

**An Investigation of the Interactions of the Androgen
Receptor with a Non-steroidal Compound and Two
Synthetic Progestins**

Tamzin Maria Tanner

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Science at the University of Stellenbosch.



Supervisor:

Prof. J.P. Hapgood

Co-supervisor:

Dr. A. Louw

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis 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

The aim of this thesis was to define the interactions of the androgen receptor (AR) with an analog of a non-steroidal plant compound, Compound A (CpdA), as well as two synthetic progestins, medroxyprogesterone acetate (MPA) and norethindrone acetate (NET-A). The data presented indicates that CpdA has anti-androgenic properties, as it represses androgen-induced activation of both specific and non-specific androgen-responsive reporter constructs. It was found that CpdA exerts these effects by a mechanism other than competition with androgen for binding to the ligand-binding domain (LBD) of the receptor. On the other hand, it is demonstrated that both MPA and NET-A compete with androgen for binding to the AR and induce partial agonist activity via the receptor. Using mammalian two-hybrid assays it was revealed that CpdA, similar to anti-androgenic compounds that are able to compete with androgens for binding to the receptor, represses the androgen-induced interaction between the NH₂- and COOH-terminals of the AR (N/C-interaction) without competing for binding to the LBD. Furthermore, it was shown that CpdA slightly represses the androgen-dependent recruitment of steroid receptor co-activator 1 (SRC1) to the activation function (AF2) domain of the AR. When the effects of MPA and NET-A on the N/C-interaction were studied, intriguing results were obtained. NET-A, as expected, induced this AR agonist-induced interaction. MPA, however, repressed this AR agonist-induced interaction, an effect previously associated with anti-androgenic activity, despite displaying partial agonist activity in transactivation experiments. On the other hand, both MPA and NET-A induced the interaction between SRC1 and the AF2 domain. In additional experiments with CpdA, it was found that CpdA did not affect the recruitment of SRC1 to the AF1 domain of the receptor; neither did it influence the constitutive activity of the NH₂-terminal domain.

The anti-androgenic activities of CpdA were confirmed by the toxic effect that this compound had on the androgen-dependent lymph node carcinoma of the prostate (LNCaP) cell-line as well as its ability to repress the androgen-induced expression of the prostate specific antigen (PSA) protein. Taken together, the results presented in this thesis, in combination with the knowledge available on AR function, contribute to an improved understanding of AR function. Furthermore, the importance of defining the precise mechanism by which individual compounds exert their effects is highlighted. In this regard it is demonstrated that two compounds (MPA and NET-A) that display partial agonist activity, can exert their effects via different mechanisms at the molecular level. Detecting such differences in the molecular mechanisms of action could facilitate the improved design of progestins as well as aid clinicians and their patients in selecting the best method of contraception. Lastly, the insights gained into the mechanisms of the anti-androgenic action of CpdA could be useful in therapeutic drug design for diseases, such as prostate cancer, that have an androgen-dependent etiology.

Samevatting

Die doel van hierdie tesis was om die interaksies van die androgeen reseptor (AR) met 'n analoog van 'n nie-steroïediese plant verbinding, Verbinding A (VbgA), sowel as met twee sintetiese progestogene, medroksiprogesteron asetaat (MPA) en noretiendron asetaat (NET-A), te definieer. Die data verskaf dui daarop dat VbgA anti-androgeniese eienskappe besit deurdat dit androgeen-geïnduseerde aktivering van beide spesifieke- en nie-spesifieke androgeen-responsiewe rapporteerderkonstruksie onderdruk. VbgA veroorsaak hierdie effekte deur 'n meganisme wat nie kompetisie met androgeen vir binding aan die ligand-bindingsdomein (LBD) van die reseptor behels nie. In teenstelling hiermee word getoon dat beide MPA en NET-A kompeteer met androgeen vir binding aan die AR en gedeeltelike agonistiese aktiwiteit induseer via hierdie reseptor. Deur gebruik te maak van 'n soogdier twee-hibried essai word getoon dat VbgA, soos ander anti-androgeniese verbindings wat kompeteer met androgeen vir binding aan die reseptor, die androgeen-geïnduseerde interaksies tussen die NH₂- en COOH-terminale van die AR (N/C-interaksie) onderdruk, sonder om te kompeteer vir binding aan die LBD. Daarby is dit bewys dat VbgA die androgeen-afhanklike werwing van steroïed reseptor ko-aktiveerde 1 (SRC1) na die aktiverings funksie (AF2) domein van die AR gedeeltelik onderdruk. Die studie van die effekte van MPA en NET-A op die N/C-interaksie het interessante resultate opgelewer. NET-A, soos verwag, het hierdie AR agonis-geïnduseerde interaksie geïnduseer. MPA, aan die ander kant, het hierdie AR agonis-geïnduseerde interaksie onderdruk, 'n effek wat tevore met anti-androgeniese aktiwiteit geassosieer is, al het die transaktiverings-eksperimente daarop gedui dat MPA 'n AR agonis is. Aan die ander kant, het beide MPA en NET-A die interaksie tussen SRC1 en die AF2 domein geïnduseer. In addisionele eksperimente met VbgA is gevind dat VbgA geen effek het op die

werwing van SRC1 na die AF1 domein van die reseptor nie en ook geen invloed het op die konstitiewe aktiwiteit van die NH₂-terminaal domein nie. VbgA se anti-androgeniese eienskappe is bevestig deur die toksiese effekte op die androgeen-afhanklike limfknoop karsinoom van die prostaat (LNCaP) sellyn sowel as deur sy vermoë om die androgen-geïnduseerde uitdrukking van die prostaat spesifieke antigeen (PSA) proteïen te onderdruk. Die resultate aangebied in hierdie tesis, in kombinasie met die beskikbare kennis oor AR funksie, dra by tot 'n verbeterde kennis van AR funksionering. Verder word die belang van die definiëring van die meganisme waardeur individuele verbindings hulle effekte veroorsaak, getoon. In hierdie verband is getoon dat twee verbindings (MPA en NET-A), wat gedeeltelike agonistiese aktiwiteit besit, hulle effekte via verskillende meganismes op die molekulêre vlak veroorsaak. Deur hierdie verskille in die molekulêre meganismes van aksie uit te wys, kan beter progestogene ontwikkel word, en verder sal dit vir dokters en hul pasiënte help om die beste voorbehoedmiddel te kies. Laastens, die insig wat verkry is ten opsigte van die meganismes van anti-androgeniese aktiwiteit van VbgA mag nuttig wees in die ontwerp van terapeutiese middels vir die behandeling van siektetoestande met androgeen-afhanklike etiologie (bv. prostaatkanker).

Format of this thesis

The supervisors of this project decided that the experimental work presented in this thesis should be written-up in manuscript format. The thesis is thus composed of:

- (i) a literature review on the appropriate background complete with references (*Chapter 1*);
- (ii) two manuscripts describing, reporting and discussing the experiments undertaken by the candidate (*Chapters 2 & 3*), each of which is followed by a discussion of additional results (data not shown in the manuscript) and/or comments and suggestions regarding the current data; and
- (iii) a discussion of the overall results with emphasis on the implications of the study and future perspectives (*Chapter 4*).

The manuscript presented as *Chapter 2* has been accepted for publication in *Molecular and Cellular Endocrinology* [In press].

The manuscript comprising *Chapter 3* has not yet been submitted for review, as experiments are currently being performed that will be included in the final version of the manuscript.

Abbreviations

| | | |
|-------------|---|---|
| ACTH | - | adrenocorticotrophic hormone |
| AF1 | - | activation function 1 |
| AF2 | - | activation function 2 |
| AP-1 | - | activator protein 1 |
| AR | - | androgen receptor |
| ARAs | - | androgen receptor activator proteins |
| ARE(s) | - | androgen response element(s) |
| C-terminal | - | carboxy- (COOH-) terminal |
| CBG | - | corticosteroid-binding globulin |
| CBP/p300 | - | CREB-binding protein complex |
| CpdA | - | Compound A |
| DBD | - | DNA-binding domain |
| DHT | - | dihydrotestosterone |
| DMPA | - | depot medroxyprogesterone acetate |
| DRIPs | - | vitamin D receptor interacting proteins |
| EGF | - | epidermal growth factor |
| EMSAs | - | electromobility shift assays |
| ER | - | estrogen receptor |
| ERAPs | - | ER-associated proteins |
| ERE | - | estrogen response element |
| FSH | - | follicle-stimulating hormone |
| GR | - | glucocorticoid receptor |
| GRE | - | glucocorticoid response element |
| hAR | - | human androgen receptor |
| HRE(s) | - | hormone response element(s) |
| hsps | - | heat shock proteins |
| hsp90 | - | 90 kDa heat-shock protein |
| LBD | - | ligand binding domain |
| LH | - | luteinizing hormone |
| LNCaP | - | lymph node carcinoma of the prostate |
| MAP kinases | - | mitogen-activated protein kinases |

| | | |
|---------------------|---|--|
| MMTV | - | mouse mammary tumor virus |
| MPA | - | medroxyprogesterone acetate |
| MR | - | mineralocorticoid receptor |
| N/C-interaction | - | interaction between the NTD and C-terminal domain |
| NCoR | - | nuclear receptor co-repressor |
| NET | - | norethindrone/norethisterone |
| NET-A | - | norethindrone/norethisterone acetate |
| NET-EN | - | norethindrone/norethisterone enanthate/oenantate |
| NF κ B | - | nuclear factor κ B |
| nGRE | - | negative glucocorticoid response element |
| NLS | - | nuclear localisation signal |
| NTD | - | amino- (NH ₂ -) terminal domain |
| PDEF | - | prostate-derived Ets factor |
| POMC | - | proopiomelanocortin |
| PR | - | progesterone receptor |
| PSA | - | prostate specific antigen |
| R1881 | - | methyltrienolone |
| RAR | - | retinoic acid receptor |
| RIPs | - | receptor-interacting proteins |
| RNA Pol II | - | RNA polymerase II |
| RXR | - | retinoic X receptor |
| SEM | - | standard error of the mean |
| SMRT | - | silencing mediator for retinoid and thyroid hormone receptor |
| SRC1 | - | steroid receptor co-activator 1 |
| SR(s) | - | steroid receptor(s) |
| TAF _{II} s | - | TBP-associating factors |
| TBP | - | TATA-binding protein |
| TR | - | thyroid hormone receptor |
| TRAC | - | T ₃ receptor-associating cofactor 2 |
| TRAPs | - | TR-associated proteins |
| TU | - | testosterone undecanoate |
| VDR | - | vitamin D receptor |

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Introduction

1.1 Steroid receptors

The androgen receptor (AR) is a member of the steroid receptor (SR) family, a subfamily of the nuclear receptor superfamily (Evans, 1988). The steroid receptors (SRs) also include the estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and progesterone receptor (PR). The cloning and sequencing of the cDNAs of these SRs, and the subsequent comparison of the deduced amino acid sequences, indicate the presence of domains that display a high degree of homology amongst the members of the SR family. Functional mapping of these domains illustrates that there is a high level of similarity between the members of the nuclear receptor superfamily, where the arrangement of the different domains is essentially the same for all the members. These functional domains are discussed in full detail in section 1.2.

The SRs are transcription factors, which are activated upon interaction with their specific ligands, the steroid hormones. The mechanism by which the SRs mediate their biological effects in target cells is comparable and is discussed thoroughly in section 1.3.

The AR is activated when bound by one of its specific ligands, namely the androgens, and AR action is repressed when the receptor is bound by anti-androgens. The molecular mechanisms by which androgens and anti-androgens exert their effects contribute to the understanding of AR action, and are explained in sections 1.4 and 1.5, respectively.

1.2 The structural and functional domains of the steroid receptors

1.2.1 Domain arrangement and subfamilies

The members of the nuclear receptor superfamily are believed to have evolved from an ancestral multi-domain gene, that, through duplication and mutation, resulted in the variety of receptors that exist today (O'Malley, 1989; Laudet *et al.*, 1992). The basic structure of these receptors involves the following arrangement of domains (reviewed in Tsai and O'Malley, 1994; and Beato *et al.*, 1995) (refer to figure 1). The highly conserved and centrally located DNA-binding domain (DBD) follows the hypervariable NH₂-terminal domain (NTD). The hinge region links the DBD to the COOH-terminal (C-terminal) domain. The C-terminal domain is also conserved between members of the family and contains the ligand-binding domain (LBD).

The nuclear receptor superfamily can be divided into subfamilies based on either the homology of their DBDs or the homology of their LBDs. The sequences of the NTDs of the receptors are hypervariable, making it impossible to group the receptors based on this region. Three subfamilies are delineated when the receptors are classed according to the similarity of their DBDs. Firstly, there is the thyroid hormone-/retinoic acid-receptor subfamily. Secondly, the orphan receptor subfamily, for which no physiological ligands have been identified. Lastly, the steroid hormone receptor family, which in turn is further divided into the GR group (including the GR, PR, MR and AR) and the ER group (including the estrogen-related receptor 1 and 2) (Laudet *et al.*, 1992). When the superfamily is divided into subfamilies according to the conserved region of the LBD, again, three subfamilies emerge with a receptor distribution similar to that found when classed according to the DBD.

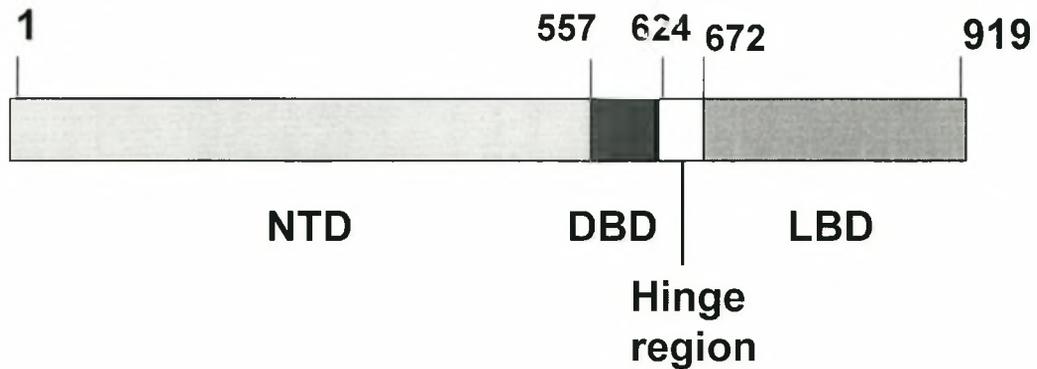


Figure 1: A schematic representation of the structural and functional domains of the human AR.

The human AR (919 amino acids), and other steroid receptors, is composed of: a variable amino terminal domain (NTD), a highly conserved DNA binding domain (DBD), a hinge region, and a ligand binding domain (LBD) at the C-terminal. The numbers indicate the numbering of the amino acid residues for the human AR.

1.2.2 The amino-terminal domain (NTD)

In comparison to the other domains of the SRs, the NTD is the domain that varies most with regards to both size and sequence (reviewed in Tsai and O'Malley, 1994). This domain is involved in the transcriptional activation of target genes, as the activation function (AF1) domain is located here. The NTD accomplishes this activation by making direct protein-protein contacts with basal and specific transcription factors.

1.2.3 The DNA binding domain (DBD)

The DBD is the most conserved domain of the steroid receptor family. The function of this domain is to mediate the interactions of the SRs with the hormone response elements (HREs) of their target genes. These interactions are specific and as such, are one of the factors contributing to the specificity of SR functioning.

The DBD of each SR consists of about 70 amino acid residues and is rich in the basic residues, arginine and lysine as well as cysteine residues. This domain also contains two zinc clusters (refer to figure 2). Each zinc cluster is composed of four cysteine residues, in a tetrahedral arrangement, and a zinc ion located in the centre of this tetrahedral arrangement (Freedman *et al.*, 1988). Together, the two zinc finger-like modules are organised in three α -helices (Luisi *et al.*, 1991), and play a role in maintaining the structural integrity of the DBD (Freedman *et al.*, 1988; Zilliagus *et al.*, 1992).

The first zinc finger, located at the NH₂-terminal side of the DBD, is responsible for binding to the DNA (Green *et al.*, 1988), and contains the so-called P-box. Three amino acid residues of the P-box are essential for recognition of the hormone response element (HRE) (refer to Table 1) and thereby responsible for the specificity of binding (Umesono and Evans, 1989).

Together with the LBD, the second C-terminally located zinc finger is involved in the dimerization of two receptor molecules (Green and Chambon, 1989). This zinc-finger module contains the D-box. The D-box is composed of the amino acid residues located between the first and second cysteine residues of this finger, and mediates the dimerization contacts.

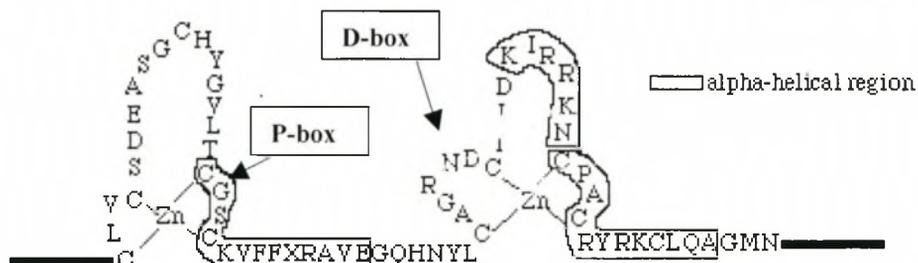


Figure 2: The DBD of the GR, characterised by two steroid hormone receptor-specific zinc clusters.

Each zinc finger is composed of four cysteines (C) which tetrahedrally co-ordinate a zinc ion. The proximal box (P-box) is responsible for specific DNA-recognition. The the distal box (D-Box) mediates DBD-dimerization. Capital letters represent the amino acid sequence, with X in the P-box representing a positively charged amino acid residue.

(<http://www.neurosci.pharm.utoledo.edu/MBC3320/steroids.htm>)

Table 1: The steroid receptors, their DBD P-box sequences, and consensus half-sites to which they bind.

| P-box sequence | Steroid receptor | Half-site |
|----------------|------------------|-----------|
| CGSCKV | GR, MR, PR, AR | TGTTCT |
| CEGCKA | ER | TGACCT |

1.2.4 The hinge region

The hinge region is a flexible region that links the DBD to the LBD and contains the nuclear localisation signal (NLS). This signal is involved in the nuclear import of the GR, PR, and AR and functions by being recognised by the nuclear pore complex (Picard and Yamamoto, 1987; Guiochon-Mantel *et al.*, 1989; Jenster *et al.*, 1993). It is composed of two basic amino acid residues, arginine and lysine, followed by a ten amino acid residue spacer and then an arginine-lysine stretch (Robbins *et al.*, 1991).

1.2.5 The ligand binding domain (LBD)

The LBD of the SRs is composed of about 250 amino acid residues. This domain has various regions involved in a number of functions. Firstly, the LBD is involved in interactions with the heat-shock proteins (Dalman *et al.*, 1989; Cadepond *et al.*, 1991). It is also important in the stabilization of homodimerization (Guiochon-Mantel *et al.*, 1989; Fawell *et al.*, 1990) as well as transactivation of transcription (Hollenberg *et al.*, 1988; Danielian *et al.*, 1992). The LBD is further involved in interactions with co-regulators (Moras and Gronemeyer, 1998). The number and location of the NLSs vary between the SRs and a second, ligand-dependent NLS may be found in the LBD. Lastly and most importantly, the LBD is the region of the receptor to which ligand binds (reviewed in Tsai and O'Malley, 1994 and references therein). In contrast to other functions that depend on small stretches of the amino acid sequence, binding of ligand requires most of the LBD.

