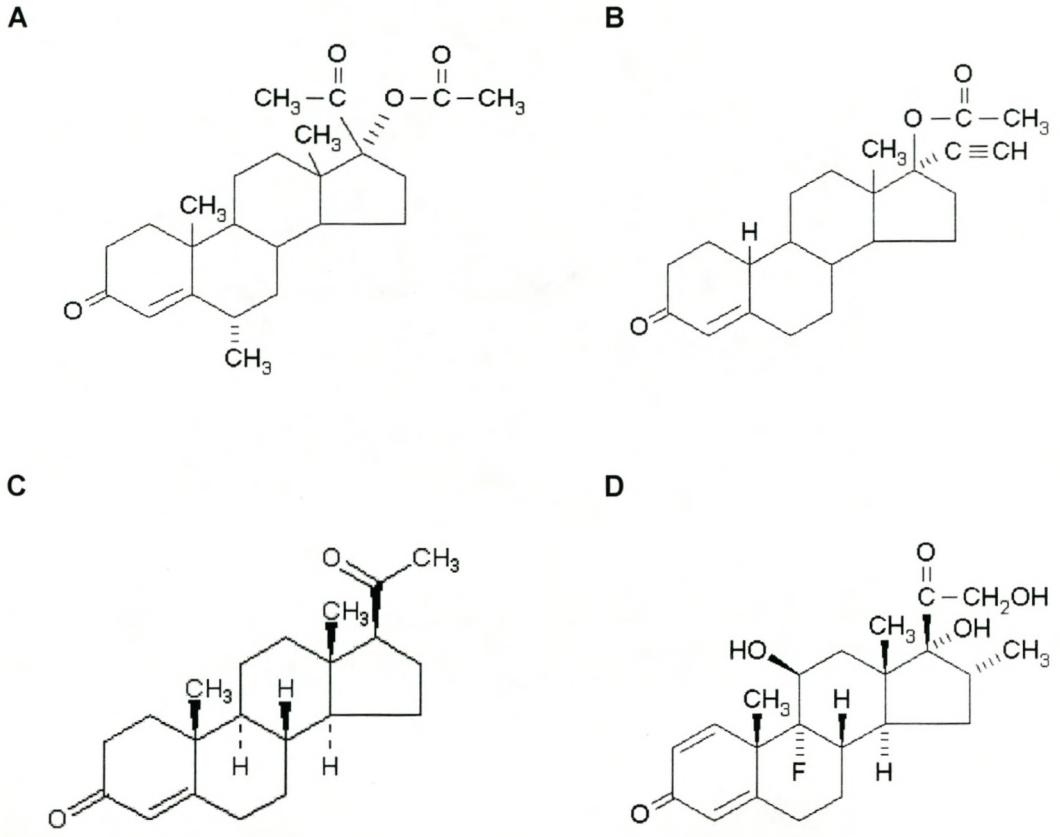


Figure 2: The chemical structures of natural and synthetic progestins, and a synthetic glucocorticoid. (A) medroxyprogesterone acetate; (B) norethisterone acetate; (C) progesterone; (D) dexamethasone.

Materials and methods

Cell culture, cytokines and inducing compounds

Human lung carcinoma A549 cells and human embryonic kidney Hek293 cells were a kind gift from Prof S. Okret (Karolinska Institute, Sweden) and were maintained in 1:1 mixture of high glucose (1g/ml) DMEM (Sigma, South Africa) and Ham-F12 (Highveld Biologicals, South Africa) medium supplemented with 10% FCS (Highveld Biologicals, South Africa) and 50 IU/ml penicillin and 50 μg/ml streptomycin (GibcoBRL, South Africa). Hek293 cells stably transfected with rat GR expression vector were also provided by Prof S. Okret (Karolinska Institute, Sweden) and were grown in complete medium described above, supplemented with hygromycin 100 μg/ml (Roche, South Africa). COS-1 cells were cultured in high glucose (1 g/ml) DMEM (Sigma, South Africa) supplemented with 10% fetal calf serum (Highveld Biologicals, South Africa), 50 IU/ml penicillin and 50 μg/ml streptomycin (GibcoBRL). [^{3}H] dexamethasone (89 Ci/mmol) was purchased from AEC-Amersham (South Africa). Dexamethasone (dex) (9-alpha-fluoro-16-alpha-methylprednisilone), medroxyprogesterone acetate (MPA) (6-alpha-methyl-17-alpha-hydroxyprogesterone acetate), progesterone (prog) (4-pregnene-3, 20-dione), and norethisterone acetate (NET-A) (17-alpha-ethynyl-19-nortestosterone 17-beta-acetate) were purchased from Sigma Aldrich, South Africa. We chose to use NET-A since the acetate 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). The origin and activity of TNF has been described previously (Vanden Berghe et al., 1998). Hydroxyflutamide was obtained from Dr C. Tendler (Schering Plough Research Institute, USA). Stock solutions were routinely dissolved in ethanol and stored at -20°C.

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 (Tanner et al., 2003). The rat GR cDNA (pSVGR1) (Delauney et al., 1996) was obtained from Prof S. Okret (Karolinska Institute, Sweden). The plasmids, pCMVβ-gal and pSVβ–gal, were obtained from Stratagene (South Africa) and Promega (South Africa) respectively. The IL-8 promoter variant, p546hIL-8luc+, was kindly provided by Dr N. Mukaida (Cancer Research Institute, Japan) (Mukaida et al., 1990).

Whole cell binding assays

Competitive whole cell binding assays were performed essentially as described by Bamberger et al., (1995) with the following modifications. Briefly, A549 and COS-1 cells were maintained as above and seeded into 12-well tissue culture plates (Nunc, South Africa) at 1.5 x 10^5 cells per well, in complete medium. On day two, COS-1 cells were transiently transfected with pSVGR1 and pSVβ–gal using FuGENE 6 according to manufacturer's instructions, whereas A549 cells remained untransfected. On day three, the cells were washed three times with PBS warmed to 37°C and then incubated for three hours for A549 cells and one hour for COS-1 cells at 37°C, with 1.25 nM for A549 cells, or 10 nM for COS-1 cells of [^{3}H] dex (89 Ci/mmol) (Amersham, South Africa), in the absence and presence of varying concentrations of unlabelled steroids diluted in medium without FCS, in a final volume of 1 ml per well. Working on ice at 4°C, cells were washed three times with ice-cold 1 x PBS containing 0.2% (w/v) BSA for 15 minutes. Cells were then lysed with 200 μl Reporter Lysis Buffer (Promega Luciferase Assay System) and total binding was determined by scintillation counting. Specific bound [^{3}H] dex was calculated as the difference between total and non-specific binding, which was

determined by incubating cells in the presence of [^{3}H] dex plus 500-fold excess unlabelled dex. For A549 cells, the Bradford assay was used to determine total protein concentration. For COS-1 cells, the β-galactosidase assay (Galacto-*Star*™ assay system) was performed in a Labsystems Luminoskan RS luminometer. Specific binding was therefore normalised to co-expressed β-galactosidase activity (for COS-1 cells) or total protein (for A549 cells). Binding data was analysed using GraphPad Prism software (see Chapter 2 Appendix for calculation of parameters).

Transfections and dose-response curves

Transient transfection of COS-1 cells was performed with FuGENE 6 (Roche Biochemicals, South Africa) according to manufacturer's instructions. Hek293 cells were transiently transfected with p546hIL-8luc+ promoter reporter construct using FuGENE 6 (Roche Biochemicals, South Africa) according to manufacturer's instructions. Hek293 cells were also transiently transfected with the pTAT-GRE-E1b-luc construct and pCMV-βgal where indicated, using polyethylenimine (PEI) (Aldrich, South Africa) transfection method (Prof S. Okret, Karolinska Institute, Sweden, personal communication). Briefly, cells were seeded at 6 x 10^4 cells per well in 24-well tissue culture plates (Nunc, South Africa) and grown in complete medium. Cells were left for at least 36 hours to reach optimal confluency of approximately 60%. For transfection of each well, 0.5 μl (0.2 mM) PEI was added to 200 ng reporter DNA and topped up to 20 μl with H_2O. The mix was then incubated for 10 minutes at room temperature, followed by addition of 500 μl complete medium and incubation for 24 hours. Cells transfected with pTAT-GRE-E1b-luc were incubated with or without test compounds for 18 hours. Cells transfected with p546hIL-8luc+ were untreated or pre-treated with dex, MPA or prog in the absence or presence of 2000 IU/ml TNF. Steroid hormone was added two hours before TNF, for a total period of eight hours, after which cells were lysed with Reporter Lysis Buffer (Promega Luciferase Assay System) and assayed for luciferase and β-galactosidase expression. The β-

galactosidase (Galacto-*Star*™ assay system) and luciferase assays (Promega Luciferase Assay System) were performed in a Labsystems Luminoskan RS luminometer. Where indicated, luciferase activity was normalised to co-expressed β-galactosidase activity to correct for differences in transfection efficiencies. Otherwise where normalising to β-galactosidase is absent, transfection efficiency was previously determined to be highly reproducible. Data was analysed using GraphPad Prism software. Briefly, the following parameters were chosen for dose-response curve analysis: Nonlinear regression (curve fit), sigmoidal dose-response curve, where the mean y value of each point was considered. A stricter (slower) criterion for convergence was used. Furthermore, runs test and residuals test were used to determine whether the curve deviated systematically from the data.

Statistical analysis

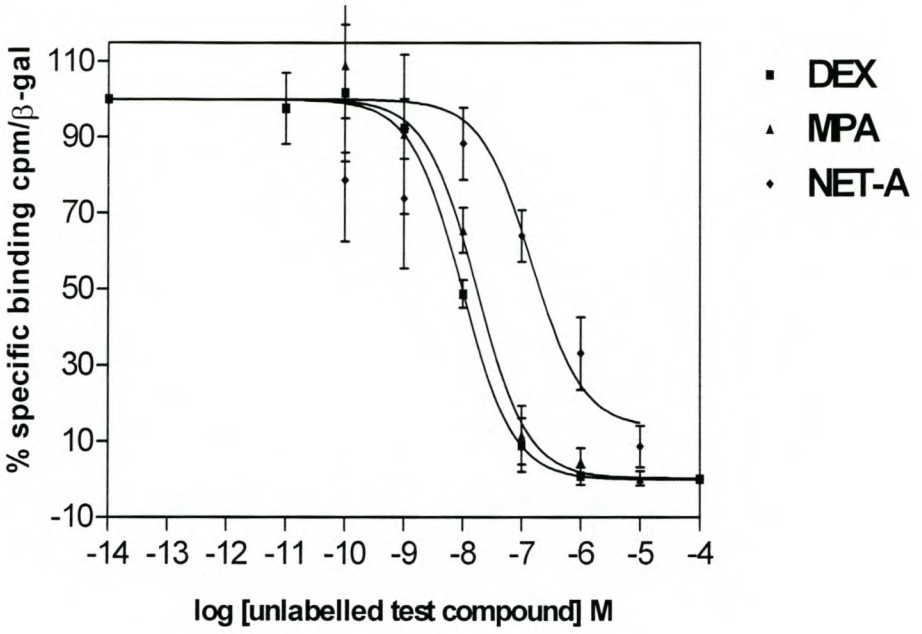
Statistical analyses were carried out using GraphPad Prism software, using one-way analysis of variance with either Bonferroni (compares all pairs of columns) or Dunnett (compares all columns vs. control column) posttests. In most figures, statistical significance of differences is denoted by *, ** or ***, to indicate $P<0.05$, $P<0.01$ or $P<0.001$, respectively. The letters a,b,c etc. are also used to denote statistically significant differences, where all those values which differ significantly ($P<0,05$) from others, are assigned a different letter.