The crystallographic structures of the LBDs of the hER α , hPR, and rAR, complexed with their natural ligands have been determined (Brzozowski *et al.*, 1997; Tanenbaum *et al.*, 1998; Matias *et al.*, 2000; Sack *et al.*, 2001) and it was found that

in such cases the ligand is completely buried within the ligand-binding pocket. The secondary structural elements of the LBD include 12 α -helices and 2 β -strands arranged in an anti-parallel orientation, creating the hydrophobic ligand binding pocket (reviewed in Beato and Klug, 2000). For the AR however, there are only 11 α -helices as helix 2 does not exist. In the unliganded state helix 12 protrudes from the LBD leaving the entrance of the ligand pocket open. Upon ligand binding a conformational change takes place in this domain. This involves helix 12 folding back towards the LBD and essentially closing the entrance to the ligand-binding pocket (figure 3). This realignment of helix 12 also generates a new surface(s) through which co-activators can interact with the LBD, and thereby mediate the activity of the activation function 2 (AF2) domain located in helix 12.

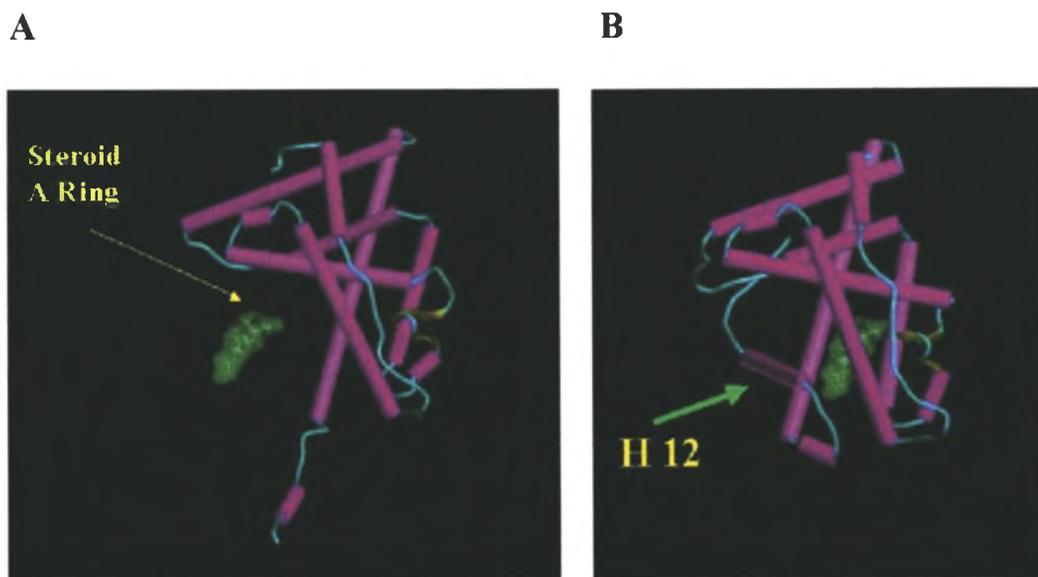


Figure 3: A generalised model of the ligand binding domain of the steroid receptors.

The secondary structural elements of the ligand binding domain, the 12 α -helices (*purple*) and 2 β -sheets (*yellow-green*) (A) An orthographic view of a steroid (green surface contour) approaching the ligand binding cavity of a steroid receptor (helices 1-11 shown). (B) An orthographic view of the ligand-binding cavity of a steroid receptor (helices 1-12 shown) after helix 12 (H12) has closed the cavity. This results in the predominantly lipophilic ligand being surrounded by the hydrophobic interior of the receptor (<http://pps9900.cryst.bbk.ac.uk/projects/taylor/LPT4PPS/Domain.htm>).

1.3 The molecular mechanisms of steroid hormone action

1.3.1 The specificity of steroid hormone/steroid receptor action

When hormones are released into the circulation, they are dispersed throughout the organism. One mechanism by which these endocrine messages exert specific effects is by interacting specifically with their respective receptors. There is little cross-responsiveness between the different SRs and their natural ligands. However, the corticosteroid hormones (aldosterone and glucocorticoid hormones) can bind to both the MR and the GR (reviewed in Trapp and Holsboer, 1996; Farman and Rafestin-Oblin, 2001).

In contrast to this somewhat stringent ligand-receptor specificity the SRs interact far less stringently with HREs. In fact, for a number of years no major differences were detected in the ability of the GR group of receptors to recognise specific response elements. Recently, however, a group of response elements that are specifically recognised by the AR have been identified (discussed in detail in section 1.4). Thus, specificity of gene regulation by this group of receptors may be achieved at a level other than DNA binding. Possible explanations include the idea of receptor distribution, as not all of the SRs are present and/or active in all cells and tissues (Sträle *et al.*, 1989). Although, it is often the case that more than one SR is expressed in a cell and in such cases, the relative levels of expression of the different receptors may play an important role. Another factor could be differing rates of metabolism for the different hormones, thereby removing specific signals by the metabolism of a single hormone to an inactive form (Funder *et al.*, 1988). Furthermore, the capacity of the receptors to modulate chromatin structure, as well as interactions of the specific

receptors with other transcription factors (Truss and Beato, 1993), have also been shown to be responsible for the steroid specificity of gene regulation.

1.3.2 Subcellular localisation of steroid receptors

It is generally accepted that in the absence of ligand, the SRs are coupled to heat-shock proteins and reside in the cytoplasm. Binding of ligand then causes dissociation of the heat-shock proteins from the SRs and the subsequent homodimerization and translocation to the nucleus (Tsai and O'Malley, 1994; Simental *et al.*, 1991).

The subcellular localisation of the SRs is, however, a controversial topic as the distribution of the receptors between the cytoplasm and nucleus appears to be the result of nuclear-cytoplasmic diffusion and ATP-dependent cytoplasmic-nuclear shuttling (Guichon-Mantel *et al.*, 1991). The majority of ER, AR and PR is in the nucleus due to the presence of the NLSs. However, the subcellular localisation of the GR and MR is less clear, because ligand-induced nuclear translocation has been reported for both receptors (Beato and Klug, 2000).

1.3.3 The heat-shock proteins (hsps)

It is well established that the heat-shock proteins (hsps), that possess a number of important 'house-keeping' functions, play an important role in SR action. Under non-stress conditions the constitutively expressed hsps are thought to function as molecular chaperones, mediating the correct self-assembly of other proteins (Ellis and Hemmingsen, 1989). Stress either enhances the expression of these hsps or induces the expression of other hsps that are better equipped to function under stress conditions (Lindquist, 1986; Welch *et al.*, 1983).

With respect to SR action, the hsps are found in complex with the unliganded SR. These complexes contain at least a SR monomer, a dimer of a 90 kDa heat-shock protein (hsp90) and the immunophilin p59 (also known as hsp56). Although the exact composition of these complexes is unknown, it is known that these proteins are mostly associated with the LBD of the receptor (Pratt *et al.*, 1988; Carson-Jurica *et al.*, 1989). Ligand binding causes the non-receptor proteins to dissociate from the complex. Hsp90 can re-associate with unliganded receptors in an ATP- and Mg^{2+} -dependent manner that involves Hsp70 and other chaperones (Smith, 1993; Bohlen *et al.*, 1995). The rate of association is faster than the rate of dissociation, and as a result most of the unliganded receptor is found associated with hsps.

A number of functions have been attributed to the heat-shock proteins that bind the SRs (reviewed in Pratt, 1993; Bohlen *et al.*, 1995). It is proposed that they are involved in the proper folding of the LBD to ensure that high-affinity ligand binding is acquired. Furthermore, it is thought that they may play a role in the transport of the SRs through the cytosol. Lastly, they could be involved in maintaining the unliganded receptors in a transcriptionally inactive state. More recent findings indicate that hsp90 may be involved in the recycling/reutilization of nuclear GRs, into a form capable of productive interactions with hormone, without the obligatory passage of the receptor to the cytoplasm (Liu and DeFranco, 1999).

1.3.4 Translocation to the nucleus

Small molecules are capable of entering the nucleus by passive diffusion through the nuclear pores. On the other hand, larger molecules, like the SRs, have to be actively transported across the nuclear envelope, through the nuclear pore complex (Feldherr *et al.*, 1983, 1984). Proteins needing to enter must first be directed towards

the nucleus and this is achieved by a nuclear targeting sequence. A general bipartite NLS has been identified that is conserved throughout the SR family (Dingwall and Laskey, 1991). The precise mechanism(s) by which the NLS sequences direct proteins into the nucleus is not known. One explanation is that these sequences interact directly with the nuclear pore complex. Another explanation would be that these sequences interact with soluble proteins, which in turn interact with the nuclear pore complex. Formation of the SR dimer may also be important, resulting in co-translocation of the receptors. Ligand-independent receptor translocation also occurs and could be dependent on other proteins for co-transportation. An example of such a protein would be hsp70, as it contains a NLS and is found in the nucleus (Koskinen *et al.*, 1991).

Recently, it has been demonstrated that the 69 amino acid DBD of the GR is necessary and sufficient for nuclear export. This domain is unrelated to any known nuclear export signals. A 15 amino acid sequence between the two zinc binding loops of the GR DBD was found to be critical for nuclear export (Black *et al.*, 2001)

1.3.5 Receptor dimerization and DNA binding

The SRs bind to their HREs either as homo- and heterodimers. For a number of years it was thought that the members of the SR family strictly form homodimers. However, in recent years it has been established that the GR and MR can form heterodimers with one another, and as a result increase the functional diversity of corticosteroid action (reviewed in Trapp and Holsboer, 1996). It is not yet clear whether dimerization takes place before DNA binding or as a consequence of DNA binding. For the PR and ER it has been shown that dimerization takes place before DNA binding (De Marzo *et al.*, 1992; Fawell *et al.*, 1990), whereas, for the GR and

AR it has been demonstrated that dimerization may be a consequence of DNA binding (Dahlman-Wright *et al.*, 1990; Schoenmakers *et al.*, 2000).

Both receptors in the SR dimer interact with the DNA (figure 4) (Luisi *et al.*, 1991). For this reason, most HREs consist of two half-sites that are organised either as direct repeats or inverted repeats. Direct repeats refer to half-site sequences on the same strand, whereas inverted repeats (also known as palindromic sequences) refer to half-site sequences on opposite strands. The division of the steroid receptors into the GR or the ER group has been based on the response elements that they recognise (refer to Table 1). The GR group recognises the glucocorticoid response element (GRE) consensus half-site TGTTCT (Truss and Beato, 1993), whereas, the ER group, together with most non-steroid receptor members of the superfamily, recognises the estrogen response element (ERE) consensus half-site TGACCT. These half-sites are all separated by a three-base pair space; that is important for receptor specificity (reviewed in De Luca, 1991; Glass *et al.*, 1991).

Receptor dimerization and DNA binding are however, not essential for the SRs to control the activity of natural promoters. These actions of the SRs are discussed further in section 1.3.7.

1.3.6 Phosphorylation

It is well established that many transcription factors are regulated by their phosphorylation status. All of the SRs are known to be such phosphoproteins. A number of studies have highlighted the importance of phosphorylation in receptor function (reviewed in Weigel, 1996), which can be summarised as follows. Initial analyses revealed that phosphorylation can substantially modify both DNA binding and transcriptional activation by the SRs. Unliganded SRs that are bound by hsp

have basal levels of phosphorylation. As a result of ligand binding, these receptors exhibit increases in phosphorylation probably by cyclin-dependent kinases and mitogen-activated protein (MAP) kinases. Some members of the family possess the ability to bind DNA in the absence of ligand, resulting from phosphorylation at sites different from those involved in ligand-dependent phosphorylation. These ligand-independent phosphorylation sites have frequently been found to be casein kinase II or protein kinase A sites.

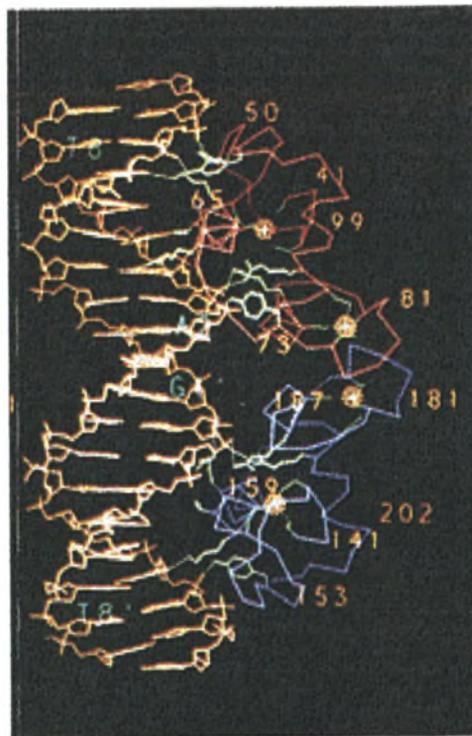


Figure 4: A steroid receptor homodimer bound to a hormone response element.

A stereoscopic view of the glucocorticoid response element (*double helix shown in yellow on the left*) with a glucocorticoid receptor homodimer (*red and blue structures on the right*) bound to it (<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/S/StcroidREs.html>).

1.3.7 Transcriptional regulation

1.3.7.1 Activation of transcription by steroid receptors

Steroid hormones acting via their respective SRs can modify the rate of transcription of their responsive genes, either positively or negatively. One way in which the steroid hormones regulate transcription, is via HREs that may be several kilobases from their target promoters. RNA polymerase II (RNA Pol II) mediates transcription at these target promoters. Initiation of transcription by RNA Pol II involves a complex hierarchy of both protein-protein and protein-DNA interactions, which starts with the regulated and ordered assembly of basal transcription factors into a pre-initiation complex at the promoter region (Buratowski, 1994). This process is generally thought to involve the stepwise assembly of factors. However, there is evidence that suggests that stable, pre-formed basal transcription complexes may also exist, which contain RNA Pol II in addition to other general transcription factors (Koleske and Young, 1994).

TFIID, a multiprotein complex composed of TATA-binding protein (TBP) and the TBP-associating factors (TAF_{II}s), appears to be the part of the pre-initiation complex that plays a central role in the communication between RNA Pol II and other activators, such as the SRs. In this case, the SRs bind to their HREs in the promoter region and communicate with the TFIID complex directly or via so-called co-activators (Tsai and O'Malley, 1994). The role of general transcription factors in mediating basal transcription is well documented and beyond the scope of this thesis (for a thorough review refer to Zawel and Reinberg, 1995). Not so well characterised is the sequence of events by which the activated, DNA-bound SR achieves

transcriptional regulation. In this regard the interactions of the SRs with general transcription factors as well as co-regulator proteins is of importance.

1.3.7.1.1 *Interactions of the steroid receptors with the pre-initiation complex*

Direct protein-protein interactions between receptors and general transcription factors such as TBP and several TAF_{II}s have been reported. For example, by using protein-protein interaction assays such as the yeast two-hybrid screen and *in vitro* binding assays with recombinant proteins, it has been shown that a region of the TBP associates with the AF-2 domain of RXR (Schulman *et al.*, 1995). Furthermore, it has been demonstrated that both the AF-1 and AF-2 domains of the ER bind TBP *in vitro* (Sadovsky *et al.*, 1995). Similarly an interaction between PR and the TAF_{II}110 subunit of TFIID has been reported (Schwerk *et al.*, 1995). Not only have the nuclear receptors been shown to interact with subunits of the TFIID multiprotein complex, but also with other general transcription factors. One such example would be the interaction between the AR and TFIIF (McEwan and Gustafsson, 1997).

All of these interactions may modulate a DNA-bound ternary complex of SR, the TFIID complex and other general transcription factors, suggesting that these interactions contribute to the assembly of final transcriptional complexes at their target promoters.

1.3.7.1.2 *The recruitment of co-regulators*

Recently it has become clear that the nuclear receptors recruit a host of co-regulators that have two functions (reviewed in McKenna *et al.*, 1999; Glass and Rosenfeld, 2000). Firstly, these co-regulators can create an environment at the promoter that either activates (co-activator) or represses (co-repressor) transcription,

depending on the activation-state of the receptor. Secondly, these co-regulators can communicate with the general transcription factors and RNA Pol II. Ultimately, it is suggested that the SRs, in association with their co-regulators, achieve transcriptional regulation at hormone-regulated promoters by influencing the rate at which the pre-initiation complex assembles at the promoter. To date a plethora of such nuclear receptor-interacting proteins have been identified. To discuss these co-regulator proteins in detail is beyond the scope of this thesis.

Briefly, the co-activator proteins can be divided into five groups. The first group consists of the ER-associated proteins (ERAPs) and receptor-interacting proteins (RIPs). The second group involves the SRC family, which is a very large group of 160-kDa proteins, also referred to as the p160 nuclear receptor co-activators. There is also a group of selective co-activators, including the androgen receptor activator proteins (ARAs) (for a thorough review refer to Heinlein and Chang, 2002). The co-integrators, such as the CREB-binding protein complex (CBP/p300), that have been shown to interact with the nuclear receptors and other co-activators, are another group. The last group includes all the other co-activators that do not fall into related families and include the TR-associated proteins and vitamin D receptor interacting proteins (TRAPs/DRIPs), positive co-factors as well as TAF_{II}s. These co-activator proteins have been shown to interact with the nuclear receptors (including the SRs) and enhance transcription.

For nuclear receptor co-repressors on the other hand, there is limited data supporting direct contacts between these proteins and the nuclear receptors. The co-repressors are involved in active repression by the thyroid hormone-/retinoic acid-receptor subfamily. In this case, the co-repressors are recruited to the unliganded receptors to create an environment that is incompatible with proper assembly of the

pre-initiation complex. A few co-repressor proteins have been identified that interact with this nuclear receptor subfamily. One such protein is NCoR (nuclear receptor co-repressor), also referred to as RIP-13, that associates with unliganded TR, RAR and RXR (Horlein *et al.*, 1995; Seol *et al.*, 1996). Another co-repressor is SMRT (silencing mediator for retinoid and thyroid hormone receptor), also identified as TRAC2 (T₃ receptor-associating cofactor 2), which has been shown to interact with RXR, RAR and TR (Chen and Evans, 1995; Sande and Privalsky, 1996). Recently, it has been demonstrated that SMRT can bind to the NH₂-terminus of the hAR when treated with the anti-androgen cyproterone acetate (Dotzlaw *et al.*, 2002). Furthermore, direct interactions between the unliganded AR and NcoR have been demonstrated (Cheng *et al.*, 2002).

1.3.7.2 Repression of transcription by steroid receptors

In addition to the members of thyroid hormone-/retinoic acid-receptor subfamily, the SRs are also involved in repression of gene expression. A number of mechanisms have been proposed for transcriptional repression by the SRs. One such mechanism would be via negative HREs. An example would be the negative glucocorticoid response element (nGRE) found in the proopiomelanocortin (POMC) gene. This nGRE shows sequence homology to the regular GRE, but instead of binding as a dimer, the GR binds as a trimer and negatively regulates expression of the gene (Drouin *et al.*, 1993). It is, however, still unclear whether these elements are actually negative or merely overlap with the sites to which other stimulatory proteins bind (Drouin *et al.*, 1993).

Another mechanism of transcriptional repression by the SRs involves the tethering of the SR to other transcription factors. Genes under positive control of the

transcription factors, activator protein 1 (AP-1) or nuclear factor κ B (NF- κ B) provide us with such examples. Mutual antagonism has been reported between the SRs and the components of AP-1, the Fos and Jun proteins (reviewed in Herrlich and Ponta, 1994). The regulatory interactions between the AP-1 complex and the SRs are likely to involve direct protein-protein interaction between the two proteins. Similarly, it is known that NF- κ B can be antagonized by the ER (Ray *et al.*, 1994), GR (Ray and Prefontaine, 1994), PR (Kalkhoven *et al.*, 1996) and AR (Palvimo *et al.*, 1996). This antagonism involves direct protein-protein interactions between the SRs and NF- κ B. Tethering is not, however, limited to the repressive functions of the SRs. In this regard it has been shown that the GR can directly interact with signal transducer and activator of transcription 5 (STAT5) and thereby act as a transcriptional co-activator for STAT5 and enhance STAT5-dependent transcription (Stocklin *et al.*, 1996).