Results

MPA displays a higher relative binding affinity than NET-A for the GR.

To determine the relative binding affinity of MPA and NET-A for the rat GR, competitive whole cell binding assays were preformed in COS-1 cells, transiently transfected with rat GR expression vector. Homologous/heterologous curves were analysed and EC50 values for each test compound determined and reported in Figure 3a. Results show EC50 values of 9.6 ± 1.2 nM for dex, 17.1 ± 1.2 nM for MPA and 153 ± 1.3 nM for NET-A. The EC50 values for dex and MPA differed 1.8-fold but were found not to be statistically significantly different ($p>0.05$), whereas those for dex and NET-A displayed a 16-fold statistically significant difference (p at least <0.05). Furthermore, the EC50 values for MPA and NET-A differed 9-fold and were also found to be statistically significantly different (p at least <0.05). No specific binding was detected in the absence of transfected GR.

A similar trend in results was obtained for competitive whole cell binding assays in A549 cells, which express GR endogenously. In order to obtain accurate Kd and Ki values, a range of experiments was first carried out to establish two important parameters. The incubation time required for equilibrium to be reached for [^{3}H] dex binding to the GR was determined as three hours, and an appropriate concentration of [^{3}H] dex, in the range two to ten times lower than the EC50, was established as 1.25 nM (data not shown). Homologous/heterologous curves with unlabelled steroids were then established and results show that dex, MPA, NET-A and prog are able to compete with [^{3}H] dex for binding to the GR (Figure 3b). The curves for each competitor steroid indicate competitive binding to the same site as dex (R^2 values for each MPA, NET-A and prog were 0.9978, 0.9728 and 0.9806 respectively, for one site competitive binding curve).

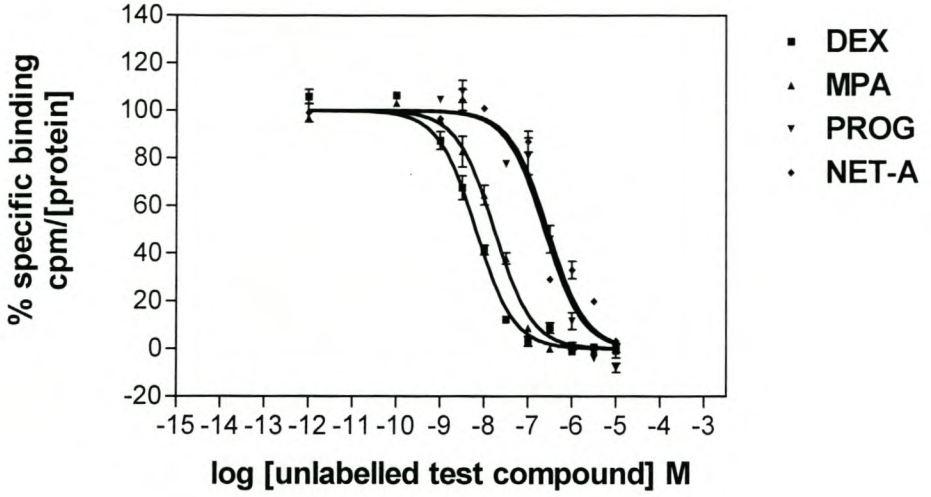


	EC50 (M) ± SEM
DEX	$9.6 \times 10^{-9} \pm 1.2$
MPA	$1.7 \times 10^{-8} \pm 1.2$
NET-A	$1.5 \times 10^{-7} \pm 1.3$

Figure 3A: MPA and NET-A both compete with [^{3}H] dex for binding to the rat GR.

COS-1 cells were transiently transfected with the pSVGR1 and pSVβ–gal expression vectors.

Cells were incubated twenty-four hours later with 10 nM [^{3}H] dex in the absence and presence of increasing concentrations of dex (■), MPA (▲) or NET-A (♦) for one hour. Competition for binding is illustrated by the percent of [^{3}H] dex specifically bound to the rGR (normalised to β-gal expression). Results shown are averages of three independent experiments, where each condition was performed in triplicate (±SEM). In table, data was analysed to obtain IC50 values ± SEM for binding curves. Dex vs. MPA ($p>0.05$); MPA vs. NET-A ($p<0.05$).



	Kd or Ki (M) ± SEM
DEX	$4.2 \times 10^{-9} \pm 1.1$
MPA	$1.08 \times 10^{-8} \pm 1.1$
NET-A	$2.7 \times 10^{-7} \pm 1.3$
PROG	$2.15 \times 10^{-7} \pm 1.1$

Figure 3B: MPA, NET-A and prog compete with [^{3}H] dex for binding to the human GR.

A549 cells were incubated with 1.25 nM [^{3}H] dex in the absence and presence of increasing concentrations of dex (■), MPA (▲), NET-A (♦) or prog (▼) for three hours. Competition for binding is illustrated by the percent of [^{3}H] dex specifically bound to the hGR (normalised to total protein). Results shown are representative of four independent experiments, where each condition was performed in triplicate (±SEM). In the table, data from four independent experiments was analysed to obtain Kd or Ki values ± SEM. Dex vs. MPA ($p<0.001$); MPA vs. NET-A ($p<0.001$); MPA vs. prog ($p<0.001$); NET-A vs. prog ($p>0.05$).

The displacement curves were analysed and Kd values determined for dex, and Ki values determined for each MPA, NET-A and prog (Figure 3b). Dex was found to have a Kd of 4.2 ± 1.1 nM for the GR, whilst MPA (Ki = 10.8 ± 1.1 nM) displayed a 2.6-fold lower binding affinity than dex ($p<0.001$). NET-A (Ki = 270 ± 1.3 nM) showed a 64-fold lower binding affinity for the GR compared with dex ($p<0.001$). The Ki values for MPA and NET-A were found to differ 25-fold and are also statistically significantly different ($p<0.001$). Prog showed a similar Ki (215 ± 1.1 nM) for the GR to that of NET-A ($P>0.05$). A549 cells express androgen receptor (AR) and GR endogenously, but have been reported not to express PR (Zhang et al., 2000). Since MPA and NET are known to bind to the AR (Kemppainen et al., 1999; Deckers et al., 2000), A549 cells were incubated with [^{3}H] dex and MPA in the presence or absence of the AR antagonist, hydroxyflutamide. Results showed no difference in [^{3}H] dex binding to the GR in the presence of MPA co-treated with or without hydroxyflutamide (data not shown). This suggests that the levels of AR are too low to interfere with the GR-binding assay (data not shown). This also rules out the involvement of the AR in our incubations with NET-A, since both MPA (EC50 = 6.79×10^{-9} M) and NET-A (EC50 = 2.73×10^{-8} M) have been shown to display statistically similar binding affinities towards the AR (unpublished data). We were unable to repeat the above binding experiments in Hek293 cells, as these cells were technically difficult to handle and would not have survived the various rigorous and necessary washes of the binding assay. We were unable to determine accurate Ki values in COS-1 cells as an excess concentration of [^{3}H] dex was used and whether binding equilibrium was reached was not determined (GraphPad Prism software).

MPA displays much greater GC agonist potency and efficacy than NET-A for transactivation.

To compare the relative GC properties of MPA and NET-A for transactivation, Hek293 cells stably transfected with a rat GR expression vector, were transiently

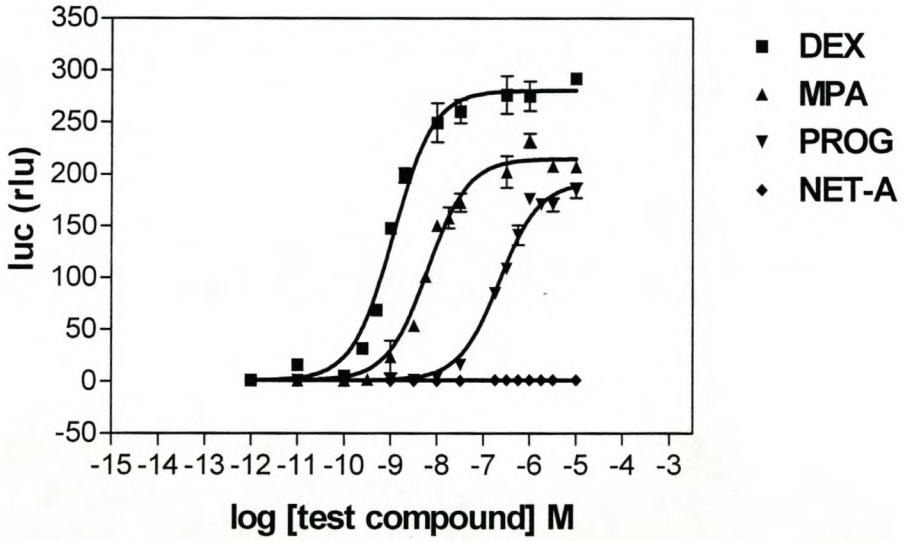
transfected with a GRE-driven reporter construct (containing two copies of the rat TAT GRE). Cells were subsequently treated with increasing concentrations of dex, MPA, NET-A or prog. Results showed greatest transactivation with dex, followed by MPA and followed by prog (Figure 4a). NET-A, even at a concentration of 10 μM was unable to transactivate the promoter reporter construct in our system (Figure 4a). To determine the relative potency and efficacy between the steroidal ligands, dose-response curves were analysed and EC50 and maximal values for each test compound were determined. Dex showed the greatest potency for transactivation with an EC50 value of 1.1 ± 1.0 nM, followed by MPA (EC50 = 7.2 ± 1.1 nM) which displayed a 6.5-fold lower potency than dex, followed by prog (EC50 = 280 ± 1.1 nM) with a 39-fold lower potency than MPA. MPA and prog were unable to transactivate to the same maximal extent as dex, showing that they are partial agonists for transactivation via the GR.

To determine whether the above transactivation results are dependent on the presence of the GR, transient transfections with the same GRE-driven reporter construct were also performed on wild-type Hek293 cells (not stably transfected with GR). Cells were treated with 10 μM dex, MPA, NET-A or prog and results showed no transactivation in the absence of stably transfected GR (Figure 4b), showing that the results obtained in Figure 4a were due to the presence of the GR.

Transactivation studies were also performed on COS-1 cells transiently transfected with a 2xGREtkluc reporter construct (containing 2 copies of a GRE upstream of the tk promoter) with and without rat GR cDNA, in the presence of steroid hormone. Results show that dex, like MPA, is able to transactivate the reporter at concentrations as low as 1nM (Figure 4c). Significant transactivation of the GRE-driven reporter by 2.6 nM, a concentration typically observed in the serum

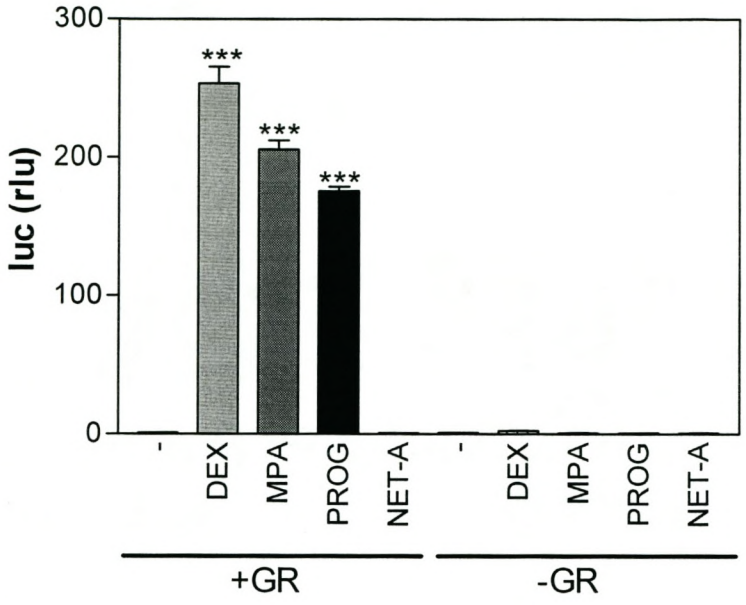
of women using MPA as a contraceptive (Mishell, 1996), was also obtained with dex or MPA (Figure 4d). Interestingly, MPA showed greater GC activity than cortisol, the endogenous ligand for the GR, at both concentrations tested (Figures 4c and 4d). Furthermore, this effect was clearly dependent on the presence of the GR. In the presence of the rat GR, no transactivation was observed with NET-A at concentrations up to 10 μM (Figures 4d and 4e).

4A

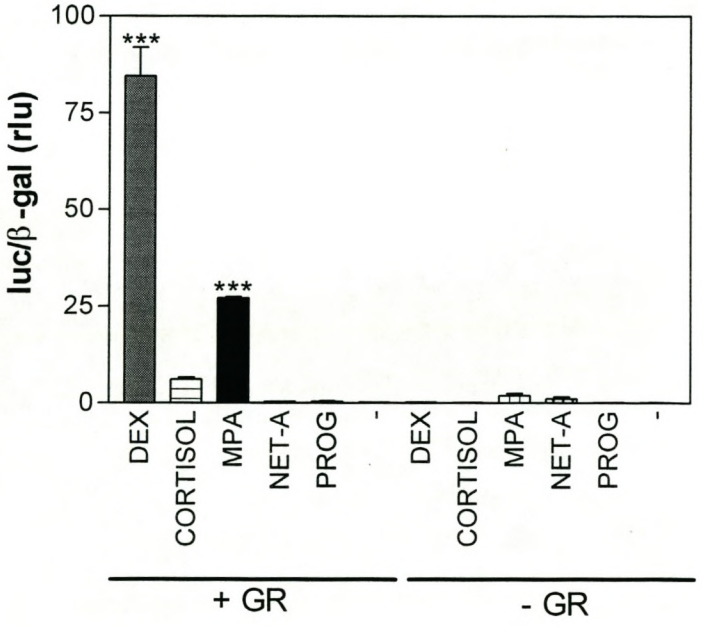


	EC50 (M) ± SEM	MAX (rlu) ± SEM
DEX	$1.1 \times 10^{-9} \pm 1.0$	287.2 ± 5.4
MPA	$7.2 \times 10^{-9} \pm 1.1$	207.6 ± 1.2
NET-A	N/A	1.2 ± 0.03
PROG	$2.8 \times 10^{-7} \pm 1.1$	177.6 ± 5.3

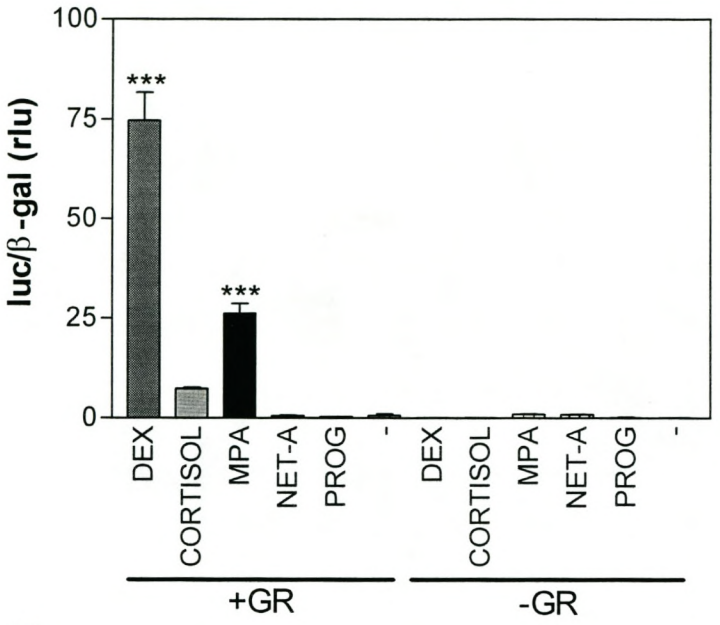
4B



4C



4D



4E

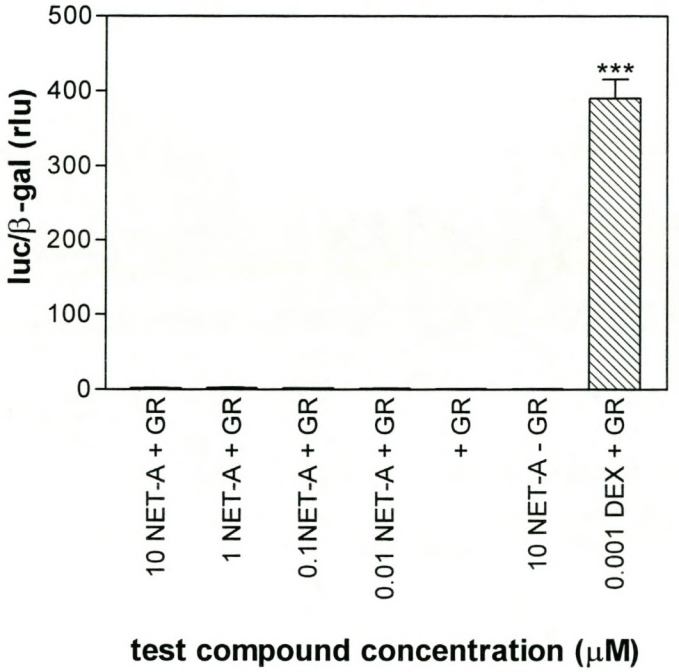


Figure 4: MPA, unlike NET-A, displays partial agonist activity via the GR.

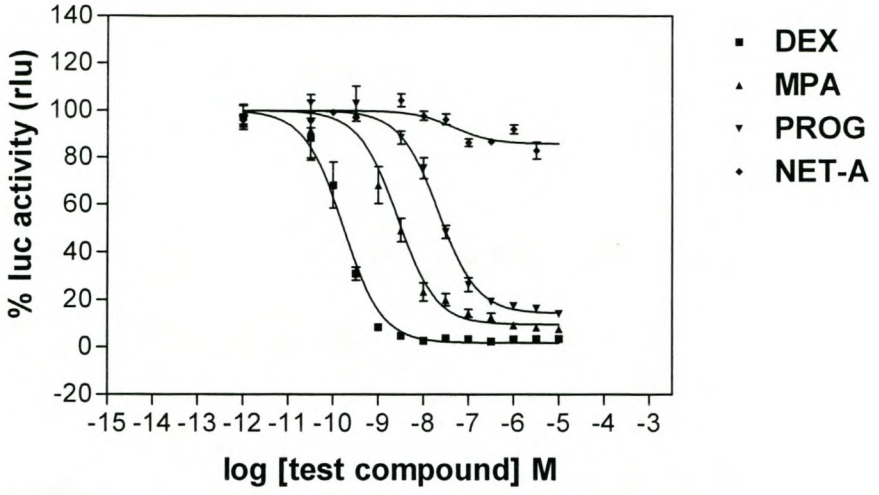
Hek293 cells, either stably transfected with the rat GR expression vector (A) or wild-type (B) were transiently transfected with the pTAT-GRE-E1b-luc reporter. For (A), cells were incubated in the absence and presence of increasing concentrations of dex (■), MPA (▲), NET-A (♦) or prog (▼) for eighteen hours. In the table, data from three independent experiments was analysed to obtain EC50 ± SEM and maximal values ± SEM for each test compound. Dex vs. MPA ($p<0.001$); MPA vs. prog ($p<0.001$). For (B) cells were incubated in the absence or presence of 10 μM dex, MPA, prog or NET-A for eighteen hours. In (C, D, and E), COS-1 cells were transiently transfected with the pTAT-GRE-E1b-luc and pCMV-βgal expression vectors, with or without pSVGR1. Cells were then incubated for 24 hours in the absence or presence of 1 nM (C), 2.6 nM (D) test compound or as indicated (E). For (A-E), results shown are representative of three independent experiments where each condition was performed in triplicate (±SEM). For (B-E), statistical significance of differences is denoted by *, ** or ***. Induction is expressed in relative light units (rlu). For some experiments (A, B), only luciferase (luc) values were determined. For others (C-E), luc values were normalised for β-galactosideas (luc/β-gal). N/A = no activity up to 10^{-5} M NET-A.

MPA displays much greater GC agonist potency and efficacy than NET-A for transrepression.

To compare the relative GC properties of MPA and NET-A for transrepression, Hek293 cells stably transfected with a rat GR expression vector, were transiently transfected with an IL-8 promoter reporter construct, p546hIL-8luc+. Cells were subsequently treated with TNF and increasing concentrations of dex, MPA, NET-A or prog. To determine the relative potency and efficacy between the steroidal ligands, dose-response curves were analysed and EC50 and maximal values for each test compound were determined. Dex showed the greatest potency for transrepression with an EC50 value of 0.21 ± 1.2 nM, followed by MPA (EC50 = 2.7 ± 1.2 nM), which showed a 13-fold lower potency than dex ($p<0.001$), followed by prog (EC50 = 26 ± 1.1 nM) with a 10-fold lower potency than MPA ($p<0.001$) (Figure 5a). NET-A showed very little repression of the promoter reporter construct (Figure 5a). MPA repressed the promoter to almost the same maximal extent as dex, while the maximal effect of prog was slightly less than that of MPA.

To determine whether the above transrepression results are GR-dependent, transient transfections with the same IL-8 promoter reporter construct were also performed on wild-type Hek293 cells not stably transfected with GR. Cells were treated with 10 μM dex, MPA, NET-A or prog and showed no transrepression in the absence of stably transfected GR (Figure 5b).