1.3.8 Chromatin remodelling in the control of transcription by the steroid

receptors

Chromatin is composed of DNA wrapped around the core histones in the nucleosome. This arrangement creates severe steric impediments for transcription factors that need to gain access to specific recognition sequences. Nuclear receptor, co-activator and co-repressor proteins all play a role in remodelling the chromatin and thereby control transcription (reviewed in Collingwood *et al.*, 1999). The role that these proteins play can be summarised as follows.

SRs such as the GR are capable of recognising and binding to response elements within the nucleosome. This is the first step towards the re-arrangement of histone-DNA contacts concomitant with the assembly of a functional transcription complex. As described above, the SRs recruit co-activator proteins in a ligand-

dependent manner. By binding to the SRs, these co-activators are brought into contact with the chromatin. Co-activators possess the ability to modify the chromatin environment by alleviating the repressive effects of histone-DNA contacts, thereby indirectly facilitating transcription. They do so by mechanisms including histone acetylation and contacts with the basal transcriptional machinery. On the other hand, the recruitment of co-repressor proteins to the nuclear receptors, either in the absence of ligand or in the presence of receptor antagonists, results in the stabilization of chromatin. This mechanism involves the targeting of histone deacetylases. Taken together, the nuclear receptors, together with the co-regulator proteins, control gene expression by reversibly modifying chromatin structure.

1.3.9 Rapid, non-genomic actions of steroids

Steroids are generally assumed to be involved in the slow regulation of cellular processes, at the genomic level. However, rapid biological responses to injected steroids were described as early as 60 years ago and recently, it has been demonstrated that steroids may modulate cellular activity at a non-genomic level (reviewed in Zinder and Dar, 1999; Sutter-Dub, 2002). Steroids have been implicated in causing specific plasma membrane effects, as well as co-ordinative effects on both membrane and intracellular receptors. Rapid cellular responses to steroids involve plasma membrane binding, changes in membrane electrical activity, G and Ras proteins, cAMP, cGMP, diacylglycerol, phosphodiesterases, and an array of kinases. With respect to membrane receptors, it has been found that for vitamin D and estrogens, both cell surface and nuclear receptors may co-exist in target cells. As a result of ligand binding, these receptors can then generate both rapid and long lasting responses (reviewed in Nemere and Farach-Carson, 1998). Furthermore, it has been

suggested that the epidermal growth factor (EGF) receptor may be involved in rapid aldosterone signalling in MDCK cells (Gekle *et al.*, 2002). Lastly, steroids can be integrated in the intracellular signalling network by cross-talk of the SRs with other signal transduction pathways (reviewed in Beato and Klug, 2000). Therefore, steroids can influence the response to other extracellular signals that are transmitted via membrane receptors and activation of protein kinase cascades. Recently, steroid stimulation of the Src/Ras/Erk signaling pathway has received much attention (reviewed in Migliaccio *et al.*, 2002). Stimulation of the pathway, or its individual members, has been observed in different cell-types. The cellular context and intracellular localisation of the receptors play a role in determining the biological effect brought about by the hormonal stimulation. It has also been shown that the steroid receptors directly interact with Src.

1.4 The molecular mechanisms of androgen action

A brief description of the molecular mechanisms of AR action will follow, but for a thorough review on the molecular biology of the AR, refer to Gelmann, 2002. The AR mediates the physiological effects of the androgen testosterone (T) and its metabolite 5α -dihydrotestosterone (DHT). Testosterone is produced and secreted by the Leydig cells in the testis and is converted to DHT by the enzyme 5α -reductase, either intratesticularly or peripherally. Androgens have a number of important functions throughout the body. These include the roles they play in the development of the genital tract of the male foetus, the full development and functional maintenance of the internal sex organs, as well as the development of the external sex

organs and secondary sex traits at puberty. Disruption of AR action can thus result in clinical phenotypes ranging from mild, to complete androgen insensitivity syndromes. Furthermore, such disruptions are also involved in the development of prostate cancer (Quigley *et al.*, 1995).

In the absence of ligand, the AR is coupled to heat-shock proteins and/or co-repressors and resides in the cytoplasm (Tsai and O'Malley, 1994; Simental *et al.*, 1991). In its unliganded state the AR is rapidly degraded. This degradation is slowed as a result of high affinity androgen binding (Zhou *et al.*, 1995). Binding of androgen causes the heat-shock proteins to dissociate from the AR, activates the bipartite nuclear localisation signal (Zhou *et al.*, 1994), and results in receptor dimerization and DNA binding (Wong *et al.*, 1993).

In addition to binding to the consensus HREs, the activated AR can also bind to more specific, complex response elements. This additional group of response elements, identified in three androgen-selective enhancers, is exclusively recognised by the AR. The recognition and binding of the AR to these specific elements, comprising direct repeats of the 5'-TGTTCT-3'-like sequences, is a determinant of AR-specificity (Claessens *et al.*, 2001). It has been demonstrated that it is the second zinc finger and part of the hinge region of the AR DBD, as opposed to the first zinc finger, that is involved in the recognition of these androgen response elements (Schoenmakers *et al.*, 1999). In fact, three AR-specific amino acids in the second zinc finger were implicated in studies using the probasin enhancer. All of these amino acids are located at the surface of the DBD and pointing away from the DNA. It has therefore been suggested that it is not a matter of sequence specificity, but rather an

alternative dimerization mechanism that explains the specificity of the AR for the probasin androgen response element (ARE) (Schoenmakers *et al.*, 2000). This alternative mechanism would involve a head-to-tail dimerization of the DNA-bound AR-DBDs. Evidence for such anti-parallel AR dimers has also been presented by Langley *et al.*, 1995. In studies using synthetic direct repeats and mutant derivatives of the 5'-TGTTCT-3' sequence, it was further demonstrated that the AR and not the GR can bind the direct repeat efficiently (Schoenmakers *et al.*, 2000). The ability of the AR to bind to elements, resembling direct repeats of the 5'-TGTTCT-3' sequence, of a number of known androgen-selective enhancers has been shown and is reviewed in Claessens *et al.*, 2001.

Another feature of the AR is the ligand-dependent interaction that occurs between the NTD and the C-terminal domain (referred to as the N/C-interaction) (Langley *et al.*, 1995; Doesburg *et al.*, 1997). A functional ligand-dependent association of these domains has also been described for the estrogen receptor (Kraus *et al.*, 1995) and progesterone receptor (Tetel *et al.*, 1995). The N/C-interaction of the AR was found to be essential for optimal AR function (Ikonen *et al.*, 1997). The AF2 domain in the LBD was identified as the region of the C-terminal that is involved in mediating this interdomain communication (Berrevoets *et al.*, 1998; He *et al.*, 1999). Furthermore, two regions in the NTD have been identified that are involved in this functional interaction. The first is located near the NH₂-terminus between amino acid residues 3 and 36, and the second is located between residues 370 and 494 (Berrevoets *et al.*, 1998). More specifically, these regions each contain an LXXLL-like motif, where L is leucine and X is any amino acid. The first region contains a FXXLF motif (sequence ²³FQNLF²⁷) whereas the second region contains a WXXLF

motif (sequence ⁴³³WHTLF⁴³⁷). The FXXLF motif binds AF2 in the C-terminal of the AR, and the WXXLF motif binds to a region of the IBD outside of AF2 (He *et al.*, 2000). As discussed earlier, binding of hormone to the SRs causes helix 12 of the LBD to undergo a conformational change, closing down over the ligand-binding pocket. Similarly, the binding of androgen to the AR causes the proper closure of the pocket by helix 12. Concomitant with this conformational change is the activation of the AF2 domain, or more specifically the formation of a new structural surface that can interact with other domains or co-factors. For this reason the N/C-interaction is ligand-dependent, in that ligand first needs to bind, thereby inducing the closure of helix 12 and exposing the AF2 domain for interaction with regions in the NTD. When helix 12 closes down over the ligand-binding pocket, it also functions to slow the rate at which ligand dissociates from the pocket. The subsequent interaction of the NTD with the AF2 domain further stabilizes helix 12 thereby assisting the decrease in the rate of androgen dissociation (Kemppainen *et al.*, 1999; He *et al.*, 1999).

The AF2 domain of the AR displays weak transcriptional activity in comparison to the AF2 domains of other SRs. It has been demonstrated that the ligand binding ability of the LBD is imperative for the functioning of the AF2 domain, and that in a yeast system the activity of AF2 is enhanced when the hinge region is present. These findings suggest that the hinge region is involved in modulating the activity of the LBD, probably by providing an interface for interacting proteins (Moilanen *et al.*, 1997). A functional interaction has been demonstrated between the AF2 domain and the p160 nuclear receptor co-activators (Alen *et al.*, 1999). The AF2 core domain located in helix 12, together with a conserved lysine residue in helix 3, were found to be mandatory for this interaction with the p160 proteins. These co-

activators include SRC1, GRIP1/TIF2, and AIB1/ACTR/TRAM1 (as described earlier and reviewed in McKenna *et al.*, 1999; Glass and Rosenfeld, 2000; Xu *et al.*, 1999; Leo and Chen, 2000). Similarly to the N/C-interaction, this interaction is ligand-dependent, in that ligand has to bind to the LBD inducing the closure of the ligand-binding pocket by helix 12. This realignment of helix 12 in the presence of ligand is believed to form a hydrophobic cleft, composed of helices 3, 5, and 12. The p160 co-activators can then bind to the hydrophobic cleft via three highly conserved α -helical LXXLL motifs that are centrally located in their nuclear receptor interacting regions (Heery *et al.*, 1997; Voegel *et al.*, 1998).

For the wild-type AR it has been shown that almost the entire NTD is necessary for full transcriptional activity. The NH₂-terminal AF1 domain has been found to be the major activating region of the AR in both mammalian and yeast cells (Moilanen *et al.*, 1997). The size and location of the active AF1 domain in the NTD is variable, in that it is dependent on the promoter context and the absence or presence of the LBD (Jenster *et al.*, 1995). Similarly to the AF2 domain, the AF1 domain of the AR interacts functionally with the p160 co-activators (Alen *et al.*, 1999). This is a ligand-independent interaction and involves a direct interaction between the AF1 domain and a glutamine-rich region of the co-activator protein, which is conserved amongst the p160 co-activator family (Bevan *et al.*, 1999).

The region of the AF2 domain that interacts with the NTD during the N/C-interaction and the region of the AF2 domain that interacts with the LXXLL motifs of the p160 co-activators, overlap (Thompson *et al.*, 2001). This, together with the ability of the p160 co-activators to interact with both the NTD and the LBD of the

AR, suggests that these co-activator proteins may play a role in bridging the N/C-interaction.

Taken together, these mechanisms of AR action provide, in part, an explanation for the specificity of the androgen response. Firstly, the AR is able to bind to response elements composed of direct repeat sequences, to which other SRs are unable to bind. Secondly, binding of androgen to the LBD of the AR induces conformational changes in this domain that result in the subsequent N/C-interaction. This interaction prolongs the occupation of receptor with low concentrations of androgen, a requirement for AR stabilization and function.

1.5 The molecular mechanisms of anti-androgen action

Anti-androgens (or AR antagonists) are compounds that prevent androgens from exerting their biological effects. Such compounds are used extensively for the treatment of androgen-based dysfunctions. Based on their structure, the anti-androgens can be divided into two groups, namely the steroidal (e.g. cyproterone acetate) and non-steroidal anti-androgens (e.g. hydroxyflutamide).

When considering the various steps involved in SR action, it is apparent that there are a number of potential targets for antagonist action. The antagonists compete with the agonists for binding to the LBD of the SR and impair the complete conversion of the receptor to a transcriptionally active form. Possible target steps where such antagonists may exert their action include the dissociation of the hsp

(Segnitz and Gehring, 1990) as well as the translocation of the receptor to the nucleus. Furthermore, dimerization of the SRs (Fawell *et al.*, 1990; Klein-Hitpass *et al.*, 1991) and binding of the SR dimer to the DNA response elements (Berry *et al.*, 1990) have also been implicated as target steps in antagonist action. Lastly, interactions of the DNA-bound SRs with transcription factors (Berry *et al.*, 1990; Klein-Hitpass *et al.*, 1991) are also potential steps where antagonists may exert their action.

With regards to antagonists that impair the dissociation of non-receptor proteins (i.e. hsps) from the inactive receptor-complex, studies using the LNCaP (lymph node carcinoma of the prostate) cell-line (Veldscholte *et al.*, 1992b) demonstrate this mechanism of anti-androgen action. Here it was shown that compounds that act as androgen agonists in this system, result in the dissociation of Hsp90 and p59, whereas in the presence of the antagonist ICI 176.334, these proteins do not dissociate from the receptor complex.

The subcellular localisation of antagonist-bound AR appears to be dependent on the antagonist used. In a study by Berrevoets *et al.*, 1993 it was found that when bound by the AR antagonists, hydroxyflutamide and ICI 176.334, the AR remains in the cytoplasm. However, when bound by cyproterone acetate, it was found that some of the AR was detected in the nucleus. Taken together, these results indicate that certain anti-androgens may impair translocation of the AR to the nucleus.

When considering the DNA-binding capacity of antagonist-bound receptor, the antagonists for the PR and ER have been divided into two classes (Klein-Hitpass *et al.*, 1991; Green, 1990). Firstly, type I antagonists have been identified that inhibit

receptor binding to DNA, and secondly, type II antagonists have been identified that induce high affinity DNA binding but prevent receptor interactions with the transcription initiation complex. Certain type II antagonists demonstrate partial agonist activity. This has been ascribed to the ligand-independent activation function in the NTD, but is also dependent on the promoter and cellular context in which it is measured (Berry *et al.*, 1990). For the AR both types of antagonists have been identified. Cyproterone acetate, which has shown partial agonist activity in transactivation assays (Kemppainen *et al.*, 1992), promotes binding of the AR to DNA, whereas hydroxyflutamide inhibits binding of the AR to DNA (Wong *et al.*, 1993).

To date many attempts have been made to characterise and distinguish AR agonists and antagonists by elucidation of their distinct mechanisms of action. Due to the broad range of AR agonists and antagonists available, no single feature has been illuminated by which these compounds can be categorised. For a number of years AR antagonists were characterised by their low affinity for the AR. Compounds that exhibited an affinity that was less than 10% of that of the synthetic androgen, methyltrienolone (R1881), were considered to be anti-androgens (Kemppainen *et al.*, 1992; Veldscholte *et al.*, 1992a).

Another feature that was explored is the ability of different SR ligands to induce different conformations of the receptor. Electromobility shift assays (EMSAs) have shown that agonist- and antagonist-receptor-DNA complexes differ in their mobility, suggesting distinct changes in the spatial structure of the receptor as a consequence of binding by either agonist or antagonist (Kallio *et al.*, 1994b). Limited

proteolysis of the PR, ER and GR has led to the hypothesis that antagonist activity can be ascribed to the induction of a non-functional conformation at the C-terminus of the SRs (reviewed in Tsai and O'Malley, 1994). Limited proteolysis studies with the AR have, however, yielded conflicting results. Firstly, Kallio *et al.*, 1994a found an unaltered conformation of the LBD, similar to that found in the absence of ligand, for the anti-androgen bound receptor. Secondly, Kuil *et al.*, 1994 found that in the presence of androgen, limited proteolysis yielded a protected fragment consisting of the entire LBD, whereas in the presence of anti-androgen a larger fragment consisting of the entire LBD and an extension of the hinge region was observed. Lastly, Zeng *et al.*, 1994 found that regardless of whether agonist or antagonists were used, there was no difference in the size of the proteolysis-resistant fragment. None of the studies performed with the AR suggest the involvement of the extreme C-terminal region, as found for the PR, ER and GR (reviewed in Tsai and O'Malley, 1994), suggesting that the mechanisms of anti-androgen action may differ from the mechanisms by which other SR antagonists exert their action.

More recently the ability of compounds to induce the ligand-dependent N/C-interaction of the AR was investigated as a marker for agonist action (Kemppainen *et al.*, 1999). In this study it was demonstrated that androgens induce this interaction and that anti-androgens inhibit it. However, it was further demonstrated that inhibition of this interaction does not necessarily imply antagonist activity. The synthetic progestin, medroxyprogesterone acetate (MPA), which displayed weak agonist activity in transactivation studies, not only failed to induce this interaction, but also inhibited the DHT-induced N/C-interaction. Prior to this study it had been observed that one property that distinguished androgen agonists from antagonists was their ability to

stabilise the AR and thereby protect against degradation (Kemppainen *et al.*, 1992). In addition, it has been observed that more potent agonist activity is associated with a slow dissociation rate and that this retention of receptor-bound agonist is enhanced by the N/C-interaction (Zhou *et al.*, 1995). When the ability of MPA to stabilise the AR was determined, it was found that 100 nM MPA was required to achieve stabilization similar to that achieved with 1 nM DHT (Kemppainen *et al.*, 1999). Furthermore, this study showed that antagonist activity is associated with the inability to induce binding of the AR to DNA as well as more rapid ligand dissociation. In Kemppainen *et al.*, 1999 it is concluded that when distinguishing between agonists and antagonist for the AR, a number of features are involved. They propose that AR agonist activity can be characterised by a slow dissociation rate of bound ligand, stabilisation of the AR with agonist concentrations of less than 10 nM, as well as the induction of the N/C-interaction. It is further suggested that AR antagonist activity would be best reflected by the inability of a ligand to stabilise the AR against degradation at concentrations of 500 nM or more.

1.6 Compound A, an analogue of a non-steroidal plant compound

Compound A (CpdA) was developed as a stable analogue of a highly labile hydroxyphenyl aziridine precursor (Louw *et al.*, 1997) found in the African shrub, *Salsola tuberculatiformis* Botsch. This synthetic compound is non-steroidal as is evident from the chemical name, 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride, and structure (figure 5).

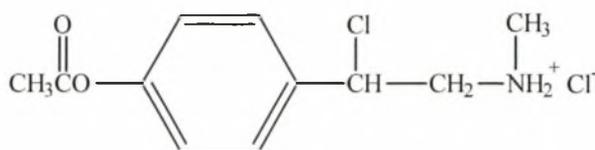
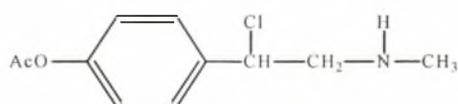


Figure 5: The chemical structure of 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride (Compound A).

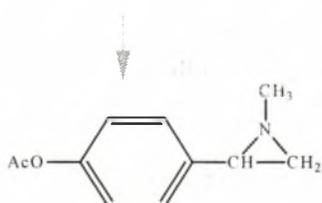
The shrub was used by the Kalahari Bushmen for traditional contraceptive purposes (Maritz, 1969). Subsequently, it was found that the shrub also causes prolonged gestation in sheep (Basson *et al.*, 1969) as well as contraception in rats (Louw *et al.*, 1997). A number of studies have been carried out in an attempt to elucidate the mechanism(s) of action underlying these activities of both CpdA and the shrub. Initial studies using female Wistar rats revealed that CpdA, like the shrub, disrupts the oestrus cycle (Louw *et al.*, 1997), while in *in vitro* studies CpdA was shown to inhibit the action of sheep adrenal cytochrome P450c11, the final enzyme involved in glucocorticoid biosynthesis, similarly to the active compound isolated from the shrub (Louw *et al.*, 1997). Furthermore, *in vitro* studies also indicate that CpdA can bind to ovine and rat corticosteroid-binding globulin (CBG), thereby displacing endogenous glucocorticoids and thus increasing free glucocorticoid levels (Louw *et al.*, 2000b). This was confirmed in studies with female Wistar rats showing that both the shrub and CpdA displace glucocorticoids from CBG (Louw and Swart, 1999). In addition, this study revealed that there are also significant decreases in CBG, luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH) levels during treatment. Taken together, the results of these studies suggest that the binding

of CpdA to CBG and the concomitant increase in the levels of free corticosterone, may offer part of the explanation for the contraceptive action of CpdA in female rats.

CpdA has been shown to cyclize to the corresponding aziridine, acetoxypheyl methylaziridine, at a physiological pH (figure 6). A study to determine whether the biologically active component is the aziridine or the open chain precursor, CpdA, revealed that CpdA is the inhibiting agent in the cytochrome P450c11 system (Louw *et al.*, 1997). This study also showed that CpdA is stabilized by CBG in both sheep and rat serum, resulting in slower cyclization to the aziridine (Louw *et al.*, 1997). Taken together, these results therefore strongly suggest that it is CpdA that exerts the biological actions in these experimental systems.



Compound A:
2-(4-acetoxyphenyl)-2-chloro-N-
methyl-ethylammonium chloride



Compound A's aziridine:
2-(4-acetoxyphenyl)-1-
methylaziridine

Figure 6: The chemical structures of Compound A and the aziridine to which it is cyclized.

The ability of CpdA to interact with glucocorticoid binding proteins such as the steroidogenic enzymes (Louw *et al.*, 2000a) and plasma steroid-binding globulins (Louw *et al.*, 2000b) has led to the following postulation. It has been suggested that CpdA could disrupt the oestrus cycle of rats by interacting with glucocorticoid-binding proteins and thereby altering the interactions between the hypothalamus, pituitary, adrenals and gonads. Furthermore, recent studies from our laboratory indicate that CpdA can affect glucocorticoid receptor-mediated activation of transcription (data unpublished), suggesting that CpdA may interact with the GR, another glucocorticoid-binding protein. This potential interaction between CpdA and the GR, together with the fact there is a high degree of homology between the members of the SR family, prompted us to investigate whether CpdA interacts with the AR, another member of the SR family. Although CpdA is a non-steroidal compound, we were not deterred from pursuing this investigation, since a number of non-steroidal compounds have been shown to interact with and exert effects via the AR (e.g. the anti-androgen, hydroxyflutamide).

1.7 Contraceptive agents administered by injection

Although the combined oral contraceptive pill is often the automatic choice for effective contraception, the long-acting progestogen-only injectable contraceptives have some advantages and as a consequence are widely used by women (reviewed in Kaunitz, 1998). These advantages include greater efficacy, easier compliance, avoidance of estrogenic side-effects, as well as greater privacy. An additional advantage is that they are an appropriate choice for postpartum or lactating women

whose status precludes them from using contraceptive doses of estrogen. Some of the disadvantages include irregular bleeding and a slow return to fertility.

Two progestogen-only injectables are offered as conventional contraceptive agents in family planning clinics throughout South Africa. They are, depot medroxyprogesterone acetate (DMPA) and norethisterone enanthate (NET-EN). Little is known of the precise molecular mechanisms via which these contraceptive agents exert their effects and side-effects. It has however, been demonstrated that these agents bind to and interact with a number of the steroid receptors. This, together with the widespread use of these contraceptive agents, prompted the investigation into the mechanism(s) by which these compounds may potentially exert effects via the AR.

1.7.1 Medroxyprogesterone acetate

DMPA has been used in clinical practice for more than 30 years by millions of women. However, for many years it was banned as a contraceptive in the United States until it obtained approval from the Food and Drug Administration for contraceptive use in 1992. Since its approval, DMPA has been, and still is, used by several million U.S. women (Kaunitz, 1998). DMPA is an aqueous suspension of 17-acetoxy 6-methyl progestin administered by intramuscular injection of 150 mg every three months. Medroxyprogesterone acetate (MPA) (figure 8) is detected in the serum 30 minutes after injection, displays long-term action due to slow release into the circulation, and has multiple targets, all factors which make MPA a very effective contraceptive (reviewed in Mishell, 1996).

MPA elicits its contraceptive effects by abolishing peak mid-cycle gonadotropin (luteinizing hormone, LH and follicle-stimulating hormone, FSH) levels

and as a consequence inhibiting follicular maturation and preventing ovulation (reviewed by Mishell, 1996 and Kaunitz, 1998). Although inhibition of ovulation is the major effect, two other mechanisms of action are also involved. These include the thinning of the endometrium, rendering it unfit to support a blastocyst, and keeping the cervical mucus thick and viscous, preventing sperm from reaching the oviduct.

MPA was synthesized as a true progestin, meaning that MPA is a 21-carbon series steroid (refer to figure 7 for carbon numbering system), consisting of the pregnane nucleus (Darney *et al.*, 1995). Almost all other contraceptive progestins are 19-nortestosterone derivatives, and as a result have varying degrees of androgenic activity (Mishell, 1996). It has, however, been demonstrated that MPA, in addition to having high affinity and agonist activity for the PR, also binds to and is an agonist for the GR and the 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). DMPA has also been shown to induce a number of side-effects, including amenorrhea, weight gain, headaches and many more (reviewed in Kaunitz, 1998). It is possible that the contraceptive actions and side-effects of MPA could be mediated via any of these receptors.

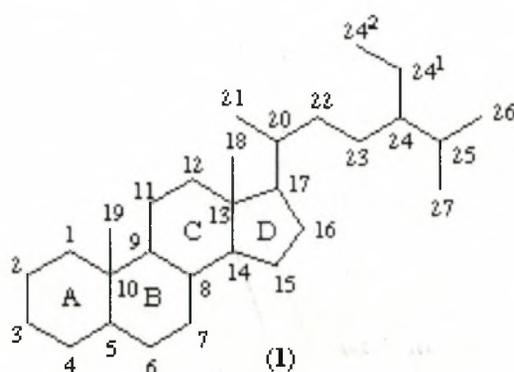


Figure 7: The steroid hormone carbon numbering system.

The carbons are numbered 1 to 27, and the rings are labelled A to D.

(<http://www.chem.qmul.ac.uk/iupac/steroid/3S01.html#3S11>)

Not only is MPA used as a contraceptive, but it is also used for the treatment of dysmenorrhea, menorrhagia, endometriosis, endometrial hyperplasia, ovulatory pain, pain associated with ovarian disease, premenstrual dysphoria and perimenopausal symptoms (Kaunitz, 1998). Furthermore, MPA is used in cancer therapy, at 500-1500 mg orally per day for about 12 weeks (Blossey *et al.*, 1996), and in hormone replacement therapy, at 10 mg per day for days 10 to 21 of the therapeutic month (Brunelli *et al.*, 1996). The actions of MPA in tumour regression are brought about partly by interactions with the PR (Blossey *et al.*, 1984), but predominantly by interactions with the GR (Bojar *et al.*, 1979) or the AR (Teulings *et al.*, 1980).

The doses of MPA administered for cancer and hormone replacement therapies are much higher than that used for contraceptive purposes. Women using MPA as an injectable contraceptive have serum concentrations of about 1ng/ml (Mishell, 1996), which translates to about 2.6 nM. The ability of MPA to exert AR agonist activity (Kemppainen *et al.*, 1999), together with the fact that little research has been carried out to define the precise mechanisms by which MPA exerts its effects, prompted the initiation of the study to define the *in vitro* interaction of circulating, contraceptive doses of MPA with the AR, the results of which are reported in chapter 3.

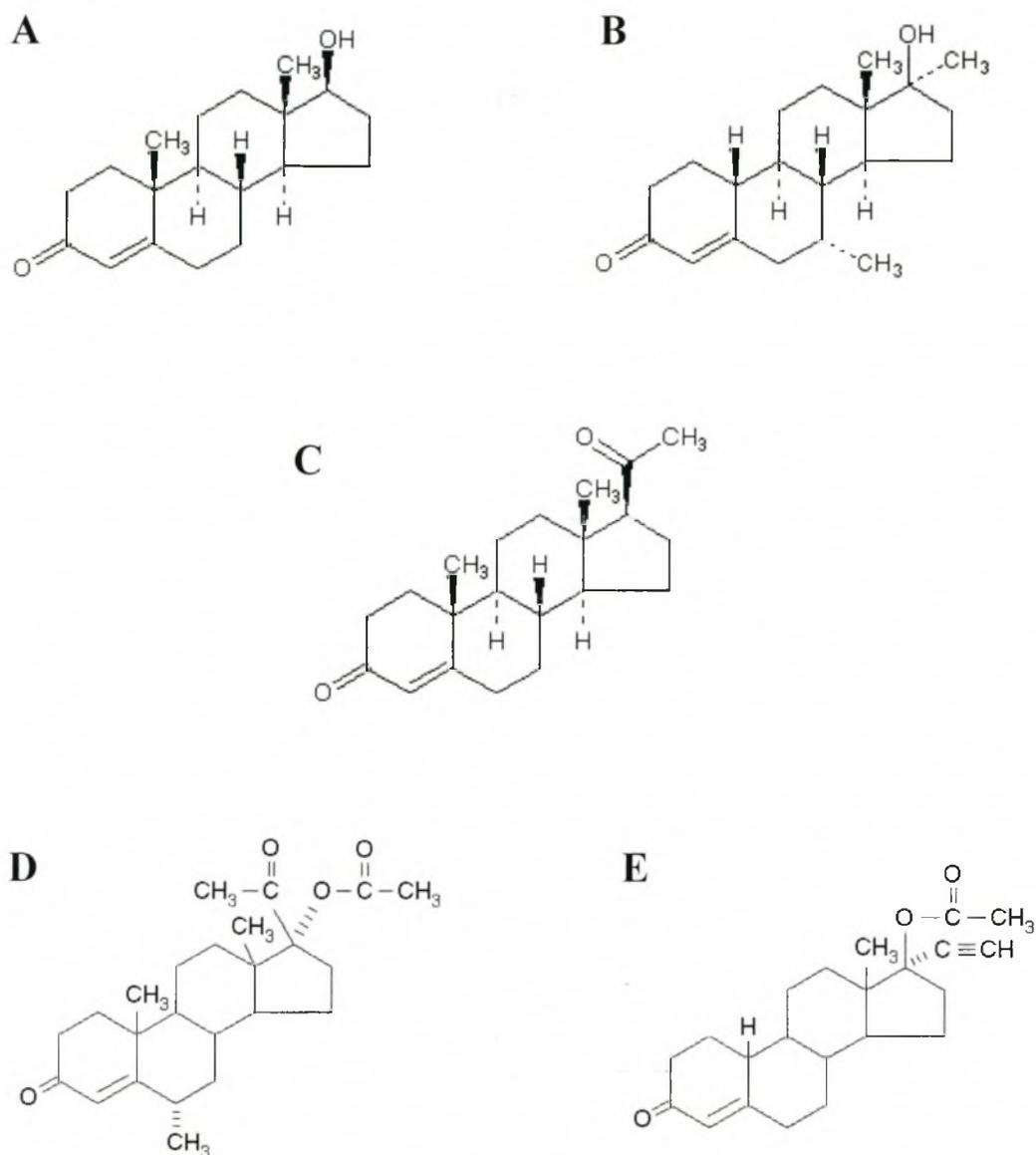


Figure 8: The chemical structures of natural and synthetic androgens and progestins.

The (A) natural androgen, *testosterone (T)*; (B) synthetic androgen, *mibolerone*; (C) natural progestin, *progesterone*; (D) synthetic progestin, *medroxyprogesterone acetate (MPA)*; and (E) synthetic progestin, *norethindrone acetate (NET-A)*.

1.7.2 Norethindrone enanthate

Similarly to DMPA, NET-EN (often referred to as either norethindrone enanthate, norethisterone enantate or nuristerate) has been used as an injectable contraceptive for many years. NET-EN is formulated in an oily suspension of benzyl benzoate and castor oil and is administered by an intramuscular injection of 200 mg every two months (reviewed in Stanczyk and Roy, 1999). After administration, NET-EN is slowly hydrolysed by blood esterases to the parent compound, norethindrone (NET). NET (structurally related to testosterone) can be further metabolised and undergoes extensive reduction of the α , β -unsaturated ketone ring A, forming the corresponding dihydro and tetrahydro reduced products. From pharmacokinetic studies (Sang *et al.*, 1981) it is evident that post-injection, the serum levels of NET and NET-EN increase rapidly, and that at all times the levels of NET exceed those of NET-EN. Furthermore, NET is detectable in the circulation for about 74 days after injection, whereas NET-EN is only detected for about 43 days. This rapid metabolism of NET-EN therefore indicates that it is NET and its metabolites that are the active contraceptive compounds. Fotherby *et al.*, 1983, found that over the 60 day interval between administrations of NET-EN, the serum levels of NET can range from 0.5 to 20 ng/ml, which translates to about 1.5 to 59 nM.

Studies in rats demonstrate that NET-EN elicits its contraceptive effects primarily through changes in the content of cervical mucus, thereby creating a progestogenic phase, which prevents sperm penetration and thus fertilisation (Bhowmik and Mukherjea, 1987). Later studies, also in rats, show that NET-EN can also block ovulation (Bhowmik and Mukherjea, 1988). This is not surprising as it was demonstrated that NET-EN has a long-lasting gonadotropin suppressive effect in women (Fotherby *et al.*, 1983).

NET-EN, like norethindrone acetate (NET-A) (figure 8) and ethyndiol diacetate, is a 19-nortestosterone progestin. This means that these progestins are 19-carbon series steroids and therefore are composed of the androstane nucleus (similar to all androgens). When carbon 19 is removed from testosterone, the androgenic hormonal effect is converted to a progestogenic one (refer to figure 7 for carbon numbering system). However, these “19-nor” steroids have been shown to retain varying degrees of androgenic activity (Darney, 1995). The ability of NET to bind to the PR and the AR, as well as display agonist activity via these receptors, has been demonstrated (Deckers *et al.*, 2000). It has also been demonstrated that in human mononuclear leukocytes, NET is virtually devoid of binding affinity towards the GR, nor does it induce glucocorticoid-like effects on the lymphocyte functions (Kontula *et al.*, 1983). Similarly to DMPA, use of NET-EN as a contraceptive agent is associated with a number of side-effects. Some of these side-effects are attributed to the lingering androgenic effects of the compound (for example lipoprotein synthesis, acne and weight gain) (Darney, 1995).

NET-EN is also used for applications other than contraception, including its use in hormone replacement therapy. At present, NET-EN is under investigation to be used as a male injectable contraceptive in combination with a testosterone ester (Kamischke *et al.*, 2002, 2000a, 2000b). In this application, NET-EN functions to suppress spermatogenesis by its strong, rapid and sustained suppression of serum FSH and testosterone levels.

Taken together, the structural relationship between NET-EN and testosterone together with the androgenic properties of NET-EN prompted us to study the interaction of circulating, contraceptive doses of NET-EN with the AR at the molecular level.

1.8 Aim and scope of this thesis

The main aim of the studies, described in the next two chapters, was firstly, to establish whether CpdA possesses any androgenic or anti-androgenic activity and secondly, to compare the androgenic activity of the two synthetic progestins, medroxyprogesterone acetate and norethindrone acetate.

In chapter 2 the potential interaction between CpdA and the AR, a steroid-binding protein, is studied. This study was prompted by the fact that CpdA has previously been shown to interact with other steroid-binding proteins such as steroidogenic enzymes (Louw *et al.*, 2000a) and plasma steroid-binding globulins (Louw *et al.*, 2000b). Furthermore, as the AR is a member of the steroid receptor family, the specificity of its actions within this family is also addressed in this study. The contraceptive activity previously displayed by CpdA (Louw *et al.*, 1997) further prompted these investigations with the steroid receptors. This study also attempts to define the precise mechanisms by which CpdA exerts its effects via the AR, as such a compound could be used as a lead compound in designing drugs that are targeted to the AR.

The third chapter describes investigations that directly compare the androgenic properties of MPA and NET-A. To date, little research has been done to compare the actions of these compounds at the molecular level. By comparing the androgenic activities of these compounds the mechanisms by which they exert their effects should become clearer, which in turn would provide women and their clinicians with more information to facilitate the selection of a method of contraception.

Both the study with CpdA and the study with the contraceptive compounds could shed some light on the general mechanisms of AR action.

In the final chapter the results of these investigations are summarised and discussed in the broader context, with emphasis placed on the implications of these findings. Lastly, suggestions are made for future investigations.

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Anti-androgenic properties of Compound A, an analog of a non-steroidal plant compound

T. M. Tanner¹, G. Verrijdt², W. Rombauts², A. Louw¹, J.P. Hapgood¹ and F. Claessens²

¹Department of Biochemistry, University of Stellenbosch, 7600 Stellenbosch, South Africa. ²Division of Biochemistry, Campus Gasthuisberg, Catholic University of Leuven, 3000 Leuven, Belgium.

2.1 Manuscript

Summary

We investigated the interactions between Compound A (CpdA), an analog of a hydroxyphenyl aziridine precursor found in an African shrub, and the androgen receptor (AR). CpdA represses androgen-induced activation of both specific and non-specific androgen DNA response elements. While a similar effect was obtained for the progesterone receptor via a non-specific hormone response element, CpdA had no effect on the actions of the glucocorticoid and mineralocorticoid receptors. CpdA represses the ligand-dependent interaction between the NH₂- and COOH-terminal domains of the AR, similarly to well-characterized anti-androgens. CpdA also interferes with the interaction of steroid receptor co-activator 1 (SRC1) with the activation domain AF2 but not with AF1. However, CpdA does not compete with androgen for binding to the AR. These results demonstrate that CpdA elicits anti-androgenic actions by a mechanism other than competitive binding for the AR.

Introduction

Compound A (CpdA), 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, is a non-steroidal compound that was developed as a stable analog (figure 1) of a highly labile hydroxyphenyl aziridine precursor (Louw *et al.*, 1997) found in the African shrub, *Salsola tuberculatiformis* Botsch. CpdA can interact with steroid-binding proteins such as steroidogenic enzymes (Louw *et al.*, 2000a) and plasma steroid-binding globulins (Louw *et*

al., 2000b). We were thus prompted to investigate the possible interactions between CpdA and other steroid-binding proteins, in particular the steroid receptors.

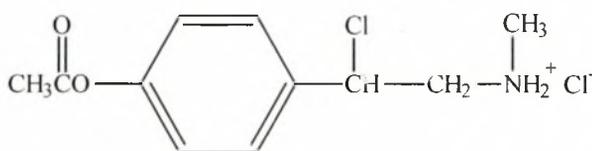


Figure 1: The chemical structure of 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride (Compound A).

The steroid receptors form a subfamily of the nuclear receptor superfamily, a large group of ligand-dependent transcription factors. The steroid receptors include the androgen receptor (AR), estrogen receptor, glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and progesterone receptor (PR). In general, the inactive steroid receptors reside predominantly in the cytosol. The steroid hormones bind to these receptors, which are then activated. After activation, the hormone receptor-complex translocates to the cell nucleus where it binds to specific DNA sequences, called response elements and subsequently regulates gene expression (Evans, 1988; Beato, 1989). There is a high level of similarity between the members of the nuclear receptor superfamily, where the arrangement of the different domains is essentially the same for all the members. This arrangement involves the highly variable NH₂-terminal domain (NTD), followed by the highly conserved and centrally located DNA-binding domain (DBD) through which the interactions with the response elements are mediated. The COOH-terminal (C-terminal) domain that is also conserved between members of the family contains the ligand binding domain (LBD), and areas

involved in stabilization of homodimerisation as well as orchestration of interactions with coregulators (Moras and Gronemeyer, 1998). The C-terminal domain is linked to the DBD by a region known as the hinge region. A hormone-independent transcription activation function 1 (AF1) is found within the NTD (Beato *et al.*, 1995), whereas a hormone-dependent activation function 2 (AF2) has been located in the LBD (Danielian *et al.*, 1992; Moilanen *et al.*, 1997).