5A



	EC50 (M) ± SEM	% repression ± SEM
DEX	$2.1 \times 10^{-10} \pm 1.2$	96.8 ± 0.4
MPA	$2.7 \times 10^{-9} \pm 1.2$	92.3 ± 1.1
NET-A	$>100 \times 10^{-9}$	22.2 ± 3.3
PROG	$2.6 \times 10^{-8} \pm 1.1$	85.8 ± 0.6

5B

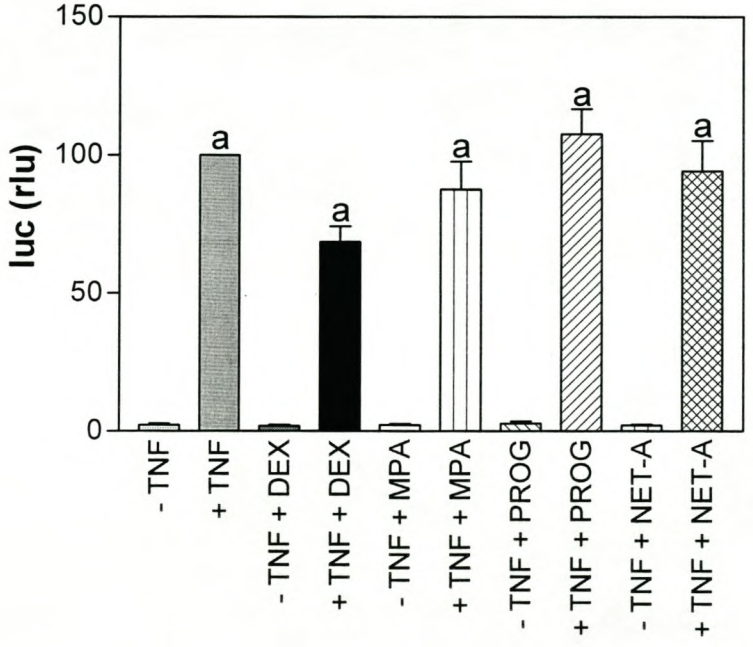


Figure 5: MPA acts as a potent agonist for transrepression via the GR, while NET-A acts as a very weak partial agonist.

Hek293 cells, either stably transfected with the rat GR expression vector (A) or wild-type (B) were transiently transfected with the p546hIL-8luc+ reporter. For (A), cells were incubated in the absence and presence of increasing concentrations of dex (■), MPA (▲), NET-A (♦) or prog (▼) whereas in (B) cells were incubated in the absence or presence of 10 μM dex, MPA, prog or NET-A. For both (A) and (B), incubations were performed in the absence or presence of 2000 IU/ml TNF. Steroid hormone was added two hours before TNF, for a total period of eight hours, after which cells were lysed and assayed for luciferase expression. In (A) luciferase activity for each test compound condition was normalised relative to the activity of the reporter in the presence of TNF and in the absence of test compounds, which was taken as 100%. In table, data was analysed to obtain EC50 ± SEM and % repression values ± SEM for each test compound. Dex vs. MPA ($p<0.001$); MPA vs. prog ($p<0.001$). For (A), results shown are representative of three independent experiments where each condition was performed in triplicate (±SEM). For (B), results shown are averages of four independent experiments with each condition in triplicate (±SEM). For (B), the letters a,b,c etc. are used to denote statistically significant differences, where all those values which differ significantly ($P<0,05$) from others, are assigned a different letter. Induction is expressed in relative light units (rlu). N/A = no activity up to 10^{-5} M NET-A.

Discussion

Ideally, a drug targeted to the PR should display similar properties as prog in terms of binding affinity and agonist activity towards the PR and other receptors. To date, no thorough comparison exists at the molecular level of the mechanism of action of MPA vs. NET or NET-A. In particular, a comparison of the relative binding affinity (relative equilibrium dissociation constants) and relative potency and efficacy for transactivation and transrepression of these two progestins and prog towards the GR has not been previously reported.

Our results show that both MPA and NET-A bind to the GR in A549 cells and that they bind to the same site as that of dex. Interestingly, prog was also observed to bind to the GR with a relative binding affinity similar to NET-A. Furthermore, we determined accurate equilibrium dissociation constants, which show that MPA displays a higher relative binding affinity than NET-A for the GR. A similar trend of results was obtained in COS-1 cells transiently transfected with the rat GR expression vector as is reflected in the relative EC50 values. The results in COS-1 cells strongly support our binding data obtained in A549 cells and confirm that the Ki values determined are due to the binding of test compounds to the GR and not to any other steroid receptors. To our knowledge, our study is the first to establish accurate Ki values for both MPA and NET-A towards the human GR. Our Kds for dex and MPA (4.2- and 10.8 nM, respectively) for the human GR, are slightly lower than the previously reported Kds of 10- and 31 nM respectively, also for the human GR (Kontula et al., 1983), and are slightly higher than the previously reported Kds for the dog GR (0.8- and 3.7 nM, respectively) (Selman et al., 1996). However, the previously established Kds of 4- and 5 nM for dex and MPA respectively, for the rat GR (Winneker and Parsons, 1981) agree well with our results, and suggest that there

are no substantial species differences between the rat and human GR towards binding affinity of MPA and dex. Our results with NET-A are consistent with the study by Schoonen et al., (2000) who show a low relative binding affinity of NET and its metabolites for the human GR (below 1%, where dex = 100%) in human leukemic IM-9 cells. It is unclear from that data whether the relative binding activity was determined from Kd values or from EC50 curves.

Previously we observed significant transactivation by MPA of a GRE-driven promoter reporter construct in both L929sA mouse fibroblast and COS-1 cells. These effects of MPA were shown to be mediated at least in part via the GR (submitted for publication, Chapter 2). In the current study, we investigated relative agonist activity of MPA and NET-A for transactivation via the GR. Our results show that MPA and prog act as partial agonists for transactivation of a GRE-driven promoter reporter construct in Hek293 cells and this effect is clearly dependent on the presence of stably transfected GR. We found however, that NET-A was unable to induce the reporter even at the highest concentrations (10 μM) used. This is consistent with results obtained in COS-1 cells transiently transfected with a GRE-driven promoter reporter construct. Interestingly, these COS-1 cell experiments were also previously carried out using a simple GRE-driven promoter reporter construct (2 x GREtkluc) yielding similar results, indicative of the absence of promoter-specific effects (data not shown). EC50 values extrapolated from the dose-response curves performed in Hek293 cells show that dex displayed the greatest potency for transactivation, followed by MPA, which showed a 6.5-fold lower potency than dex, followed by prog which had a 39-fold lower potency than MPA (Table 1).

	MPA	NET-A	PROG
relative binding affinity in A549 cells (%)	41.0 ± 3.39	1.68 ± 0.42	2.00 ± 0.10
relative potency for transactivation (%)	15.5 ± 1.73	N/A	0.40 ± 0.05
relative efficacy for transactivation (%)	72.2 ± 0.85	0.40 ± 0.01	64.3 ± 2.46
relative potency for transrepression (%)	7.90 ± 1.29	0.21[1]	0.80 ± 0.10
relative efficacy for transrepression (%)	95.4 ± 1.16	23.0 ± 3.44	88.7 ± 0.66

Table 1: Relative binding affinity from Kd or Ki values in Figure 3b, relative EC50 (potency) and relative maximal values (efficacy) for both transactivation (from Figure 4a) and transrepression (from Figure 5a). All values are percentages, calculated relative to 100% activity of dex ± SEM. N/A = no activity up to 10^{-5} M NET-A. [1] This EC50 could not be determined with as much accuracy as the other values, due to the shallow slope.

We also investigated the relative agonist activity of MPA and NET-A for transrepression of IL-8 via the GR. IL-8 belongs to a chemotactic cytokine family and is responsible for the chemotactic migration and activation of neutrophils, lymphocytes, monocytes and other cell types at sites of inflammation (Mukaida et al., 1994; Feghali and Wright, 1997). Furthermore, GC-mediated repression of IL-8 genes has been shown to involve the GR (Mukaida et al., 1994). We have shown previously that MPA and prog, like dex, significantly represses TNF-stimulated IL-8 promoter reporter constructs at the transcriptional level in L929sA cells, an effect thought to be mediated at least in part via the GR. In this study, our results show that dex, MPA and prog act as agonists for transrepression of the IL-8 promoter reporter construct in Hek293 cells and this effect is clearly dependent on the presence of stably transfected GR. We found however, that NET-A only repressed the induced reporter by 22% at the highest concentrations (10 μm) used. Furthermore, EC50 values extrapolated from the dose-response curves performed in Hek293 cells showed that dex displayed the greatest potency for transrepression. In addition, MPA showed a 13-fold lower potency than dex, followed by prog and NET-A, which had a 10-fold and about 38-fold lower potency than MPA respectively (Table 1).

When assessing the dissociative properties of each test compound, the relative efficacies of MPA, NET-A and prog for transrepression were found to be significantly greater than their respective efficacies for transactivation. The difference in efficacy of MPA relative to dex for transrepression is 95% and for transactivation is 75% (Table 1). The transrepression/transactivation ratio for MPA therefore is 1.3 which is similar to that of dex used as the standard (transrepression/transactivation = 1) (Bamberger and Schulte, 2000). We conclude that MPA discriminates only slightly between transactivation and transrepression for the rat GR in Hek293 cells. This result is similar to our previous findings for the endogenous mouse GR in L929sA cells where the ratio for MPA was calculated as 1.5 (submitted for publication,

Chapter 2). However, in the studies by Bamberger et al., (1999) and Bamberger and Schulte, (2000), MPA behaved as a dissociated GC, as it only marginally transactivated a GRE-reporter construct in human HeLa cells and in normal human lymphocytes, via the endogenous GR in both cell types. In normal lymphocytes, MPA showed a transrepression/transactivation ratio of 6.6, compared with dex where the ratio was 1 (Bamberger and Schulte, 2000). The differences between these results and those of the present study could be due to tissue-specific effects as previously discussed (submitted for publication, Chapter 2). Similarly to MPA, we found the transrepression/transactivation ratio for prog to be 1.4. However, for NET-A, the transrepression/transactivation ratio was calculated as 55 showing significantly greater dissociative GC properties compared with MPA and prog, although it was only a very weak partial agonist for transrepression.

When comparing the relative binding affinities of MPA and NET-A towards the GR with their relative potencies and relative efficacies for transactivation and transrepression, the fold differences relative to dex display interesting findings (Table 1). In terms of rank order for dex, MPA and prog, the ligand that binds with greatest affinity to the GR is the more potent agonist for transactivation and transrepression and displays the greatest efficacy. However, the extent to which each ligand binds relative to dex does not reflect the extent to which it is an agonist for transactivation or transrepression. MPA, prog and NET-A showed 41%, 2% and 1.7% binding affinities respectively, relative to 100% binding by dex, towards the GR. However, both MPA and prog showed significantly lower relative potencies for transactivation with 15.5% (2.6 times lower) for MPA and 0.4% (5 times lower) for prog, relative to 100% potency for dex. Although NET-A is able to bind to the GR (binding affinity 1.7%, relative to 100% binding with dex), it failed completely at transactivating the promoter reporter construct at 10 μM. Similarly, there is no exact correlation between Kds and relative efficacies for transactivation, nor between Kds and relative

potencies or efficacies for transrepression. Interestingly, for MPA the relative potency observed for transactivation (15.5%) was almost 2-fold higher than its potency reported for transrepression (7.9%), relative to 100% potency for dex. In contrast, the trend for prog was the other way around where the relative potency for transactivation (0.4%) was 2-fold lower than the potency for transrepression (0.8%), relative to 100% potency of dex. Although NET-A showed no activity for transactivation, the relative EC50 for transrepression was estimated as about 0.21%. Thus, for MPA, prog and more so for NET-A, binding affinity appears to be a poor indicator of the extent of agonist potency and efficacy for transactivation and transrepression.

Determinants of ligand-specific bioactivity for the GR are not fully understood, but appear to be influenced by a number of factors, which modulate activation and activity of the GR in a multi-step process. The first step involves the ability of the ligand to recognise and bind the GR, which is influenced by the chemical structure (discussed below) of the ligand as well as the affinity of the ligand for the GR. The affinity of a ligand for its receptor has been shown to be one of several determinants of potency (Chen et al., 2002). Ligand binding induces a conformational change of the GR, suggested to be to be dependent on the affinity of the ligand for the GR (Schaaf and Cidlowski, 2003). The GR has been shown to undergo a differential conformational change upon binding of different ligands (Vicent et al., 2002). These ligand-specific differences in GR conformation may affect the potency of the GR-ligand complex, possibly due to the recruitment of different co-factors. The stability of steroid-GR complexes, which is dependent on specific steroid receptor contacts, also influences the ability of GCs to stimulate the transactivation function of the GR (Hellal-Levy et al., 1999). Furthermore, the dissociation rate of the ligand from the GR (as has been shown for dex) is a reflection of the transactivation function of the GR (Hellal-Levy et al., 1999). Once bound to ligand, receptor phosphorylation occurs

and the GR translocates into the nucleus where it either transactivates or transrepresses specific genes with the assistance of various co-factors. Differences in nuclear translocation, in terms of distinct pathways of receptor trafficking and processing within the nucleus, and retention of the liganded-GR in the nuclear compartment, are also thought to influence ligand-specific responses (Defranco, 2002; Vicent et al., 2002). Furthermore, it has been shown for some agonists and antagonists that the amount of ligand-induced phosphorylation of Ser211 in the liganded GR correlated with the transcriptional activity of the GR (Wang et al., 2002). There is also evidence in the literature that ligand-specific co-factor recruitment may also influence transcriptional regulation (Coghlan et al., 2003; Miner, 2002). Finally, the dissociation rate of liganded-GR from DNA may reflect ligand potency (Pandit et al., 2002). It is thus possible that additional information on other influencing factors as described above may reveal a more direct correlation for agonist potency and/or efficacy for dex, MPA, NET-A and prog via the GR.

Clearly the chemical structure of a ligand influences its binding specificity towards and transcriptional potency via the GR. This is now better understood since the crystal structure of the GR LBD has recently been solved (Bledsoe et al., 2002). Although structurally the GR has been shown to share over 50% identity with the androgen receptor (AR), progesterone receptor (PR) and estrogen receptor (ER) in its amino acid sequence and three-dimensional structure, many subtle differences exist in their secondary structure and topology of their ligand-binding pockets (Bledsoe et al., 2002). Regions encompassing helices 6 and 7 have been shown to be key determinants of ligand binding and transactivation potential of receptors (Wan et al., 2001). Helices 6 and 7 of the GR have been shown to deviate significantly from the ER, AR and PR and produce a unique side pocket in the GR. This pocket is thought to account for the GR selectivity of GCs, which have larger substituents at the C17 (carbon 17) position compared with estrogen, prog, and testosterone

(Bledsoe et al., 2002). Steroid selectivity appears to be influenced by the complementarity of shape and hydrogen bonding between ligands and the ligand-binding pocket in the GR.

The main determinant of GR binding affinity appears to be dependent on tight association between the steroidal A-ring and the GR, with the presence of a 4-en-3-one moiety in the A-ring being essential (Duax et al., 1988). The orientation of the A-ring relative to the B-, C- and D-rings also appears to be particularly important for the GR, since there is evidence that the GR prefers an A-ring that is turned towards the alpha-face, i.e. where the A-ring is bent underneath the plane defined by the B-, C- and D-rings (Duax et al., 1988). The C3 ketone (A-ring) of steroid hormones is also a prominent feature of steroid A-ring recognition and has been shown to accept hydrogen bonds from Q(glutamine)570 and R(arginine)611 in the GR crystal structure (Bledsoe et al., 2002; Hammer et al., 2003). While functional groups at the B-, C- and D-rings play a minor role in affinity, they appear to play an important role in determining whether a ligand is an agonist or an antagonist, as well as agonist potency (Duax et al., 1988). In the case of the GR, the hydroxyl groups at positions C11, C17 and C21, as found in dex and cortisol, are particularly important in contributing to agonist potency, most likely due to their hydrogen bonding properties. They are not required for binding, but for subsequent activation functions. The presence of an 11-beta hydroxyl appears to slow down the rate of dissociation of the ligand from the GR (Duax et al., 1988), which could affect its transactivation function (Hellal-Levy et al., 1999). In addition, the N564 of the GR forms a hydrogen bond to the C11-hydroxyl group (C-ring) of GCs, and is shown to be important for target gene activation, as well as ligand binding (Hammer et al., 2003), although the latter effect appears to be controversial. The 17-alpha-hydroxyl group (D-ring) of dex forms a specific hydrogen bond with Q642 in the GR structure (Bledsoe et al., 2002). Furthermore, the presence of C17- and C21-hydroxyl groups, allows the D-ring to

accept one hydrogen bond to oxygen (e.g. O at C20), and to donate two hydrogen bonds (e.g. at C17 and C21) (Duax et al., 1988).

When interpreting our binding and activity data in light of the above, it seems not surprising that dex binds to the GR with a high affinity and is a potent agonist, while progesterone binds with a lower affinity and is a weak partial agonist. While both steroids contain a 4-en-3-one in the A-ring, the tighter binding of dex as apposed to prog is most likely due to the bending of its A-ring towards the alpha-face, facilitated by the presence of the 9-alpha-fluoro group, and the extra double bond in the A-ring (Duax et al., 1988), plus possibly the C11-hydroxyl, features which are all absent in prog. The relatively weak GR-agonist properties of prog, similar to what has been found for testosterone (Duax et al., 1988), most likely stems from the absence of the hydroxyl groups at positions C11, C17 and C21, which are present in dex.

It is however more difficult to understand why MPA, unlike NET-A, binds so tightly and is an agonist for the GR. They both contain a 4-en-3-one in the A-ring, which most likely accounts for their binding to the GR. The presence of the two methyl groups on the A- and B-ring, respectively, for MPA, which are absent in NET-A, may explain the differences in binding affinity. It is possible that these two methyl groups result in a more optimal orientation of the A-ring with respect to the other rings, than for NET-A. Indeed it has been shown that the C6-methyl group of MPA plays a role in increasing the relative binding affinity of MPA for the GR, relative to prog (Kontula et al., 1983). MPA, like NET-A, lacks a C11-hydroxyl group, and the presence of hydrogen bond donors at C17 in the D-ring. Thus while this could contribute to the lack of agonist activity for NET-A, it is hard to understand why MPA is an agonist. The reason most likely lies in the differences between their functional groups at C17. At this position, NET-A has an ethynyl group, which is a large inflexible hydrophobic group, and may pose a steric hindrance and interfere with

optimal interactions in the GR ligand-binding pocket, further contributing to the lack of agonist activity of NET-A. One explanation for the agonist potency of MPA could be that the acetate may be rapidly hydrolysed, either enzymatically or non-enzymatically by participation of the neighbouring functional groups, resulting in a free C17-hydroxyl group, which could donate a hydrogen bond. Even if this occurs, MPA would still lack the C11-hydroxyl, which thus appears not to be essential for agonist activity. It is interesting to speculate that, since MPA has a greater efficacy for transrepression than transactivation, the contribution to conformation facilitated by hydrogen bonding to the C11-hydroxyl is not as important for transrepressioin as compared to transactivation. While NET-A, as for MPA, could also be hydrolysed in our experiments, it may be that the rate of hydrolysis of MPA is greater than that for NET-A, since the ketone at C20 of MPA (absent in NET-A), may assist in the hydrolysis of the acetate. If both MPA and NET-A are predominantly in the hydrolysed form in our assays, then the differences in agonist activity could be due to the non-optimal steric orientation of the C17-hydroxyl of NET-A, which is in the beta orientation compared with the alpha orientation of that of MPA and dex. Furthermore, the presence of the ketone as a hydrogen bond acceptor for hydrolysed MPA, compared with the hydrophobic ethynyl group of NET-A described above, may also explain the greater agonist activity of MPA. Further experiments using radiolabelled steroids could be performed to investigate these hypotheses.

Taken together, our data show that MPA displays significantly higher potency and efficacy than prog for transactivation of GREs and for transrepression of IL-8. In contrast, NET-A shows no agonist activity for transactivation of GREs while it acts as a very weak partial agonist for transrepression in our system. Given the multifunctional nature of IL-8 (Feghali and Wright, 1997; Mukaida et al., 1994; Barclay et al., 1993), our results suggest that MPA may exert significant side effects via repression of this gene on inflammation or on the immune response. Other genes

involved in immune function which are also repressed by GCs via similar mechanisms may also be targets for MPA regulation via the GR. Moreover, several genes involved in other physiological responses are also negatively regulated by GCs, and may be targets for MPA, such as the α-subunit of glycoprotein hormones (Chatterjee et al., 1991), prolactin (Sakai et al., 1988) and osteocalcin (Stromstedt et al., 1991). Our results also suggest that MPA may exert side effects in various cells via transactivation of target genes containing GREs. This may be of particular importance since transactivation may be the predominant mechanism by which GCs exert many of their metabolic and cardiovascular side effects (Imai et al., 1993; Brasier and Li, 1996).

When considering the potential physiological implications of our results for patients taking various doses of MPA or NET-A for therapy, or MPA and NET-EN for reproductive intervention, it is likely that free concentrations of MPA as low as 10 nM in a target cell could potentially occupy a significant percentage of GRs. The total serum concentrations of up to 65 nM MPA in contraceptive users could thus potentially result in significant levels of GR occupancy in some target cells, although the free concentrations of MPA would also depend on the concentrations of serum binding proteins, their affinity for MPA as well as the concentrations and affinities of endogenous competing steroids. The extent of GR occupancy would also naturally depend on the concentrations and affinities of endogenous steroids competing for GR binding, as well as the nature and extent of steroid metabolism in the cell. The potential for GR mediated side effects in HRT users would most likely be even greater than for contraceptive users, given the higher cumulative doses. With respect to NET, it would appear that a concentration of about 25-fold greater than that for MPA would be required for similar GR occupancy, and that such occupancy would not result in GR activity, but rather in antagonistic effects. It is also possible that higher concentrations of MPA and NET are achieved in selected target tissues than

in serum, such as their storage in fatty tissue, which may also be dependent on their relative stability. Although we cannot predict from our in vitro data to what extent MPA and NET will cause significant effects via the GR in vivo, our results certainly provide a molecular basis for formulating hypotheses, which can be tested in further studies. Most importantly however, these results show clearly that MPA and NET-A differ greatly from each other and from prog in their GC-like effects in vitro and thus potentially also in their physiological effects via the GR in vivo.

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Appendix 2

Data not included in manuscript

We showed in Chapter 2 that MPA repressed IL-6 and IL-8 promoter reporter constructs at the transcriptional level, via interference with NFκB and AP-1. We also showed that MPA, like dex, did not affect NFκB DNA-binding activity. Since interference with NFκB DNA-binding activity has been shown to be one mechanism that may account for inhibition of NFκB (Vanden Berghe et al., 2000; De Bosscher et al., 2003)[1] and since NET-A, like MPA has been shown to bind to the GR (Chapter 3), we were interested to compare its effects relative to dex and MPA at the NFκB DNA-binding level. Figure 4 shows the results of a gel shift experiment in L929sA cells where 1 μM NET-A like MPA and dex, had no effect on the DNA-binding activity of NFκB. Similar results were obtained for HeLa cells (data not shown). These results are discussed in Chapter 4.