For the AR, the size and location of the active AF1 domain in the NTD is variable, in that it is dependent on the promoter context and the absence or presence of the LBD (Jenster *et al.*, 1995). The transcriptional activity of the AF2 domain of the AR is weak in comparison to many of the other steroid receptors (Moilanen *et al.*, 1997). It has been demonstrated that there are functional interactions between these activation domains of the AR and the 160-kDa (p160) nuclear receptor co-activators (Alen *et al.*, 1999). The closely related p160 steroid receptor co-activators that include SRC1, GRIP1/TIF2, and AIB1/ACTR/TRAM1 (reviewed in: McKenna *et al.*, 1999; Glass and Rosenfeld, 2000; Xu *et al.*, 1999; Leo and Chen, 2000), have distinct regions that interact with the AF1 and AF2 domains of the AR. Firstly, they interact in a ligand-dependent manner with the LBD (AF2) of steroid receptors via three highly conserved α -helical LXXLL motifs that are centrally located in their nuclear receptor interacting regions (Heery *et al.*, 1997; Voegel *et al.*, 1998). Secondly, it has also been demonstrated that p160 co-activators such as steroid receptor co-activator-1 (SRC1) directly interact with the NTD of the AR. This interaction is ligand-independent and occurs between AF1 of the AR and a glutamine-rich region of the co-activator. This region is conserved amongst the members of this family of co-activators (Bevan *et al.*, 1999). Furthermore, a ligand-dependent interaction between the NTD and LBD (N/C-interaction) of the AR has been demonstrated (Langley *et al.*, 1995; Doesburg *et al.*, 1997) and found to be essential for

optimal AR function (Ikonen *et al.*, 1997). Studies to reveal the subdomains involved in this interaction indicate that it is AF2 in the LBD that mediates this interdomain communication (Berrevoets *et al.*, 1998; He *et al.*, 1999). Two LXXLL-like motifs have also been identified in the NTD of the AR (He *et al.*, 2000), which have been shown to form, in part, the interface for the interaction of the NTD with AF2. The ability of the p160 co-activators to interact with both the NTD and LBD indicates that they may play a role in bridging this N/C-interaction.

Prostate cancer is one of the most commonly diagnosed, and leading causes of death from cancer in men in westernized society. To date, the most effective therapy available for the treatment of prostate cancer is androgen deprivation, which involves either surgical or chemical castration (often anti-androgen therapy). This treatment is unable to completely eliminate prostate cancer cell populations because, although there is an initial response, this is followed by the predictable pattern of relapse, progression to androgen independence and eventual death (Sadar *et al.*, 1999 and references therein). The anti-androgenic properties displayed by CpdA in our experiments are thus of potential importance.

Materials and methods

Plasmids

The plasmid pMTV-luc, which contains a luciferase reporter gene driven by the mouse mammary tumour virus long terminal repeat, and the plasmid pC3(1)-TATA-luc, which contains a luciferase reporter gene downstream of a 204-base pair *PvuII/SstI* fragment of the first intron of the C3(1) gene of prostate binding protein are described in Claessens *et al.*, 1993. Luciferase reporter constructs driven by the TK minimal promoter and containing

the *slp* enhancer (pSLP-TATA-Luc), or the *sc* enhancer (pSC-TATA-Luc), or driven by the *pb* proximal promoter (pPB-Luc) are as described in Verrijdt *et al.*, 2000. The plasmid pTAT-GRE-E1b-luc, is driven by the E1b promoter containing two copies of the rat TAT-GRE (a gift from G. Jenster). Plasmids expressing human steroid receptors: pSVARo, pRSV-hGR, pRSV-hMR and pSG5-hPR were gifts from A. Brinkmann, R. Evans and H. Stunnenberg. The hAR DBD-LBD expression vector, pSG5-hAR(DBD-LBD), as well as the hAR NTD-VP16 fusion protein expression vector, pSNATCH-II(hAR-NTD), were previously described in Alen *et al.*, 1999. The expression vector for SRC1, pSG5-SRC1, was obtained from M.G. Parker. The luciferase reporter driven by five Gal4 binding sites, p(Gal4)₅-tata-luc, was a gift from G. Folkers (described in Folkers and van der Saag, 1995). The Gal4-DBD and SRC1 fragment fusion protein expression vector, pGal4DBD-SRC1(989-1240), was previously described in Kalkhoven *et al.*, 1998. The expression vector for the hAR NTD, pSG5-NTD, comprises the entire hAR NTD, cloned in frame with three copies of the Flag-peptide, into the *EcoRI/BglII* site of the pSG5 vector. The expression vector of residues 1 to 147 of the yeast transcription factor GAL4 fused to the hAR NTD (M1 to R538), pAB-Gal4-NTD, was described in Alen *et al.*, 1999.

Preparation of test compounds

R1881, dexamethasone, aldosterone, progesterone and dihydrotestosterone were obtained from Sigma. [³H]mibolerone as well as unlabelled mibolerone were obtained from Dupont, NEN. All test compounds (including CpDA) were dissolved in ethanol. These compounds were then added to the culturing medium such that the final concentration of ethanol did not exceed 0.1%. Control incubations (no test compounds) were performed in the presence of only 0.1% ethanol.

Transfections

Monkey kidney CV1 or COS-7 cells (gifts from M.G. Parker) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum (Gibco-BRL Life Technologies), penicillin (100 IU/ml) and streptomycin (100 µl/ml) in a humidified 5% CO₂ incubator. For transfection experiments, cells were seeded into 96-well tissue culture plates (Nunc, Roskilde, Denmark) at 10⁴ cells per well and grown in phenol red free DMEM supplemented with 5% (v/v) dextran-coated charcoal-stripped serum. On day 2, cells were transfected with FuGENE6 transfection reagent (Roche Molecular Biochemicals) in accordance with the manufacturer's instructions. The DNA mixture for transfections consisted of (per well): 100 ng of the appropriate luciferase reporter constructs, 10 ng of the appropriate mammalian steroid receptor expression vector, and 10 ng of the cytomegalovirus (CMV)-driven β-galactosidase expression vector (Stratagene). On day 3, media were replaced, either with or without the addition of the appropriate hormones or CpdA (synthesis of CpdA described in Louw *et al.*, 1997). On day 4, cells were harvested by incubation for 10 min in 25 µl of 1 X passive lysis buffer (Promega). A 2.5 µl aliquot of cellular extract was used for the quantification of luciferase in a Luminoskan Ascent Luminometer with luciferase assay reagent (Promega) in accordance with the manufacturer's instructions. The β-galactosidase activity in a 2.5 µl aliquot of extract from each sample was measured with the β-galactosidase chemiluminescent reporter gene assay system (Tropix Inc., Bedford, MA, U.S.A.). β-Galactosidase readings were used to assess transfection efficiency and normalise luciferase readings. The reported values are averages of at least three independent experiments, with each condition performed in triplicate.

Whole Cell Binding Assays

COS-7 cells were maintained as above and seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at 5×10^4 cells per well and grown in phenol red free DMEM supplemented with dextran-coated charcoal-stripped serum (5%). On day 2, cells were transfected (FuGENE6 transfection reagent) with 0.75 μg pSVARo expression vector and 0.15 μg pCMV- β -galactosidase expression vector. On day 4, the cells were incubated for 90 min at 37°C with 1nM [^3H]mibolerone in the absence and presence of an increasing concentration of unlabelled mibolerone or CpdA. Working on ice, cells were washed three times with ice-cold 1 X PBS for 15 min. Cells were then lysed with 200 μl 1 X passive lysis buffer (Promega). Lysates were briefly centrifuged to remove cellular debris and total binding was determined for 150 μl of cellular extract by scintillation counting. Non-specific binding was determined as the counts obtained when cells were incubated with 1 nM [^3H]mibolerone in the presence of 10 000 X unlabelled mibolerone. Specific bound mibolerone was calculated as the difference between total and non-specific binding. β -Galactosidase activity of 5 μl cellular extract was determined as described above. The reported values are averages of three independent assays, with each condition performed in triplicate. Data were plotted as the percentage [^3H]mibolerone specifically bound and normalised by β -galactosidase activity.

Western Blots

LNCaP cells (obtained from the American Type Culture Collection, Rockville, Md.) were maintained in RPMI medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μl /ml). Cells were seeded into 6-well tissue culture plates at 2.5×10^5 cells per well. On day 2, cells were incubated with the indicated test compounds at 37°C for 48 hours. On day 4, proteins were extracted by scraping cells off in ice-cold 1 X PBS, pelleting cells by centrifugation at 1400 rpm, and dissolving the cellular pellet in 100 μl

SB-DTT (110 mM SDS, 110 mM DTT, 80 mM Tris pH 6.9 and 10% glycerol). Protein concentrations were determined using BCA protein assay reagent (Pierce). An aliquot (3,3 μ g) of the total protein extract was separated on a SDS-10%-polyacrylamide gel and blotted onto a nitrocellulose membrane, Hybond ECL (Amersham Life Science). Membranes were incubated with the relevant primary rabbit anti-bodies, followed by incubation with goat-anti-rabbit (HRP) anti-bodies as secondary anti-body. Expression of the relevant proteins was determined using the Western Blot chemiluminescence reagent (NEN, Life science products).

Data manipulation and statistical analysis

The Graph Pad Prism[®] programme was used for data manipulations, graphical representations, and statistical analysis. One-way ANOVA and Dunnet's multiple comparison's test (post-test) were used for statistical analysis. P-values are represented as follows: $P < 0.001$ by ***; $P < 0.01$ by ** and $P < 0.05$ by *. Non-linear regression and one site competition were used in whole cell binding assays. For all experiments, the error bars represent the SEM of three independent experiments, each performed in triplicate.

Results

Compound A represses ligand-induced transcriptional activation of both specific and non-specific androgen regulatory DNA regions

The ability of CpdA to repress ligand-induced activation of specific and non-specific androgen responsive reporter genes was determined in CV1 cells transiently transfected with the corresponding reporter gene and a full-length human androgen receptor (hAR) expression vector. The transfected cultures were subsequently exposed to the synthetic, non-metabolizable androgen, R1881, in the absence and presence of increasing amounts of CpdA.

Two reporters driven by non-specific androgen regulatory regions were used in these experiments: pMTVluc and pC3(1)-TATA-luc. It has previously been shown that both of these reporter constructs are induced by androgens as well as glucocorticoids (Verrijdt *et al.*, 2000; Claessens *et al.*, 1993; Cato *et al.*, 1987; Ham *et al.*, 1988). In figure 2 we demonstrate that transcriptional activation of these reporter constructs by 1nM R1881 is significantly repressed by 10 μ M CpdA.

In order to test for androgen specificity, we examined the effect of CpdA on ligand-induced activation of pSLP-TATA-luc, pSC-TATA-luc and pPB-luc, three reporter constructs that are driven by androgen-specific regulatory DNA regions (Verrijdt *et al.*, 2000). In figure 2 we show that induction of these reporter constructs by 1 nM R1881 is significantly repressed by 10 μ M CpdA. The results show that repression of androgen-induced activation by 10 μ M CpdA is similar for androgen specific and non-specific reporters. At 1 μ M, however, CpdA only significantly repressed activation of the pSLP-TATA-Luc reporter construct.

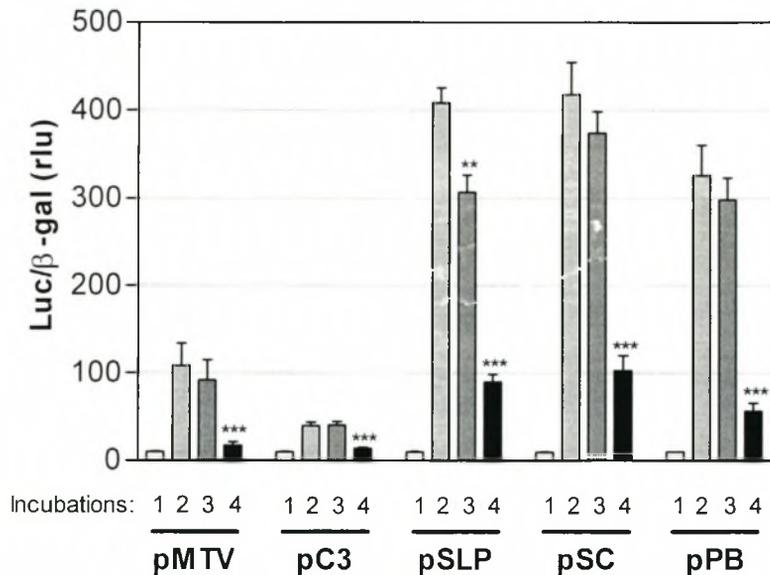


Figure 2: CpdA (10 μ M) represses R1881-induced (1 nM) activation of both non-specific (pMTV and pC3) and specific (pSLP, pSC and pPB) androgen regulatory regions by \pm 70 – 80%.

CV1 cells were transiently transfected with the respective reporter, the human AR (pSVARo) and pCMV- β -galactosidase expression vectors. Subsequently, the cells were incubated for 24 hrs with: (1) no hormone, (2) 1 nM R1881, (3) 1 nM R1881 plus 1 μ M CpdA, and (4) 1 nM R1881 plus 10 μ M CpdA. Reporters used: (i) pMTV – a mouse mammary tumour virus promoter; (ii) pC3 – promoter of component C3 of prostate (steroid-) binding protein; (iii) pSLP – liver-specific sex-limited protein promoter; (iv) pSC – secretory component promoter; and (v) pPB – rat probasin promoter. Induction is expressed in relative light units (rlu). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

The ability of Compound A to repress ligand-induced transcriptional activation is not receptor specific

To determine whether or not the repressive action of CpdA was specific for the androgen receptor, COS-7 cells were transiently transfected with a GRE-driven reporter construct and the relevant human, steroid receptor expression vectors. Again, it is demonstrated that 10 μ M CpdA significantly represses R1881-induced activation via the AR (figure 3A). We also show that CpdA does not repress dexamethasone- or aldosterone-induced transcriptional activation via the GR and MR, respectively (figure 3B and 3C). In figure 3C there appears to be an increase in reporter activity when the cells were incubated with both aldosterone and CpdA. However, statistical analysis of these results indicates that the observed increase is not significant. On the other hand, 10 μ M CpdA significantly represses progesterone-induced transcriptional activation via the PR, although to a lesser extent than via the AR (figure 3D).

Compound A does not compete for ligand binding to the human androgen receptor

In a competitive whole cell binding assay where COS-7 cells were transfected with a full-length hAR expression vector, we show that CpdA up to a concentration of 10 μ M is unable to fully compete with [3 H]mibolerone for binding to the ligand binding domain of the hAR (figure 4). Surprisingly, there appears to be a maximal 10-20% decrease in specific binding of [3 H]mibolerone in the presence of as little as 10 nM CpdA.

To determine whether the CpdA incubation conditions used in the transactivation assays resulted in any change in the binding properties of CpdA, the ability of [3 H]mibolerone to bind the hAR after incubation of the cells with CpdA for 24 hours was examined by the whole cell binding assay. It was found that under these conditions, CpdA (or a potential metabolite thereof) still does not compete with [3 H]mibolerone for binding to the hAR (data

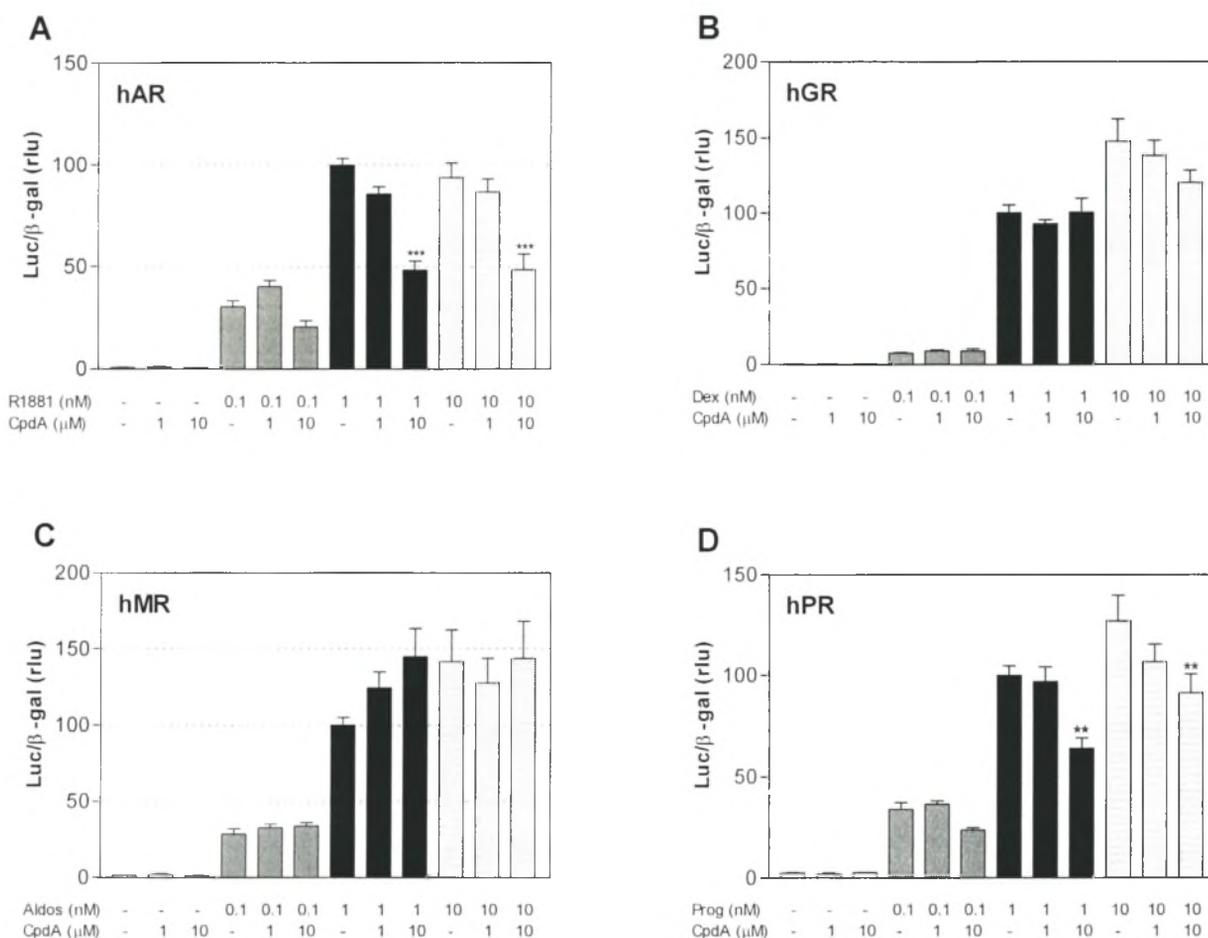


Figure 3: CpdA (10 μ M) represses ligand-induced activation of a GRE-driven reporter construct via the androgen and progesterone receptors by about 50% and 30% respectively, but has no effect on ligand-induced activation via the glucocorticoid and mineralocorticoid receptors.

COS-7 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter, the relevant full-length human steroid receptor and the pCMV- β -galactosidase expression vectors. Subsequently, the cells were exposed to the cognate ligand in the absence and presence of increasing concentrations of CpdA for 24 hrs. Steroid receptors and ligands used: (A) the human androgen receptor with R1881 as ligand; (B) the human glucocorticoid receptor with dexamethasone (Dex) as ligand; (C) the human mineralocorticoid receptor with aldosterone (Aldos) as ligand; and (D) the human progesterone receptor with progesterone (Prog) as ligand. Induction is expressed in relative light units (rlu). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

not shown). Furthermore, we have found that in a cell-free system, using whole-cell extracts from COS-7 cells transfected with a hAR expression vector, CpdA does not compete for binding to the LBD of the AR (data not shown). We therefore conclude that CpdA exerts its effects by a mechanism that does not involve competitive binding for the LBD of the AR.