[1] For References, see Chapter 2.

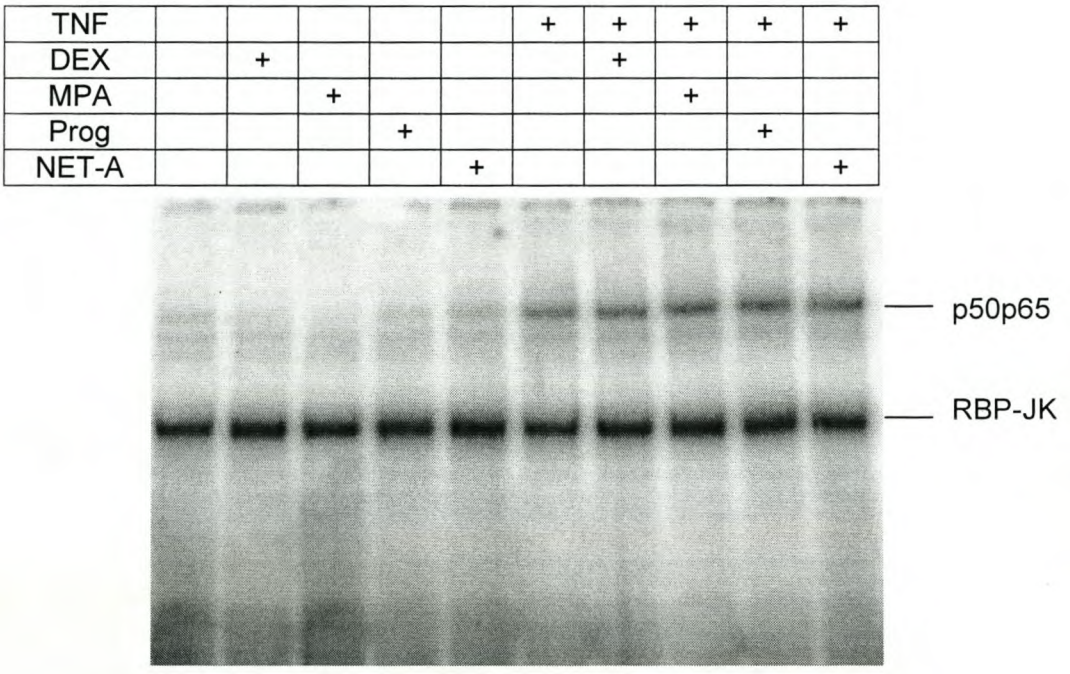

Figure 4: MPA and NET-A do not interfere with the DNA-binding activity of NFκB.

L929sA cells were untreated or pre-treated with 1 μM dex, MPA, prog or NET-A for two hours in the absence or presence of 2000 IU/ml TNF for 30 minutes. Total cell extract was incubated with an NFκB-specific oligonucleotide and protein-DNA complexes were analysed in an electrophoretic mobility shift assay (EMSA) (see Chapter 2 for method). The result shown is typical of two independent experiments. RBP-JK is the constitutively expressed recombination binding protein. p50p65 is the activated NFκB complex.

Discussion and concluding remarks

4.1 Summary and discussion of results presented in this thesis

In this thesis the interaction of MPA and NET-A with the GR, and their effects on interleukin gene expression were investigated. MPA at high doses, such as those used in HRT (Brunelli et al., 1996; Malarkey et al., 1997) and cancer therapy (Yamashita et al., 1996; Mallmann et al., 1990; Scambia et al., 1988), has been shown to exert immunosuppressive side effects in patients. Furthermore, Bamberger et al., (1999) recently reported that MPA at $2.5x10^{-7}$ M repressed transcription of a human IL-2 promoter reporter construct in normal human lymphocytes, to the same extent as dex, suggesting GR involvement.

The first part of this thesis was therefore aimed at investigating the effects of MPA relative to dex and prog, on interleukin gene expression, and the molecular mechanism of action thereof, with a view to shedding light on its immunosuppressive effects. As shown in Chapter 2, MPA, like dex and prog, significantly repressed TNF-stimulated IL-6 protein production, and IL-6 and IL-8 promoter reporter constructs at the transcriptional level. This appears to be the first report showing repression of IL-8 promoter activity by MPA. Towards further investigating the molecular mechanism of repression of the IL-6 and IL-8 promoters by MPA in L929sA cells, a number of experiments were carried out which revealed interesting and novel findings. Firstly, as a number of pro-inflammatory genes contain NFκB and/or AP-1 sites in their promoters or regulatory regions (De Bosscher et al., 2003), whether the repressive effect of MPA could affect activity of these transcription factors was investigated. Results in this study showed for the first time that the inhibitory action of MPA on interleukin gene expression involved interference with NFκB and AP-1 transcription factors (Chapter 2).

The molecular mechanism whereby MPA interferes with NFκB activity was further investigated, in particular its effects on NFκB DNA-binding activity. Previous studies have demonstrated that the GR represses NFκB-driven genes without affecting DNA-binding activity of NFκB (De Bosscher et al., 1997; Vanden Berghe et al., 1999). Since MPA has a high affinity for the GR, and since we proposed that MPA may be acting at least partly via the GR, its effects relative to dex and prog at the NFκB DNA-binding level were compared. As NET-A is also used widely in reproductive therapy, its effect was also investigated (Chapter 3 Appendix). Results showed that, like dex, MPA, prog (Chapter 2) and NET-A (Chapter 3 Appendix) treatment of TNF-induced L929sA cells could not disrupt DNA-binding by NFκB. Similar results were observed in HeLa cells. These findings are consistent with a mechanism of action of MPA and NET-A via the GR, since previous reports have shown that the GR bound to dex also does not disrupt DNA-binding by NFκB in vitro (De Bosscher et al., 1997; Vanden Berghe et al., 1999; Nissen and Yamamoto, 2000) and in vivo (Nissen and Yamamoto, 2000). However, this result does not exclude the involvement of other receptors. Interestingly, others have shown that although dex-activated GR does not inhibit TNF-stimulated pre-initiation complex assembly at the IL-8 and ICAM-1 promoters, it does interfere with phoshorylation of serine 2 of the RNA polymerase II carboxy-terminal domain (Nissen and Yamamoto, 2000). Similar mechanisms may therefore be responsible for MPA- or NET-A-mediated repression of TNF-stimulated IL-6 and IL-8 gene expression in L929sA cells.

The effects of MPA on the activity of MAPK pathways were investigated with a view to further understanding its molecular mechanism of action. TNF-activated p38 and ERK MAPK pathways have been shown to contribute to transcriptional

activation of the IL-6 promoter (Vanden Berghe et al., 1998). TNF-activated p38 and ERK are known to activate mitogen and stress activated protein kinase-1 (MSK-1), which associates with and phosphorylates the p65 subunit of NFκB, thus activating the transcription factor (Vermeulen et al., 2003). TNF-activated JNK is thought to play a role in IL-6 promoter activity by phosphorylating c-jun and thus activating AP-1 transcriptional activity (De Bosscher et al., 2003). To determine whether the mechanism of MPA repression involves interference with MAPK pathways, the effects of MPA on JNK, p38 and ERK signalling were investigated in Chapter 2 (Appendix). Results showed that MPA, in contrast to dex and prog, was unable to inhibit the amount of phosphorylated JNK p46 and p54 isoforms. Furthermore, the amount of phosphorylated p38 and ERK MAPK was not affected by dex, MPA or prog. The result with dex is consistent with the results of De Bosscher et al., (2001) who showed that dex inhibits the amount of phosphorylated JNK, but not phoshorylated p38 or ERK in L929sA cells. Similarly, dex was reported to prevent phosphorylation and subsequent activation of JNK in HeLa cells (Caelles et al., 1997; Gonzalez et al., 2000) and in human and murine endothelial cells (Gonzalez et al., 1999). Also consistent with the result for dex is the report of Gonzalez et al., (1999) showing that dex is unable to inhibit TNF-activated p38 signalling pathways in endothelial cells. Our results indicate that, although MPA appears to mimic the effects of dex and prog in repression of IL-6 and IL-8 promoters, subtle differences in the mechanisms of action of these compounds may occur. The finding that MPA behaves differently as compared to prog, while prog behaves similarly to dex, in their effects on JNK is very interesting. If the effects of dex, MPA and prog are mediated predominantly via the GR in these cells, then one would expect that MPA would give a similar result to dex, and greater than prog, since MPA is a more potent GC than prog (see later). However, if the effects of prog were mediated predominantly via the PR, then one would expect MPA to give a similar response to that of prog, since MPA is at least as potent an activator of the PR as prog. These results suggest that

receptors other than the GR and PR may be involved in the JNK response. A recent exciting report in the literature provides evidence that differences in the effects of MPA vs. prog on MAPK signalling may play an important role in neurological processes. Estrogen has been shown to have neuroprotective effects against glutamate neurotoxicity through activation of MAPK signalling, resulting in nuclear ERK activation. Not only was MPA shown to be an ineffective neuroprotectant, it also attenuated estradiol-induced neuroprotection when co-administered. In contrast to prog and estradiol, MPA-activated ERK was shown to remain cytosolic with no nuclear signal, resulting in a lack of neuroprotection (Nilsen and Brinton, 2003). These observed differences indicate that all progestins are not alike in their mechanism of action and may have serious health implications in vivo.

In order to determine which steroid receptors are involved in the above effects of MPA on regulation of interleukin genes in the L929sA cells, a combined approach involving the use of receptor-specific antagonists, as well as nuclear translocation studies were performed. Since high concentrations of ligand were required to obtain significant transrepression, we were unable to use receptor-specific antagonists to determine the receptors mediating the transrepressive effects of MPA, because at these concentrations, RU486 and hydroxyflutamide acted as agonists for transrepression. However, these receptor-specific antagonists did not display agonist properties for transactivation. In MPA-mediated transactivation of a GRE-driven promoter reporter construct, we were able to exclude the involvement of the AR. Since we observed no PR activity for transactivation using R5020, we were also able to exclude involvement of the PR, supporting a role for the GR in mediating in part the transcriptional effects of MPA (Chapter 2). This was further supported by our immunofluorescence results showing that MPA causes nuclear translocation of the GR (Chapter 2). In our transrepression studies, we were however, unable to exclude the involvement of the AR or the PR-A isoform, which is a poor activator of

transactivation, but a potent activator of transrepression (Kalkhoven et al., 1996). Furthermore, we could not exclude the possible participation of other receptors like the MR or pregnane X receptor (PXR) in MPA-mediated transactivation and transrepression. Further experiments would therefore be necessary to determine the possible involvement of other receptors (see 4.2).