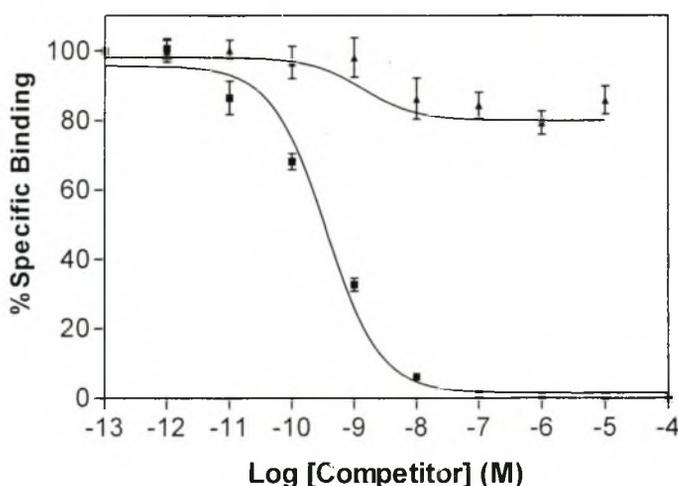


Figure 4: CpdA does not compete with ^3H mibolerone for binding to the human androgen receptor.

COS-7 cells were transiently transfected with the pSVARo and pCMV- β -galactosidase expression vectors. Forty-eight hours later they were incubated with 1 nM ^3H mibolerone in the absence and presence of increasing concentrations of either mibolerone (■) or CpdA (▲) for 90 minutes. Competition for binding is illustrated by the percent of ^3H mibolerone specifically bound to the hAR (normalised to β -galactosidase expression). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

Compound A interferes with the interaction between the NH₂-terminal domain and ligand binding domain of the human androgen receptor

The functional, *in vivo*, ligand-dependent interaction between the NTD and LBD of the AR has previously been demonstrated in both yeast and mammalian cells (Langley *et al.*, 1995; Doesburg *et al.*, 1997). Here we used a mammalian two-hybrid assay to determine whether CpdA interferes with the N/C-interaction of the hAR. In transient transfections, COS-7 cells were cotransfected with a GRE-driven reporter construct, an expression vector encoding the DBD and LBD of the hAR, as well as an expression vector encoding the NTD of the hAR fused to a VP16 activation domain. In this experiment (figure 5A) we show that CpdA on its own is not able to induce transcription and therefore we can conclude that CpdA does not induce the N/C-interaction of the hAR. This result would be expected, and is consistent with the fact that CpdA does not exhibit androgenic characteristics or compete with mibolerone for binding to the hAR. We also show that 0,1 μ M DHT induces the N/C-interaction and that this effect is partially repressed by 1 μ M CpdA and almost completely repressed by 10 μ M CpdA. These results indicate that CpdA prevents the AR from adopting the necessary stable conformation that is required to render the receptor transcriptionally active, even in the presence of androgen. This anti-androgenic characteristic has been previously reported for anti-androgens such as hydroxyflutamide (Langley *et al.*, 1995; Kemppainen *et al.*, 1999) and cyproterone acetate (Kemppainen *et al.*, 1999), however, the difference being that these anti-androgenic compounds bind competitively to the LBD of the AR.

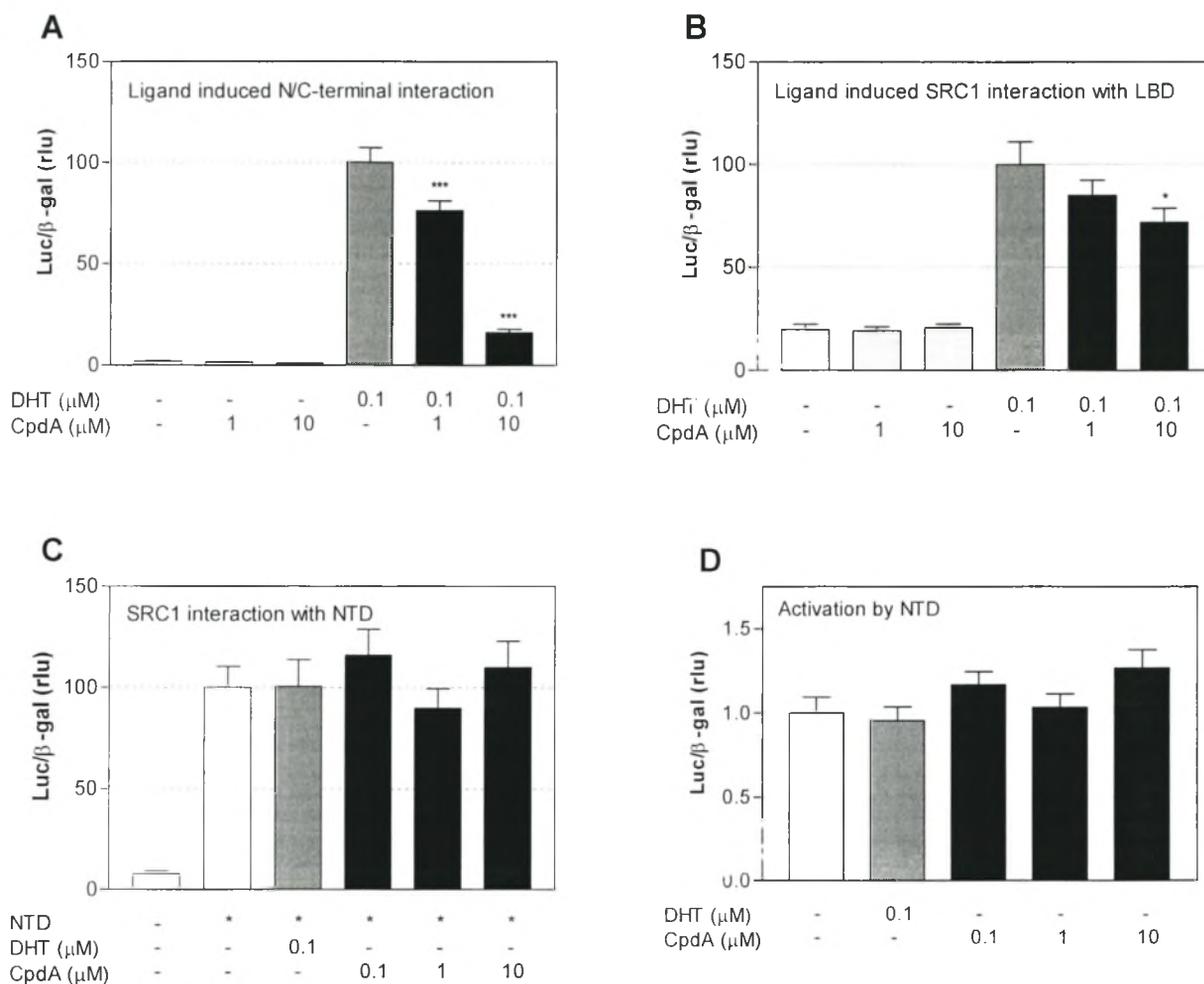


Figure 5: CpdA interferes with the N/C-interaction of the hAR, it also interferes slightly but significantly with the interaction between SRC1 and the AF2 domain of the hAR but not with the interaction between SRC1 and the AF1 domain of the hAR nor the transactivating potential of the hAR NTD.

COS-7 cells were transiently transfected with: (A) the pTAT-GRE-E1b-luc reporter, the pSG5-hAR(DBD-LBD), the pSNATCH-II(hAR-NTD) and the pCMV- β -galactosidase expression vectors; (B) the pTAT-GRE-E1b-luc reporter, the pSG5-hAR(DBD-LBD), the pSG5-SRC1 and the pCMV- β -galactosidase expression vectors; (C) the p(Gal4)₅-tata-luc reporter, the pGal4DBD-SRC1(989-1240), the pSG5-NTD and the pCMV- β -galactosidase expression vectors; and (D) the p(Gal4)₅-tata-luc reporter, the pAB-Gal4-NTD and the pCMV- β -galactosidase expression vectors. Subsequently, the cells were exposed to the indicated test compounds for 24 hrs. Induction is expressed in relative light units (rlu). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

Compound A impairs SRC1 activation of the AF2 domain, but not the AF1 domain, of the androgen receptor

To further elucidate the mechanism by which CpdA represses androgen-induced transcriptional activation via the hAR, transient transfections were carried out to determine whether or not CpdA interferes with the recruitment of the p160 co-activator, SRC1, to the hAR. Firstly, we examined the effect of CpdA on the ligand-dependent recruitment of SRC1 to the AF2 domain. The ability of the AR LBD to interact with SRC1 in a ligand-dependent manner has previously been demonstrated, in a mammalian (Alen *et al.*, 1999) and yeast (Bevan *et al.*, 1999) two-hybrid system. In these experiments, the mammalian COS-7 cell-line was cotransfected with the GRE-driven reporter construct, the vector expressing the DBD-LBD of the hAR, and a SRC1 expression vector. In figure 5B we demonstrate that 10 μ M CpdA weakly but significantly ($P < 0.05$) interferes with the DHT-induced activation of the AF2 domain of the hAR by SRC1.

Secondly, it has also been shown that SRC1 interacts with the AF1 domain of the AR, in both a mammalian (Alen *et al.*, 1999) and yeast (Bevan *et al.*, 1999) system. In both these cases, this interaction was demonstrated to be ligand-independent. To investigate the effect of CpdA on this interaction, we cotransfected COS-7 cells with a reporter gene driven by five Gal4 response elements, the Gal4-DBD and SRC1 fusion protein and hAR NTD expression vectors. In figure 5C (bars 1 and 2) we show that the interaction between SRC1 and the NTD is sufficient to induce a transcriptionally active complex in the absence of ligand, as has been previously reported (Bevan *et al.*, 1999). We also show that CpdA, up to a concentration of 10 μ M, does not interfere with the interaction between SRC1 and the NTD (figure 5C; bars 3-6).

In a similar experiment, COS-7 cells were cotransfected with the Gal4 reporter and a Gal4-DBD plus hAR NTD fusion protein expression vector. The recruitment of basal transcription machinery to the activation domain of the hAR NTD allows this fusion protein

to activate transcription at the Gal4 response element (Alen *et al.*, 1999). Incubations with varying concentrations of CpdA had no effect on transcription. This result indicates that CpdA does not interact with the AF1 activation domain in any way that would interfere with the establishment of a transcriptionally competent complex (figure 5D).

Compound A represses basal expression, and inhibits androgen-induced expression, of the prostate specific antigen protein in LNCaP cells

Having shown that CpdA can repress androgen-induced transcriptional activation of transiently transfected reporter constructs via the AR, we were prompted to study the effect of CpdA on the expression of androgen-dependent, endogenously expressed proteins. For this experiment we made use of the androgen-dependent, lymph node carcinoma of the prostate (LNCaP) cell-line. We looked at the effect of CpdA on the expression of the prostate specific antigen (PSA) protein. PSA, is a clinically important androgen-stimulated gene that is used to monitor treatment responses, prognosis and progression of prostate cancer (reviewed by Polascik *et al.*, 1999).

In figure 6A the androgen-dependence of PSA expression is illustrated clearly by the effects of the incubation with both R1881 and hydroxyflutamide (which functions as an androgen in this cell-line, Veldscholte *et al.*, 1992). We also show that 10 μ M CpdA does not induce PSA expression but rather represses even basal expression levels of the protein. When the cells are incubated with both R1881 and CpdA, there is an apparent repression of the androgen-induced expression of PSA by CpdA. These effects of CpdA on PSA protein expression are consistent with the anti-androgenic actions of CpdA observed in the transfection experiments. Incubation of the androgen-dependent LNCaP cell-line with CpdA appeared to influence the viability of the cells and thus we performed an MTT-based proliferation assay. Results from these experiments indicated that both CpdA on its own and

in the presence of androgen significantly suppressed proliferation of and/or was toxic for this cell-line (data not shown), providing additional evidence of the anti-androgenic activity of CpdA. On the other hand, when this assay was performed on the COS-7 cell-line we found that CpdA had no effect on cell viability (data not shown). Thus it is possible that the anti-androgenic effects of CpdA seen in the transfection experiments may be due to the anti-androgenic capacity of CpdA.

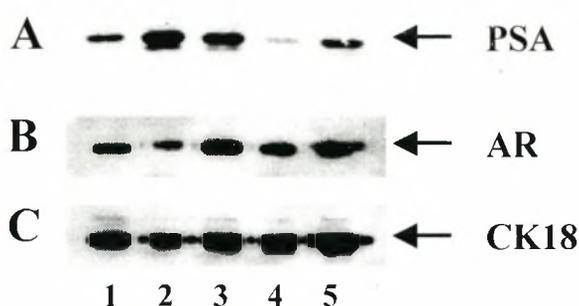


Figure 6: CpdA (10 μ M) abolishes even basal expression levels of PSA (prostate specific antigen) protein.

LNCaP cells were incubated with the indicated test compounds for 48 hrs and extracts made. Total protein (3,3 μ g) was separated on a SDS-10%-polyacrylamide gel followed by Western blotting (described in Material and Methods). (A) Rabbit-anti-PSA, (B) rabbit-anti-AR and (C) rabbit-anti-CK18 primary anti-bodies were used. Test compounds used: (1) no hormone, (2) 1 nM R1881, (3) 1 μ M hydroxyflutamide, (4) 10 μ M CpdA, and (5) 1 nM R1881 plus 10 μ M CpdA.

Figure 6B illustrates the effects that these compounds have on AR protein levels. After a 24 hour incubation period, R1881 appears to repress the AR protein levels which is consistent with reports in the literature (Yeap *et al*, 1999). Hydroxyflutamide, CpdA and CpdA together with R1881, do not seem to influence AR protein expression levels. Although CpdA itself does not regulate AR protein levels, it does appear to inhibit the repressive effect of R1881, once again illustrating the anti-androgenic properties of this compound. Figure 6C

is a control blot showing the expression of cytokeratin 18 (CK18), a constitutively expressed protein.

Discussion

There are a number of diseases that have an androgen-dependent etiology, and/or show an undesirable response to circulating androgens. Anti-androgens are used clinically for the treatment of such diseases because of their ability to inhibit androgen action. Conventional anti-androgenic compounds compete with androgens for occupancy of the AR, without eliciting androgen activity. In this investigation we describe a compound that displays anti-androgenic potential but does not compete with canonical ligands for occupancy of the AR.

The anti-androgenic action of CpdA was first noted when we found that CpdA was able to repress androgen-induced transcriptional activation of enhancer regions specifically regulated by androgens (figure 2A). This capability of CpdA to regulate anti-androgenic actions at the molecular level was later confirmed by its ability to repress the expression of the androgen-dependent PSA protein (figure 6A). We have also found that regardless of which AR agonist is used (R1881, mibolerone or DHT), CpdA represses agonist-induced transcriptional activation of a GRE-driven reporter via the AR to a similar extent (data not shown). Together, these results clearly indicate that CpdA has the ability to interfere with the action of liganded AR. However, the ability of CpdA to repress ligand-induced transcriptional activation via a steroid receptor is not specific for the AR, as CpdA is also capable of repressing PR activity to some extent (figure 3D). On the other hand, there is no indication that CpdA interferes with activation of transcription via the GR or MR (figure 3B and 3C).

In a whole cell binding assay, we show that CpdA is unable to compete effectively with [³H]mibolerone for binding to the AR (figure 4). Thus, in the presence of CpdA, the ligand remains bound to the AR. In light of this unique property, attempts to define the mechanism(s) of action of CpdA via the AR led to the finding that CpdA, like anti-androgens such as hydroxyflutamide and cyproterone acetate (Kemppainen *et al.*, 1999), is unable to induce the interaction between the NH₂-terminal and COOH-terminal domains of the AR (figure 5A). However, CpdA does inhibit the N/C-interaction that is normally induced when the ligand is bound to the receptor, most likely resulting in its anti-androgenic actions. Taken together, our results could thus be explained by the following hypothesis. In the presence of ligand, CpdA interacts with a region of the AR that inhibits the N/C-interaction, resulting in a ligand-receptor complex with an altered, non-optimal conformation that impairs the transactivating capacity of the AR.

Experiments were thus carried out to identify the region(s) of the AR with which CpdA interacts. Previous studies have shown that it is the two LXXLL-like motifs in the NTD (He *et al.*, 2000) and the AF2 domain of the LBD (Berrevoets *et al.*, 1998; He *et al.*, 1999; Alen *et al.*, 2000) that are the subdomains involved in the N/C-interaction. The inhibitory effect of CpdA on the N/C-interaction is therefore indicative of a potential interaction between CpdA and one or more of these subdomains.

Firstly, because the repressive effect of CpdA on the N/C-interaction suggests that CpdA may impair the ligand-induced activation of the AF2 domain, we investigated the effect of CpdA on a second interaction that relies on the ligand-induced activation of this domain. For this reason, we made use of the assay that demonstrates the androgen-induced interaction between the p160 co-activator, SRC1, and the AF2 domain. If CpdA were to interfere with

the activation of the AF2 domain, we would expect it to inhibit the interaction between SRC1 and this domain. Our results show that the ability of CpdA to repress the interaction between SRC1 and the AF2 domain (figure 5B) is not of the same magnitude as its ability to repress the N/C-interaction and we therefore suggest that it is unlikely that CpdA exerts its anti-androgenic activities by a mechanism that involves the absolute inhibition of AF2 domain activity. However, although the regions of the AF2 domain involved in the N/C-interaction and the interaction with SRC1 overlap, they are not identical. It is therefore possible that CpdA may interact with or even modify amino acid residues that are essential for the N/C-interaction but not essential for the interaction between SRC1 and the AF2 domain.

Secondly, we investigated the possibility of CpdA interfering with one of the known functions of the NTD of the AR. In these experiments we looked at the effect of CpdA on the interaction between SRC1 and the AF1 domain of the NTD. A direct, ligand-independent interaction between SRC1 and the AF1 domain has been established (Bevan *et al.*, 1999). In our experiments we found that CpdA had no effect on this interaction (figure 5C) and it is therefore unlikely that CpdA interacts with the sites of the AF1 domain through which regions of SRC1 interact. This result, however, does not rule out the possibility that CpdA interacts with a region(s) of the NTD other than the AF1 domain. For example, it is possible that CpdA could interact with the LXXLL-like motifs of the NTD. However, no conclusion on this matter can be drawn from our data. To summarise, we show that it is unlikely that CpdA exerts its effects by interactions via the AF2 domain in the LBD or the AF1 domain in the NTD. On the other hand, our results do not exclude the possibility that CpdA may interact with other regions in the LBD or the NTD.

Although it is not possible to conclude from our data whether CpdA interacts directly or indirectly with the AR, and what the exact nature of this interaction(s) may be, one possibility is that CpdA, a hydroxy-phenylaziridine precursor, could be cyclized to the aziridine. The aziridines are highly reactive alkylating agents that can react with nucleophilic amino acid residues. Thus, incubations with CpdA could result in the modification of nucleophilic amino acid residues of the AR that are essential for the N/C-interaction

Taken together, our results indicate that the anti-androgenic actions of CpdA are mediated via the AR by a mechanism other than competitive binding for the same site as androgen agonists. More research towards elucidating these new mechanism(s), by which CpdA and other anti-androgenic compounds elicit their anti-androgenic functions, may be helpful in unraveling the molecular mechanisms of action of the AR in normal and pathological conditions like prostate cancer.

Acknowledgements

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2.2 Data not shown / Comments and suggestions

Compound A exerts similar effects in whole cell binding experiments, regardless of the length of the incubation time.

In transactivation assays the cells were incubated in the presence of CpdA for 24 hours, whereas in whole cell binding assays the cells are only incubated with CpdA for 1,5 hours. CpdA has been shown to cyclize to the corresponding aziridine, acetoxypheyl methylaziridine, at physiological pH. Thus the effects seen in these two experiments could be brought about by different active species of CpdA that may be active under the specific conditions (e.g. CpdA itself or the aziridine). For this reason, a whole cell binding assay was performed where COS-7 cells, transfected with a full-length hAR expression vector, were incubated for either 24 or 1,5 hours with CpdA, prior to incubation with [³H]mibolerone.

This experiment demonstrates that, regardless of the length of time that the cells are incubated with CpdA (e.g. 24 hours as in the transfection experiments or 1.5 hours as in the whole cell binding experiments), neither CpdA nor other potential species thereof are able to compete with [³H]mibolerone for binding to the hAR (figure I). It is therefore accepted that the inability to compete for binding to the AR as well as the repression of androgen-induced transcriptional activation are actions of the same species of CpdA. In conclusion, CpdA (or the active species thereof) does not exert its effect by competing for binding to the AR.

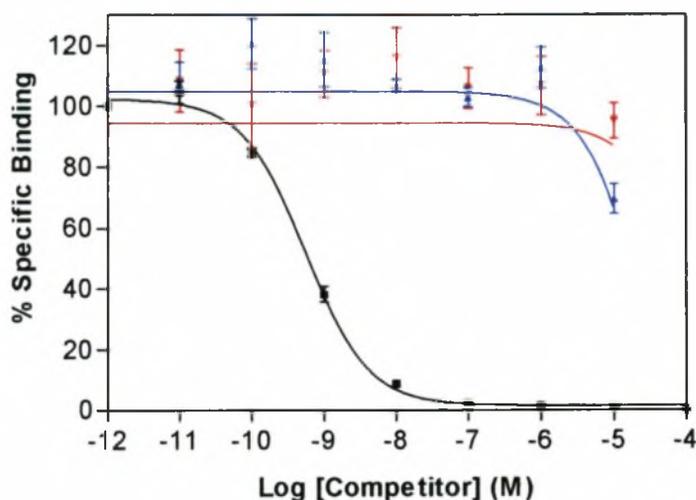


Figure (I): Regardless of the duration of the exposure of the cells to Cpda, androgen is still able to bind to the receptor.

COS-7 cells were transiently transfected with the pSVARo and pCMV- β -galactosidase expression vectors. Twenty-four hours later cells were exposed to one of the following conditions. Firstly (■), medium was changed and 24 hours later cells were incubated with 1 nM [3 H]mibolerone in the presence of increasing concentrations of unlabelled mibolerone for 90 minutes (*black*). Secondly (▲), medium was changed and 22,5 hours later cells were exposed to increasing concentrations of Cpda for 90 minutes, followed by an incubation with 1 nM [3 H]mibolerone for 90 minutes (*blue*). Lastly (▼), the cells were incubated with increasing concentrations of Cpda for 24 hours, followed by an incubation with 1 nM [3 H]mibolerone for 90 minutes (*red*). Competition for binding is illustrated by the percent of [3 H]mibolerone specifically bound to the hAR (normalised to β -galactosidase expression). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

Compound A suppresses the proliferation of the androgen-dependent LNCaP cell-line, but not that of the COS-7 cell-line.

Upon incubation of LNCaP cells with CpdA (Western blot experiments), it was noted that CpdA has an effect on the viability of the cell-line. After an incubation period of 48 hours a number of the cells had become rounded and detached from the tissue-culture plates. It was thus decided to investigate the potential of CpdA to influence the proliferation/viability of these cells under these culturing conditions. Here an MTT-based proliferation assay was used. The results obtained indicate that CpdA in the absence and presence of androgen can significantly suppress the proliferation/viability of these cells. This action of CpdA is consistent with its ability to exert anti-androgenic effects. To determine whether the repressive actions of CpdA observed in experiments using the COS-7 cell-line were not a result of anti-proliferative effects or decreased cell viability induced by CpdA, the assay was also performed for this cell-line, which lacks endogenous AR. It was found that CpdA did not influence the proliferation/viability of this cell-line, an indication that the anti-androgenic effects of CpdA seen in the transfection experiments are attributed to the anti-androgenic capacity of CpdA.

It is of importance to note that the MTT assay used in these studies is based on the reduction of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial enzymes associated with metabolic activity. Reduction of MTT thus reflects both cell viability and cell proliferation. The results obtained in the study with CpdA therefore indicate a decrease in the number of viable cells upon treatment with CpdA. To discriminate whether this is a result of less proliferation or increased toxicity, assays using tritiated

thymidine incorporation or trypan blue exclusion, respectively, would have to be performed.

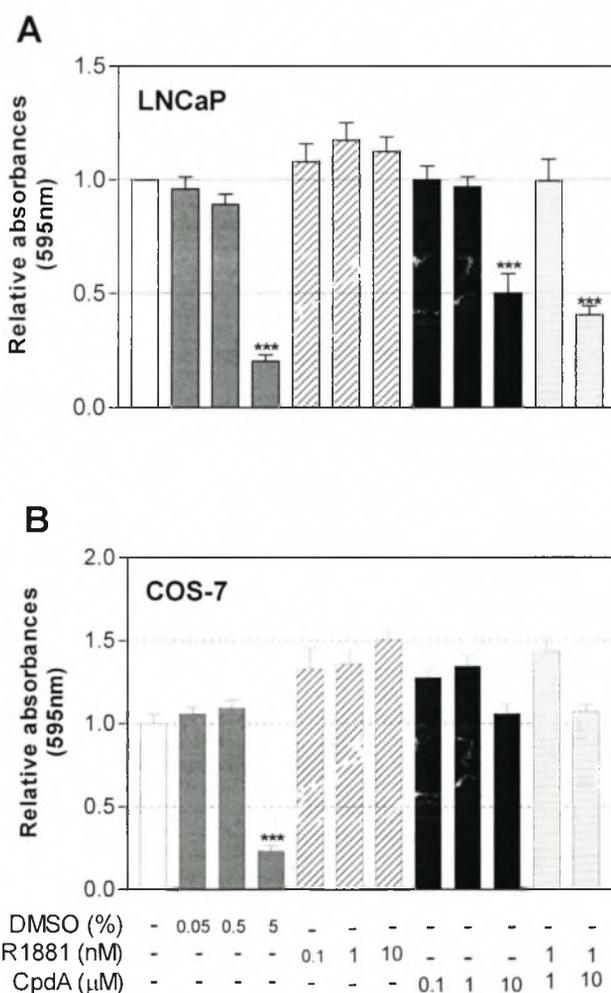


Figure (II): CpdA (10 μM) displays anti-proliferative and/or cytotoxic activity in the LNCaP cell-line, but not the COS-7 cell-line.

The (A) lymph node carcinoma of the prostate (LNCaP) cells, and (B) monkey kidney (COS-7) cells were seeded into 24-well and 96-well tissue culture plates at 5×10^5 and 1×10^4 cells per well, respectively. Cells were then incubated with the indicated test compounds for 24 hrs. Subsequently, MTT (5 mg/ml) was added as one tenth of the volume per well and incubated for 3 hrs at 37 °C. Medium was aspirated, crystals dissolved in DMSO and OD₅₉₅ measured. Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

Compound A represses, to a similar extent, the transcriptional activation induced by different androgens.

Since the whole cell binding assays were performed with mibolerone as reference androgen, and other experiments (transfections and Western blots) were carried out with either R1881 or DHT as reference androgen, it was difficult to directly compare the anti-androgenic effects of CpdA in the various experiments. For this reason the ability of CpdA to repress androgen-induced transcriptional activation via the full-length AR was directly compared for the different androgens used (DHT, R1881 and mibolerone). In figure (III) it is shown that CpdA represses, to a similar extent, the DHT-, R1881-, and mibolerone-induced transcription of the GRE-TAT reporter construct.

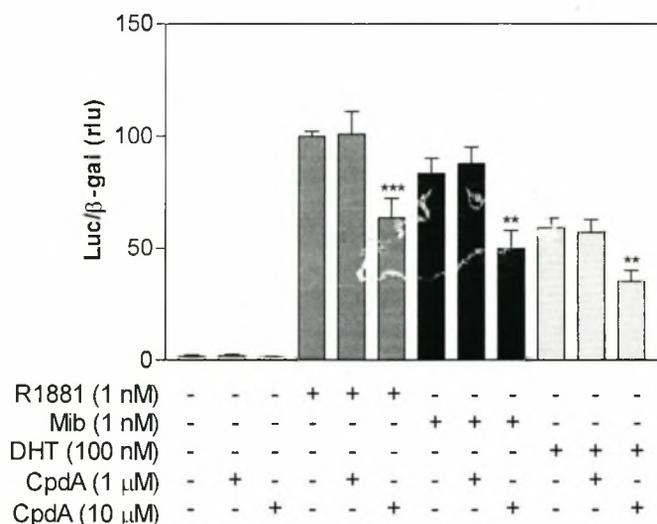


Figure (III): The degree to which CpdA represses DHT-, R1881-, and mibolerone-induced transcriptional activation is similar.

COS-7 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter, and the pSVARo and pCMV-β-galactosidase expression vectors. Subsequently, the cells were exposed to DHT, R1881 or mibolerone (at the indicated concentrations) in the absence and presence of increasing concentrations of CpdA for 24 hours. Induction is expressed in relative light units (rlu). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

Additional comments/suggestions

There are a few parameters that could still be measured to confirm or validate the data presented in this study with CpdA. Firstly, the repressive effects of CpdA on transcriptional activation could be compared with those of a known androgen antagonist like hydroxyflutamide. Secondly, the repressive effects of CpdA seen on the N/C-interaction as well as the recruitment of SRC1 may reflect an effect of CpdA on an event occurring downstream of the actual interaction, as these assays both measure the downstream response of these interactions. Therefore, it is suggested that GST pull-down assays be performed to determine whether the effect of CpdA on these two interactions is in fact direct or indirect.

**A comparison of the androgenic properties of the synthetic
progestins, medroxyprogesterone acetate and
norethindrone acetate**

T.M. Tanner¹, W. Rombauts², A. Louw¹, F. Claessens² and J.P. Hapgood¹

¹Department of Biochemistry, University of Stellenbosch, 7600 Stellenbosch, South Africa. ²Division of Biochemistry, Campus Gasthuisberg, Catholic University of Leuven, 3000 Leuven, Belgium.

3.1 Manuscript

Summary

The aim of the current study was to directly compare the relative activities and mechanisms involved in the androgenic actions of the synthetic progestins, medroxyprogesterone acetate (MPA) and norethindrone acetate (NET-A). These compounds and/or their metabolites exert contraceptive actions and have been shown to interact with a number of the steroid receptors. It is shown that MPA and NET-A both elicit partial agonist activity via the androgen receptor. Furthermore, it is demonstrated that these progestins have a similar relative binding affinity for the androgen receptor when compared to the androgen, mibolerone. NET-A, like well-characterised androgens, induces the ligand-dependent interaction between the NH₂- and COOH-terminal domains. Contrary to this, MPA not only fails to induce but also represses this androgen-induced interaction. However, both MPA and NET-A promote the interaction of steroid receptor co-activator 1 (SRC1) with the AF2 domain of the AR. These results thus demonstrate that MPA and NET-A exert their androgenic actions by different mechanisms.

Introduction

In many parts of the world, long-acting contraceptives constitute an important option in family planning services. For more than 20 years two long-acting injectable contraceptives, containing only a synthetic progestin, have been in clinical use. These are

depot-medroxyprogesterone acetate (DMPA) and norethindrone enanthate (NET-EN; frequently referred to as norethisterone enantate) (Garza-Flores *et al.*, 1991). DMPA is a 150 mg aqueous suspension administered every three months, whereas NET-EN is a 200 mg oily suspension administered every two months. Both formulations are administered as intramuscular injections. There are apparent differences with respect to the stability and metabolism of the two contraceptive agents. Medroxyprogesterone acetate (MPA) is relatively stable and is itself the active contraceptive compound. On the other hand, NET-EN is rapidly hydrolysed to norethindrone (NET) and its metabolites (Stanczyk and Roy, 1990) many of which, together with NET, exert the contraceptive action. Contraceptive doses of DMPA and NET-EN result in serum concentrations of about 2.6 nM of MPA (Mishell, 1996) and 1.5 – 59 nM of NET (Fotherby *et al.*, 1983). These agents are, however, also used in therapy for the treatment of cancer or as replacement hormones, and are then administered at much higher doses. The contraceptive mechanism of MPA action involves the prevention of ovulation by abolishing peak mid-cycle gonadotropin levels (reviewed by Mishell, 1996 and Kaunitz, 1998). NET-EN has also been shown to block ovulation (Bhowmik and Mukherjea, 1988). However, the primary contraceptive action of NET-EN involves changing the content of cervical mucous to a progestogenic phase that prevents sperm penetration (Bhowmik and Mukherjea, 1987). The mechanisms of action of MPA and NET have been assumed to be elicited by their ability to interact with the progesterone receptor (PR), as both compounds are progestins. However, it has been demonstrated that MPA has high affinity and is an agonist for the PR, glucocorticoid receptor (GR), and androgen receptor (AR) (Teulings *et al.*, 1980; Bentel *et al.*, 1999; Kempainen *et al.*, 1999; Bojar *et al.*, 1979; Bergink *et al.*, 1983; Feil and Bardin, 1979). Such activity for NET has only been demonstrated for the PR and AR (Decker *et al.*, 2000).

The AR, GR and PR, together with the estrogen and mineralocorticoid receptors, constitute the steroid receptor family. They are ligand-dependent transcription factors that display a high level of similarity with regards to their structure and mechanism of action. The structural arrangement of the different domains of these receptors involves the highly variable NH₂-terminal domain (NTD), followed by the highly conserved and centrally located DNA-binding domain (DBD), which directly interacts with the DNA response elements. The DBD is linked to the COOH-terminal (C-terminal) domain by the hinge region. The C-terminal domain contains the ligand binding domain (LBD) to which the ligand, a steroid hormone, binds. Furthermore, these receptors also possess transcriptional activation functions located in the NTD (AF1 domain) and the LBD (AF2 domain). Once bound by steroid 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).

For the AR an interaction between the NTD and LBD (N/C-interaction), that is dependent on ligand binding, has been demonstrated (Langley *et al.*, 1995; Doesburg *et al.*, 1997), and found to be essential for optimal AR function (Ikonen *et al.*, 1997). Studies to reveal the subdomains involved in this interaction implicate the AF2 domain in the LBD (Berrevoets *et al.*, 1998; He *et al.*, 1999), and two LXXLL-like motifs in the NTD (He *et al.*, 2000) as being important. These LXXLL-like motifs form, in part, the interface for the interaction of the NTD with the AF2 domain. Functional interactions between the transcriptional activation domains of the AR and the 160-kDa (p160) nuclear receptor co-activators have also been demonstrated (Alen *et al.*, 1999). These co-activators have distinct regions that interact with the AF1 and AF2 domains of the AR. It has been

demonstrated that the p160 co-activator, steroid receptor co-activator 1 (SRC1), directly interacts with the NTD (AF1 domain) of the AR via a glutamine-rich region that is conserved amongst the members of this family of co-activators (Bevan *et al.*, 1999). These co-activators also interact, in a ligand-dependent manner, with the LBD (AF2 domain) of steroid receptors via three highly conserved α -helical LXXLL motifs that are centrally located in the nuclear receptor interacting region (Heery *et al.*, 1997; Voegel *et al.*, 1998).

MPA and NET have both been shown to possess androgenic activity. However, little research has been done to directly compare their relative activities and the precise mechanisms involved. Furthermore, both contraceptive agents have a number of contraindications. Since these compounds are not specific for the PR, but also bind to other steroid receptors, they may exert side-effects via any of these receptors. This highlights the importance of understanding the interactions of these compounds with steroid receptors. In the present study we focus on the AR and compare the relative androgenic properties of MPA and norethindrone acetate (NET-A) with those of androgens (refer to figure 1 for structures). NET-A was used in these studies since, similarly to NET-EN, NET-A is rapidly hydrolysed to the parent compound, NET, and its metabolites (Stanczyk and Roy, 1990). We investigate the effects that these compounds have on binding to the AR, transcriptional activation via the AR, the N/C-interaction of the AR as well as the interaction of SRC1 with the AF2 domain of the AR, to directly compare the androgenicity of MPA and NET-A.

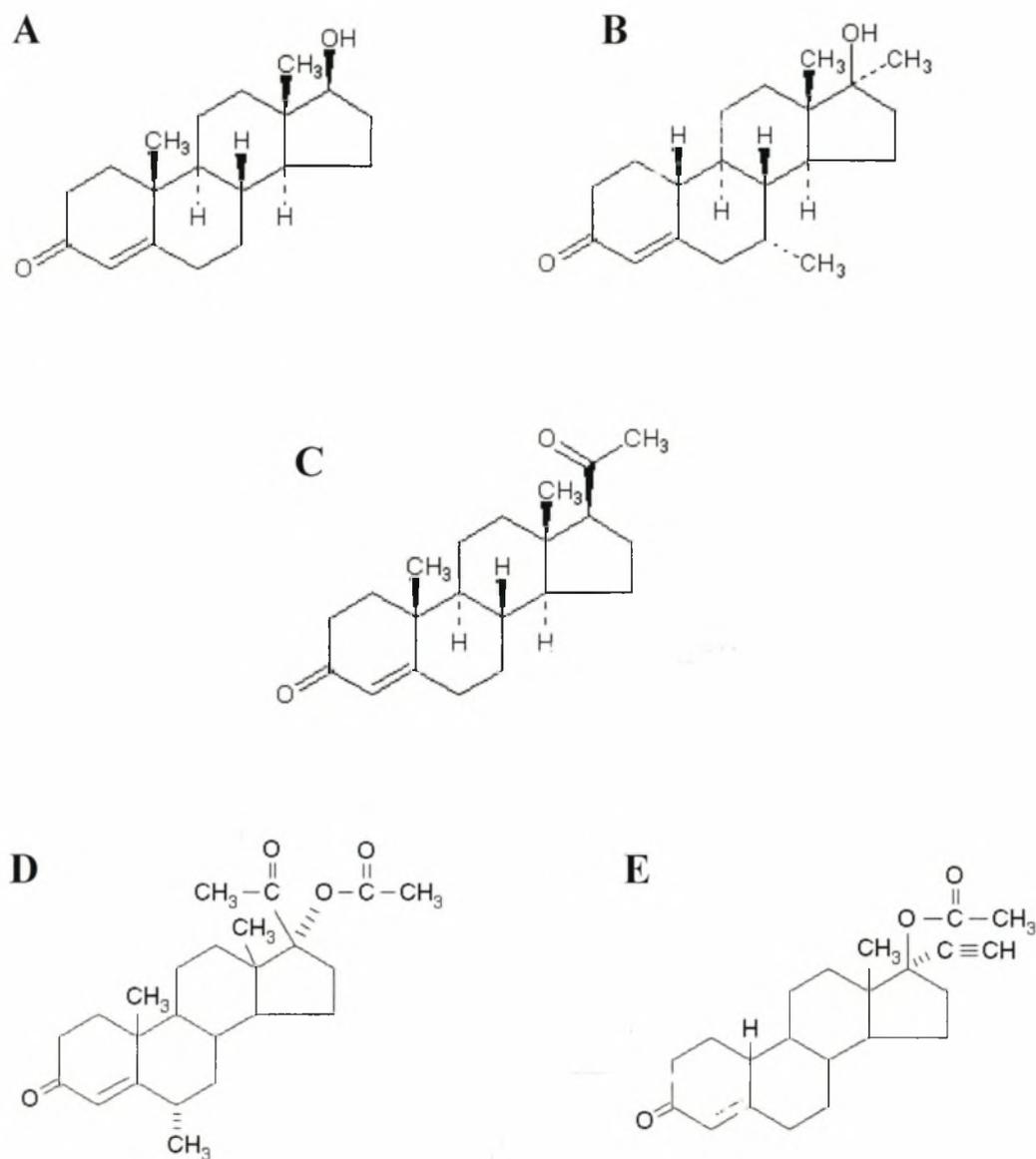


Figure 1: The chemical structures of natural and synthetic androgens and progestins.