Although NET-EN (or NET-A) is as widely used as MPA in reproductive therapy, to date no proper pharmacological characterisation of the relative efficacy and potency of the two progestins on gene regulation via the GR has been reported. Furthermore, no thorough comparison exists on the relative binding affinity of MPA vs. NET (or NET-A) towards the GR. Considering that MPA has been shown to bind with high affinity to the GR, AR, and PR (Teulings et al., 1980; Bentel et al., 1999; Kemppainen et al., 1999; Bojar et al., 1979; Bergink et al., 1983; Feil and Bardin, 1979) and as the above results in L929sA cells in Chapter 2 could not show conclusively that the transrepressive effects of MPA were mediated exclusively via the GR, the next part of the thesis was performed in a more suitable experimental model system. In Chapter 3 therefore, most of the work was carried out in Hek293 and COS-1 cells transfected with the rat GR. These cells do not express significant levels of endogenous steroid receptors, and allow the possibility of doing negative control experiments in the absence of transfected GR, unlike L929sA cells which express endogenous GR, PR and AR (Jung-Testas et al., 1976). To investigate and compare meticulously the agonist properties for both transrepression and transactivation of MPA, NET-A and prog via the GR, EC50 curves were established where possible, each with a wide range of concentration points, and reproducible EC50 values were extrapolated from each curve to determine the relative potency of each progestin. The results (Table 1) clearly indicate that MPA displays a significantly greater GC agonist potency and efficacy than NET-A and prog for both transactivation and transrepression via the rat GR, in Hek293 cells stably transfected

with rat GR. For clarity of discussion these cells will now be referred to as 'Hek293 + GR' in this chapter.

Hek293 + GR cells	MPA	NET-A	PROG
Relative potency for transactivation (%)	15.5 ± 1.73	N/A	0.40 ± 0.05
Relative efficacy for transactivation (%)	72.2 ± 0.85	0.40 ± 0.01	64.3 ± 2.46
Relative potency for transrepression (%)	7.90 ± 1.29	0.21	0.80 ± 0.10
Relative efficacy for transrepression (%)	95.4 ± 1.16	23.0 ± 3.44	88.7 ± 0.66
L929sA cells	**MPA**	**NET-A**	**PROG**
Relative potency for transactivation (%)	62.5 ± 14.5	N/D	6.64 ± 2.80
Relative efficacy for transactivation (%)	48.0 ± 1.44	N/D	16.0 ± 0.74
Relative potency for transrepression (%)	27.5 ± 9.80	N/D	5.22 ± 1.52
Relative efficacy for transrepression (%)	74.4 ± 1.57	N/D	75.4 ± 2.20

Table 1: Relative EC50 (potency) and relative maximal values (efficacy) ± SEM, for both transactivation and transrepression in L929sA cells (Chapter 2) and Hek293 + GR cells (Chapter 3). All values are percentages, calculated relative to 100% activity of dex ± SEM. N/A = no activity up to 10^{-5} M NET-A. N/D = not determined.

Equilibrium binding studies were carried out to determine for the first time precise Ki values as a measure of relative binding affinity of MPA, NET-A and prog for the GR. Extensive optimisation experiments were initially performed, including the determination of the time required to obtain binding equilibrium as well as a suitable concentration of radiolabelled ligand, to enable Kds to be obtained from competitive binding curves. It is often easier to obtain relative binding affinity values from EC50 curves instead, which provide less accurate relative binding affinity values than Kd or Ki values.

Results showed that MPA, NET-A and prog were able to compete with dex for binding to the GR in A549 cells, and MPA (Ki = 1.08×10^{-8} M) displayed a higher relative binding affinity than NET-A (Ki = 2.7×10^{-7} M) and prog (Ki = 2.15×10^{-7} M) towards the receptor. A similar trend of results for MPA and NET-A was obtained in COS-1 cells. Collectively, these results show that MPA behaves as a GR agonist in our experimental model system, whereas antagonist-like properties of NET-A towards the GR were observed, since it binds to the GR but displayed no or very weak partial agonist activity for transrepression only. However, due to its low binding affinity for the GR, NET-A would only be able to compete with endogenous GCs and thus antagonise the GR when present at relatively higher concentrations. Taken together, our results with MPA and NET-A may have significant physiological implications in vivo, since many genes in various biological processes are regulated by the GR, which is ubiquitously expressed. Such processes include genes involved in reproduction (Chatterjee et al., 1991; Sakai et al., 1988), immune (Spangelo and Gorospe, 1995; Galon et al., 2002) and adrenal function (Drouin et al., 1993) and bone metabolism (Ishida and Heersche, 2002).

A comparison of the results obtained in L929sA (Chapter 2) and Hek293 + GR cells (Chapter 3) provide interesting insights into the dissociative properties of MPA, prog and NET-A, and possible cell-specific effects (Table 1). Although GCs have been widely used since the late 1940s for the treatment of excessive inflammation, the molecular mechanisms responsible for their anti-inflammatory activity are still under investigation. Transactivation is considered to be the predominant mechanism by which GCs exert many of their side effects (Imai et al., 1993; Brasier and Li, 1996). This has led to a search for novel GCs, referred to as 'dissociated GCs', which selectively transrepress pro-inflammatory genes with relatively little transactivation of other genes, thus reducing the potential risk for systemic side effects (Adcock, 2003). MPA showed significant transactivation of a GRE-driven promoter construct in both L929sA (via the endogenous mouse GR) and Hek293 + GR cells (via expressed rat GR), whereas prog only showed significant transactivation in Hek293 cells. Unlike MPA and prog, NET-A showed no transactivation in Hek293 cells. When comparing the dissociative GC properties of MPA as compared to dex in both L929sA and Hek293 + GR cells, similar results were observed. In L929sA cells, the fold differences relative to dex for transrepression and transactivation efficacy are 0.74- and 0.48-fold respectively. The transrepression/transactivation ratio for MPA therefore is 1.5, which is similar to that of dex used as the standard (transrepression/transactivation = 1) (Bamberger and Schulte, 2000). Similarly, in Hek293 + GR cells the fold differences relative to dex efficacy for transrepression are 0.95-fold, and for transactivation 0.72-fold, yielding a transrepression/transactivation ratio of 1.3. From this data we may conclude that MPA discriminates only marginally between transactivation and transrepression in both mouse L929sA and Hek293 + rat GR cells in this study. These findings are in contrast to the results of Bamberger et al., (1999) and Bamberger and Schulte, (2000), where MPA behaved as a dissociated GC, as it showed only marginal transactivation of a GRE-reporter construct in HeLa cells and in normal human

lymphocytes, via the endogenous human GR in both cell types. MPA showed a transrepression/transactivation ratio of 6.6 in normal human lymphocytes, compared with dex where the ratio was 1 (Bamberger and Schulte, 2000). Similarly, in mouse hepatoma BTWG3 cells, MPA was not able to transactivate the $p(GRE)_2$50hluc+ promoter construct via endogenous GR, in contrast to the results for dex (Allie et al., unpublished results). These differences could, as has been shown for GCs, be due to cell-specific differences as described in Chapter 2. In addition, differences in receptor expression levels (Bamberger et al., 1996) may also affect the transrepression/transactivation ratio and thus the dissociative GC properties of a compound. As discussed in Chapter 1, transactivation requires dimerisation of the GR (Adcock, 2000), whereas a GR monomer is sufficient for transrepression (Radoja et al., 2000). Therefore, under conditions where receptor levels are limited, transactivation may be inhibited to a greater extent than transrepression. This could explain the results observed by Bamberger et al., (1999), where the dissociative effect of MPA was dependent on GR expression levels. When normal lymphocytes were co-transfected with human GR, the transactivating effect of MPA was enhanced, and a subsequent loss of dissociative GC properties was observed (Bamberger et al., 1999). It is therefore possible that L929sA and Hek293 + GR cells express higher levels of GR than lymphocytes or hepatoma cells. Although MPA has been implicated as a promising agent for the treatment of autoimmune and/or inflammatory disease (Bamberger et al., 1999), the results of the present study indicate that further investigations into its potential dissociative GC activity are necessary. When assessing the dissociative GC properties of NET-A compared to dex in Hek293 + GR cells in the current study, the transrepression/transactivation ratio was calculated as 55, showing significantly greater dissociative GC properties compared with MPA and prog, although it displayed very weak partial agonist activity for transrepression only at high concentrations. Based on these findings, NET-A does not appear to be a potential compound for use as an anti-inflammatory drug. When

comparing the dissociative GC properties of prog relative to dex in both L929sA and Hek293 + GR cells, the transrepression/transactivation ratio in L929sA cells was determined as 0.75/0.16 = 4.7. In Hek293 + GR cells, this ratio was calculated as 0.89/0.62 = 1.4. These results indicate that prog acts as a stronger dissociated GC in L929sA cells, whereas its dissociative profile in Hek293 + GR cells is more similar to that of MPA. This observed difference may be due to a number of cell-specific variations as described above for MPA. Furthermore, prog may also be metabolised in a cell-specific manner. Another possible explanation for this may involve the presence of different receptor isoforms. Prog may therefore be mediating its transactivation effects via the PR-A isoform (a poor activator of transactivation, but a potent activator of transrepression) present in L929sA cells, resulting in a greater dissociative ratio. This theory holds only if PR-A is present at low levels in L929sA cells relative to GR protein. If greater levels of PR-A were present, then MPA would, like prog, show significantly greater dissociative GC properties in L929sA cells (a greater ratio than 1.5) compared with Hek293 + GR cells. Although prog displays dissociative GC properties in one model system in this study, it is involved in many biological processes in vivo and would not be an ideal candidate for anti-inflammatory treatment. More suitable compounds like non-steroidal selective GR agonists (SEGRAs) have recently been reported to show dissociative properties in human cells, several of which are now in clinical development (Adcock, 2003).

Dose-response experiments performed in L929sA (Chapter 2) and Hek293 + GR cells (Chapter 3) revealed further interesting cell-specific differences in response to the same steroids. When comparing the relative potencies (to dex) and relative efficacies (to dex) of both MPA and prog in L929sA vs. Hek293 + GR cells for transactivation and transrepression, a statistically significant trend was observed (Table 1). Firstly, the potency of MPA (relative to dex as 100%) for both transactivation and transrepression was significantly greater (4- and 3.5-fold

respectively) in L929sA cells compared with Hek293 + GR cells. Similarly, the potency of prog (relative to dex as 100%) for both transactivation and transrepression was also significantly greater (16.6- and 6.5-fold respectively) in L929sA cells compared with Hek293 + GR cells. In contrast, the efficacies of MPA and prog (relative to dex as 100%) for both transactivation and transrepression were greater (1.5- and 1.3-fold respectively for MPA, 4- and 1.2-fold respectively for prog) in Hek293 + GR cells compared with L929sA cells. The above results may be explained by a number of cell-specific factors thought to influence ligand potency and/or efficacy. To begin with, such factors may include variations in receptor content, which could influence the relative potency and relative efficacy for transrepression in L929sA cells compared with Hek293 + GR cells. L929sA cells have been reported to express GR, AR and ER (Jung-Testas et al., 1976), and we also detected PR mRNA and protein in our cells by RT-PCR and Western blotting (data not shown). Furthermore, MPA has been shown to bind to the GR, PR and AR (Teulings et al., 1980; Bentel et al., 1999; Kemppainen et al., 1999; Bojar et al., 1979; Bergink et al., 1983; Feil and Bardin, 1979) and prog has been shown to bind to the PR and GR (Chapter 3; Kontula et al., 1983). A role for the ER in L929sA cells can be excluded, as it is well established that MPA does not bind to the ER (Teulings et al., 1980). Although our results in Chapter 2 suggest that the GR is responsible for mediating the transactivation effects of MPA and prog in L929sA cells, additional receptors may be responsible for mediating the transrepressive activity of these compounds in these cells. In Hek293 + GR cells however, we showed that all the observed transcriptional effects of MPA were exclusively due to the presence of the GR (Chapter 3).

Intracellular hormone availability may also be an influencing factor in the observed differences in relative efficacy and relative potency in the two cell types investigated. As the Hek293 cell line was derived from human embryonic kidney cells and since L929sA cells are of mouse fibroblast origin, there may be significant

variation in steroid metabolism within these cell types. Intracellular hormone availability may also be regulated cell-specifically by variations in expression levels of the transporter proteins LEM1 or MDR1, which actively and specifically export GCs from the cell (Kralli et al., 1995; Bourgeois et al., 1993). Furthermore, variations in the receptor protein such as mutations, or ratio of GRα-A/B isoforms (de Lange et al., 1997; Yudt and Cidlowski, 2001), may affect ligand-binding affinity of the receptor or ligand-receptor complex stability (Hurley et al., 1991; Ashraf and Thompson, 1993) and thus may influence ligand potency vs. efficacy. In addition, post-translational modifications (Wallace and Cidlowski, 2001; Bamberger et al., 1996; Galigniana et al., 1999; Poukka et al., 2000; Kyriakis, 2000), alternative receptor dimerisation (Savory et al., 2001) and receptor co-chaperones (Schneikert et al., 2000) may also affect ligand potency and/or efficacy in a cell-specific manner. Expression levels of hsp proteins (Bamberger et al., 1996) may also influence GR function. Furthermore, cell-specific cross-talk between MAPK signalling and steroid receptors (Kyriakis, 2000), DNA-binding of activated GR complexes and recruitment of other nuclear factors (Bamberger et al., 1996; Chen et al., 2002) may also play influencing roles. All these above points highlight the importance of characterising pharmacologically new or potential drugs in various model systems to gain an improved understanding of potential biological effects.

4.2 Future perspectives

One area of possible future investigation would be to determine which steroid receptors play a role, and to what extent, in the responses to MPA and NET-A in L929sA cells, by using receptor-specific antagonists. This approach would also be useful in other model cell systems, but can be limited by the availability and specificity of such antagonists, as well as their cell-specific effects. For example, RU486 acts as an agonist in some cells and as an antagonist in others (Bamberger and Chrousos, 1995). Another useful approach could be the use of antibodies, where available, to identify specific receptor isoforms. In the first part (Chapter 2) of this thesis, we were unable to determine conclusively which receptor(s) was responsible for mediating the IL6 and IL8 transrepressive effects of MPA in L929sA cells. Although we observed no PR activity for transactivation using R5020, a strong PR agonist, the transrepressive effects of MPA may have been mediated in part via the PR-A isoform. Furthermore, the AR may also have been implicated in the transrepressive effects of MPA. These possibilities remain to be investigated. A GR-specific antagonist (DO6) has recently been developed (Miner et al., 2003) which could be used in the place of RU486, which does not discriminate between PR and GR, to determine which receptor is involved in mediating the transrepressive effects of MPA. In addition, it would be interesting to determine which of the different isoforms of the GR (Yudt and Cidlowski, 2001) might also be involved. Other likely receptor candidates, which could be investigated as potential mediators of transcription by MPA, may include the MR, as MPA has been shown to bind the receptor (Wambach et al., 1979), or PXR (Moore et al., 2002). Therefore, a more thorough investigation in L929sA cells in terms of establishing receptor content and presence of receptor isoforms would be applicable.

It would be interesting to investigate in more detail the mechanism whereby MPA, NET-A and prog inhibit NFκB and AP-1 driven promoters and how this relates to their effects on JNK, p38 and ERK. Furthermore, it would be of interest to determine what possible differences exist between the effects of MPA, NET-A and prog on these pathways. Further experiments in L929sA cells or other suitable model systems could include an investigation into the effects of MPA, NET-A and prog on MAPK activation including phosphorylation, nuclear translocation and genomic/non-genomic effects. Time course studies could also be included. Furthermore, GC-dependent repression of NFκB-driven genes has been proposed to be mediated by increased synthesis of IκB-α, which could subsequently sequester NFκB in the cytoplasm in an inactive form. The effects of MPA and NET-A on mRNA and protein levels of IκB-α, the cytoplasmic inhibitor of NFκB could be studied, in L929sA cells or other model systems.

In the second part of this thesis (Chapter 3), the relative binding affinity of MPA towards the GR was investigated in A549 cells, as Hek293 cells could not be used due to technical complications. In addition to being easy to handle technically (a necessary requirement for this type of assay), another advantage to using A549 cells was that they express GR endogenously, which eliminated the requirement for transfection. Although suspected to be present at low levels, one limitation to using these cells was thought to be the presence of endogenously expressed AR (Zhang et al., 2000), which was controlled for in this study by using the AR antagonist, hydroxyflutamide. It would therefore be appropriate to perform the binding experiments in a more robust batch of Hek293 + GR cells, thus ensuring that all the assays (competitive binding, transactivation, transrepression) are performed within the same system. In this way the results would be influenced by the same cell-

specific factors. Failing to perform the binding experiments in Hek293 + GR cells, all the assays described above could be carried out in a new system, responsive to low concentrations of steroid and where only GR is endogenously expressed. In addition to investigating the equilibrium dissociation constant for each MPA and NET-A, their dissociation rates from the GR could also be investigated. These results may better reflect their relative agonist potency and efficacy. Such studies may provide more insights into the relationship between binding parameters and biological potency.

Prior to this thesis, no thorough pharmacological characterisation of the relative efficacy and potency of MPA vs. NET (or NET-A) on gene regulation via specific steroid receptors had been performed. Furthermore, no thorough comparison exists in the literature on the relative binding affinity of MPA vs. NET (or NET-A) towards the AR, PR, MR or PXR. Additional experiments could therefore be performed where the relative binding affinity and dissociation rates of MPA and NET-A as well as their relative agonist properties for transactivation and transrepression on target genes are investigated via various receptors within the same system. This could be done for each of the PR, AR, MR and PXR. Such work would be vital in gaining a better understanding at the molecular level of the immunosuppressive and other possible side effects of MPA vs. NET (or NET-A).. PXR is a promiscuous nuclear receptor that has evolved to protect the body from toxic chemicals by responding to a wide variety of prescription drugs, xenobiotics and endogenous compounds (Watkins et al., 2002) including dex, prog and DHT (Moore et al., 2002). PXR stimulates the transcription of cytochrome P450 3A monooxygenases (CYP3A) and other genes involved in the detoxification and elimination of these potentially harmful chemicals (Goodwin et al., 2002). It would be interesting to determine whether MPA and/or NET-A used in reproductive therapy have the capacity to modulate PXR activity, and thus potentially influence the metabolism and elimination of other drugs taken concurrently.

In addition to the results obtained in this thesis, work in this area has already been initiated in our laboratory on the AR and MR. Both MPA and NET-A have been shown to compete with [^{3}H] mibolerone for binding to the human AR co-transfected into COS-7 cells (Africander et al., unpublished results; Tanner et al., unpublished results). However, further investigations are currently underway to obtain precise Ki values for both MPA and NET-A. MPA and NET-A also displayed similar androgen agonist activity in COS-7 cells transiently co-transfected with a GRE-driven promoter construct and human AR, and EC50 values for each progestin are currently being established (Africander et al., unpublished results). Furthermore, MPA, unlike NET-A and DHT, was unable to induce the ligand-dependent N/C interaction of the AR, a result in agreement with that reported by Kemppainen et al., (1999). Yet similarly to DHT, MPA and NET-A were both able to induce the ligand-dependent interaction of the co-activator, SRC-1, with the AF2 domain (Tanner, MSc Thesis). Further studies could be performed to determine the relative agonist potency for transrepression of MPA vs. NET-A of various interleukin genes, such as IL-6 and IL-8, via the AR. Furthermore, the relative binding affinity of MPA and NET-A in COS-1 cells for co-transfected MR has also been investigated. In contrast to Wambach et al., (1979), results showed that MPA and NET-A were unable to compete with [^{3}H] aldosterone for binding to the MR. Furthermore, transactivation experiments in COS-1 cells transfected with an MR expression vector and the MMTV-luciferase reporter construct demonstrated that MPA and NET-A were devoid of either agonist or antagonist activity (Africander et al., unpublished results). Such studies will serve to improve our understanding of the potential role of various steroid receptors in the responses of various cells to MPA and NET-A, since most cells contain a mixture of steroid receptors, present at varying relative concentrations.

4.3 Concluding remarks

Although their precise molecular mechanisms of action are not well defined, millions of women worldwide use MPA and NET-EN as contraceptives, and MPA and NET-A in HRT, while MPA is also used in cancer treatment. The importance of understanding the mechanism of action of different synthetic progestins is becoming increasingly evident, especially in light of recent clinical trials showing that long-term use of MPA in combination with estrogen in HRT presents more risks than benefits (Rossouw et al., 2002). Recent evidence suggests that MPA, used as contraceptive, increases viral shedding (Mostad et al., 1997), raising concerns about the impact of its use on sexually transmitted diseases. Another important recent finding is that MPA, unlike prog, may have deleterious effects on neurological processes (Nilsen and Brinton, 2003). Clearly, further investigations are required at a molecular level to shed light on the mechanism of action of synthetic progestins, including their interaction with steroid-binding proteins such as serum proteins, steroidogenic enzymes and steroid receptors. In addition, their tissue-specific intracellular signalling mechanisms, including genomic and non-genomic effects, metabolism, activation of kinase pathways, and target genes involved, need to be further investigated.

The experiments presented in this thesis focus on the effects of MPA and NET-A on interleukin gene expression and their interaction with the GR. These results imply that MPA, unlike NET-A, use in vivo may exert GC-like immunosuppressive side effects, via genes with NFκB or AP-1 in their promoters, via the GR. The results also suggest that MPA, unlike NET-A, use in vivo may result in the upregulation of various genes with GREs in their promoters. Considering that serum concentrations of MPA in contraceptive users can reach up to 65 nM, and that

MPA was shown in Chapter 3 to have a Kd of 10 nM, it could potentially occupy a significant fraction of GRs in vivo and elicit various side effects.

By a careful comparison of binding, agonist potency and efficacy data, the results obtained in this study enabled GR ligand-specific relationships between these parameters to be examined, revealing that binding affinity is not a reliable indicator of agonist potency or efficacy for transactivation or transrepression of these compounds. These, as well as the JNK results, also highlight the point that all progestins are not alike, nor similar to prog, in their mechanism of action. Furthermore, the results obtained in this study also revealed interesting cell-specific differences for MPA, NET-A and prog.

Clearly much more work needs to be done to further our understanding of the mechanism of action of synthetic progestins. However, combining the existing physiological and molecular information available on MPA and NET-A action with the results presented in this thesis, can contribute to a better understanding of the effects of these progestins in vivo. Such insights may provide women and their clinicians with more information to facilitate the selection of method of contraception or HRT. Furthermore, these insights into mechanisms of action could also facilitate improved drug design for contraceptive agents and reproductive therapy regimes.

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