The (A) natural androgen, *testosterone (T)*; (B) synthetic androgen, *mibolerone*; (C) natural progestin, *progesterone*; (D) synthetic progestin, *medroxyprogesterone acetate (MPA)*; and (E) synthetic progestin, *norethindrone acetate (NET-A)*.

Materials and methods

Plasmids

The plasmid pTAT-GRE-E1b-luc, was used as a reporter and is driven by the E1b promoter that contains two copies of the rat TAT-GRE (a gift from G. Jenster). A plasmid expressing the human androgen receptor, pSVAR α (Brinkmann *et al.*, 1989), was used. The hAR DBD-LBD expression vector, pSG5-hAR(DBD-LBD), as well as the hAR NTD-VP16 fusion protein expression vector, pSNATCH-II(hAR-NTD), were previously described in (Alen *et al.*, 1999). The expression vector for SRC1, pSG5-SRC1, was obtained from M.G. Parker.

Preparation of test compounds

5 α -androstane-17 β -ol-3-one (dihydrotestosterone; DHT), 6 α -methyl-17 α -hydroxyprogesterone acetate (medroxyprogesterone acetate; MPA), and 17 α -ethynyl-19-nortestosterone 17 β -acetate (norethindrone acetate; NET-A) were obtained from Sigma-Aldrich. Mibolerone was obtained from Dupont, NEN. All test compounds were dissolved in ethanol. These compounds were then added to the culturing medium such that the final concentration of ethanol did not exceed 0.1%. Control incubations (no test compounds) were performed in the presence of only 0.1% ethanol.

Transfections

Monkey kidney COS-7 cells (a gift from M.G. Parker) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum (Gibco-BRL Life Technologies), penicillin (100 IU/ml) and streptomycin (100 IU/ml) in a humidified 5% CO₂ incubator. For transfection experiments, cells were seeded

into 96-well tissue culture plates (Nunc) at 10^4 cells per well and grown in phenol red free DMEM supplemented with 5% (v/v) dextran-coated charcoal-stripped serum. On day 2, cells were transfected with FuGENE6 transfection reagent (Roche Molecular Biochemicals) in accordance with the manufacturer's instructions. The basic DNA mixture for transfections consisted of (per well): 100 ng of the appropriate luciferase reporter constructs, 10 ng of the appropriate expression vectors for either a human steroid receptor or relevant fusion proteins, and 10 ng of the cytomegalovirus (CMV)-driven- β -galactosidase expression vector (Stratagene). On day 3, media were replaced, either with or without the addition of the appropriate hormones. On day 4, cells were harvested by incubation for 10 min in 25 μ l of 1 X passive lysis buffer (Promega). A 2.5 μ l aliquot of cellular extract was used for the quantification of luciferase in a Luminoskan Ascent Luminometer with luciferase assay reagent (Promega) in accordance with the manufacturer's instructions. The β -galactosidase activity in a 2.5 μ l aliquot of extract from each sample was measured with the β -galactosidase chemiluminescent reporter gene assay system (Tropix Inc.). β -Galactosidase readings were used to assess transfection efficiency and normalise luciferase readings. The reported values are averages of at least three independent experiments, with each condition performed in triplicate.

Whole Cell Binding Assays

COS-7 cells were maintained as above and seeded into 24-well tissue culture plates (Nunc) at 5×10^4 cells per well and grown in phenol red free DMEM supplemented with dextran-coated charcoal-stripped serum (5%). On day 2, cells were transfected (FuGENE6 transfection reagent) with 0.75 μ g pSVARo expression vector and 0.15 μ g pCMV- β -galactosidase expression vector. On day 4, the cells were incubated for 90 min at 37°C with 1 nM [3 H]mibolone (Dupont, NEN) in the absence and presence of an increasing

concentration of unlabelled mibolerone, MPA, or NET-A. Working on ice, cells were washed three times with ice-cold 1 X PBS for 15 min. Cells were then lysed with 200 μ l 1 X passive lysis buffer. Lysates were briefly centrifuged to remove cellular debris and total binding was determined for 150 μ l of cellular extract by scintillation counting. Non-specific binding was determined as the counts obtained when cells were incubated with 1 nM [3 H]mibolerone in the presence of 10 000 X unlabelled mibolerone (10 μ M). Specific bound mibolerone was calculated as the difference between total and non-specific binding. β -Galactosidase activity of 5 μ l cellular extract was determined as described above. The reported values are averages of two independent assays, with each condition performed in triplicate. Data was plotted as the percentage [3 H]mibolerone specifically bound, after normalizing with β -galactosidase activity, where 100% specific binding was taken as non-specific binding subtracted from total binding.

Data manipulation and statistical analysis

The Graph Pad Prism[®] programme was used for data manipulations, graphical representations, and statistical analysis. One-way ANOVA and Bonferroni test (as post-test) were used for statistical analysis. In graphical representations, bars that are significantly different ($p < 0.05$) according to the statistical test are labelled with different letters of the alphabet. Non-linear regression and one site competition were used in whole cell binding assays. For all experiments, unless otherwise indicated, the error bars represent the SEM of three independent experiments, where each point was performed in triplicate.

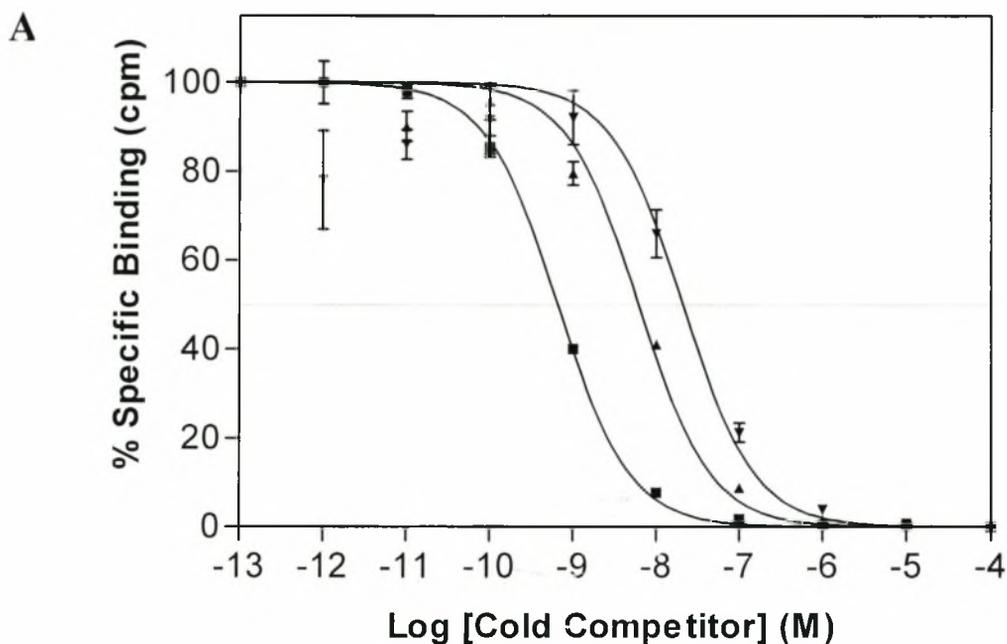
Results

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

In a competitive whole cell binding assay where COS-7 cells were transiently transfected with a full-length hAR expression vector, we show that both MPA and NET-A are able to compete with [³H]mibolerone for binding to the hAR (figure 2A). The curves for both MPA and NET-A are consistent with competitive binding to the same site as mibolerone. These homologous/heterologous displacement curves were analysed and the EC50 values for each compound were determined and are reported in figure 2B. The results show that MPA and NET-A bind to the AR with about a 10- and 40-fold lower relative affinity than mibolerone, respectively. Although it appears that MPA binds with a greater relative affinity than NET-A, this difference is not statistically significant. It is therefore concluded that MPA and NET-A have similar, but lower relative affinities than mibolerone for the AR.

MPA and NET-A display similar androgen agonist activity.

To directly compare the androgenic properties of MPA and NET-A, COS-7 cells were transiently transfected with a GRE-driven reporter construct (containing two copies of the rat TAT GRE) and a human androgen receptor (hAR) expression vector. Subsequently the cells were exposed to increasing amounts of DHT, MPA or NET-A. Figure 3 clearly indicates that MPA and NET-A have similar and relatively strong agonist potency for the AR. Even at 10^{-15} M, both MPA and NET-A show apparent agonist action. Furthermore, the similar levels of maximal induction displayed by MPA and NET-A also indicates that these compounds have a similar efficacy for agonist activity via the hAR. MPA and NET-A appear to have about a 50% lower efficacy compared to that of DHT.



B

| Binding curve data | | | Differences in log EC50-values | |
|--------------------|------------------------|----------|--------------------------------|--------------|
| Competitor | EC50 (M) | (+/-)SEM | Competitors | Significance |
| Mibolerone | 6.53×10^{-10} | 1.0 | Mib vs MPA | * |
| MPA | 6.79×10^{-9} | 1.1 | Mib vs NET-A | ** |
| NET-A | 2.73×10^{-8} | 1.6 | MPA vs NET-A | ns |

Figure 2: MPA and NET-A both compete with [³H]mibolerone for binding to the human androgen receptor.

A. COS-7 cells were transiently transfected with the pSVARo and pCMV- β -galactosidase expression vectors. Forty-eight hours later they were incubated with 1 nM [³H]mibolerone in the absence and presence of increasing concentrations of either mibolerone (■), MPA (▲) or NET-A (▼) for 90 minutes. Competition for binding is illustrated by the percent of [³H]mibolerone specifically bound to the hAR (normalised to β -galactosidase expression). Results are averages of three independent experiments with each condition in triplicate (\pm SEM). **B.** Analysis of data to obtain EC50-values for binding curves, as well as the corresponding SEM-values and statistical significance. P-values < 0.5 and 0.01 are represented by * and ** respectively, and 'ns' stands for no significant difference.

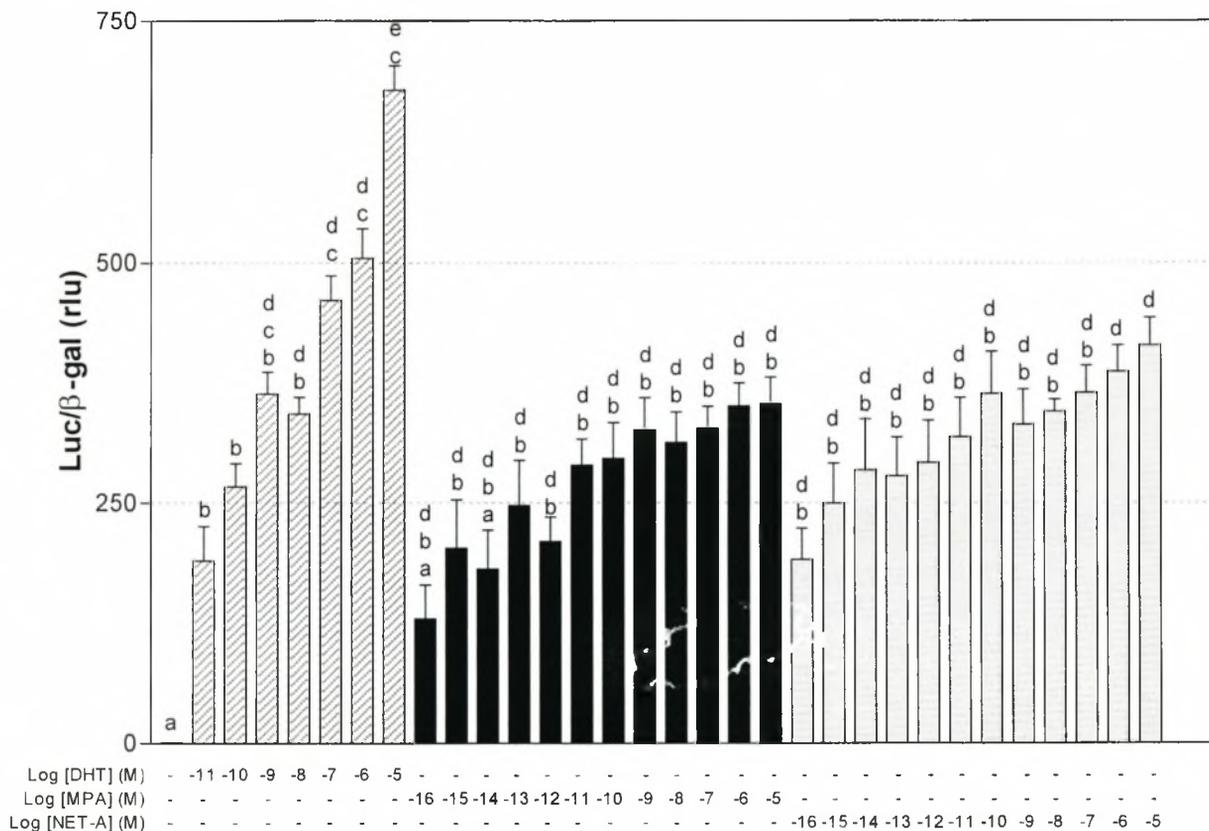


Figure 3: MPA and NET-A display similar androgen agonist activity.

COS-7 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter, the pSVARo and pCMV-β-galactosidase expression vectors. Subsequently, the cells were exposed to no hormone, DHT (hatched bars), MPA (solid black bars) or NET-A (grey bars) at various concentrations for 24 hours. Induction is expressed in relative light units (rlu). Results are averages of three independent experiments with each condition in triplicate (±SEM).

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

The functional, *in vivo*, androgen-dependent interaction between the NTD and LBD of the AR has previously been demonstrated in both yeast and mammalian cells (Langley *et al.*, 1995; Doesburg *et al.*, 1997). Here we used a mammalian two-hybrid assay to investigate the potential of MPA and NET-A to induce the N/C-interaction of the hAR. In transient transfection assays, COS-7 cells were cotransfected with a GRE-driven reporter construct; an expression vector encoding the DBD and LBD of the hAR; as well as an expression vector encoding the NTD of the hAR fused to a VP16 activation domain. In figure 4A we show that, unlike DHT (bars 1 to 4), MPA is not able to induce the N/C-interaction of the AR (bars 5 to 8). Furthermore, we demonstrate that with increasing concentrations, MPA is able to repress the DHT-induced interaction (bars 9 to 12).

In figure 4B, in contrast to MPA, we show that NET-A (bars 5 to 8) has the ability to induce the N/C-interaction of the AR, similarly to DHT (bars 1 to 4). When the cells are incubated with both androgen and NET-A, this level of induction is maintained (bars 9 to 12). Therefore, when comparing the two contraceptive compounds it would appear that NET-A displays behaviour that is more characteristic of classical strong androgen agonists (Kemppainen *et al.*, 1999).

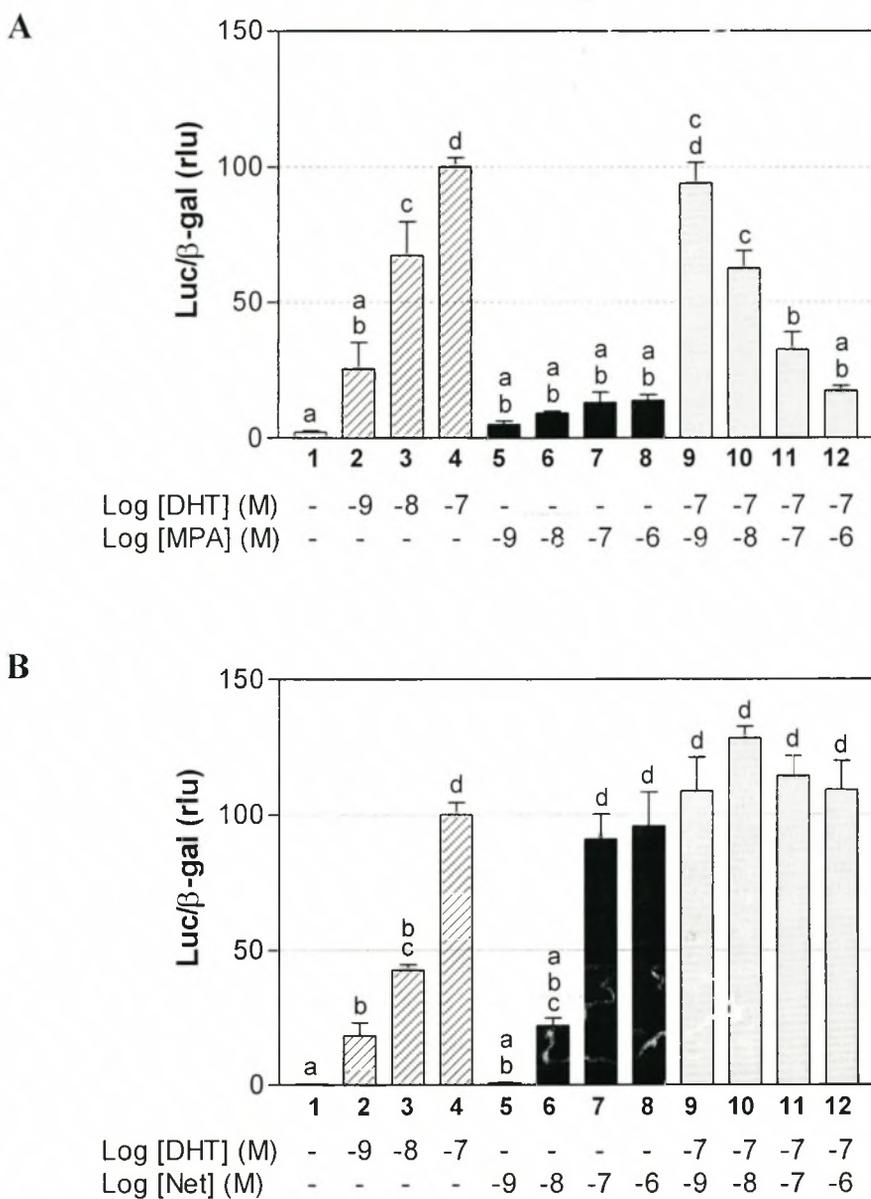


Figure 4: In contrast to DHT and NET-A, MPA does not induce the ligand-dependent N/C-interaction of the hAR.

COS-7 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter, the pSG5-hAR(DBD-LBD), the pSNATCH-II(hAR-NTD) and the pCMV-β-galactosidase expression vectors. Subsequently, the cells were exposed to the indicated test compounds, (A) MPA and (B) NET-A, for 24 hours. Induction is expressed in relative light units (rlu). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

MPA and NET-A both facilitate the ligand-dependent recruitment of the co-activator, SRC1, to the AF2 domain.

To further characterise the mechanism by which MPA and NET-A elicit their androgenic actions, transient transfections were performed to determine the effect of each compound on the recruitment of the coactivator, SRC1, to the AF2 domain of the hAR. The ligand-dependent interaction between SRC1 and the AR LBD has previously been demonstrated in a mammalian (Alen *et al.*, 1999) as well as a yeast (Bevan *et al.*, 1999) system. In these experiments, the mammalian COS-7 cell-line was cotransfected with the GRE-driven reporter construct, the vector expressing the DBD-LBD of the hAR, and a SRC1 expression vector. In figure 5A we show that MPA, similar to DHT, facilitates recruitment of SRC1 to the LBD of the hAR. Since it is shown in figure 2 that MPA binds to the AR, the result in figure 5A together with that of figure 2 implies that MPA binds to the LBD and thereby induces the conformational changes in this domain that allow SRC1 to interact with the AF2 domain of the hAR (bars 5 to 8), with a similar efficiency to that of DHT (bars 1 to 4). When the cells were incubated with both DHT and MPA (bars 9 to 12), the level of induction was maintained. Similarly, figure 5B indicates that NET-A is also able to induce the changes necessary to allow SRC1 to interact with the AF2 domain (lanes 5 to 8), with a similar efficiency to that of DHT (bars 1 to 4). This level of induction was maintained when the cells were incubated with both DHT and NET-A (bars 9 to 12).

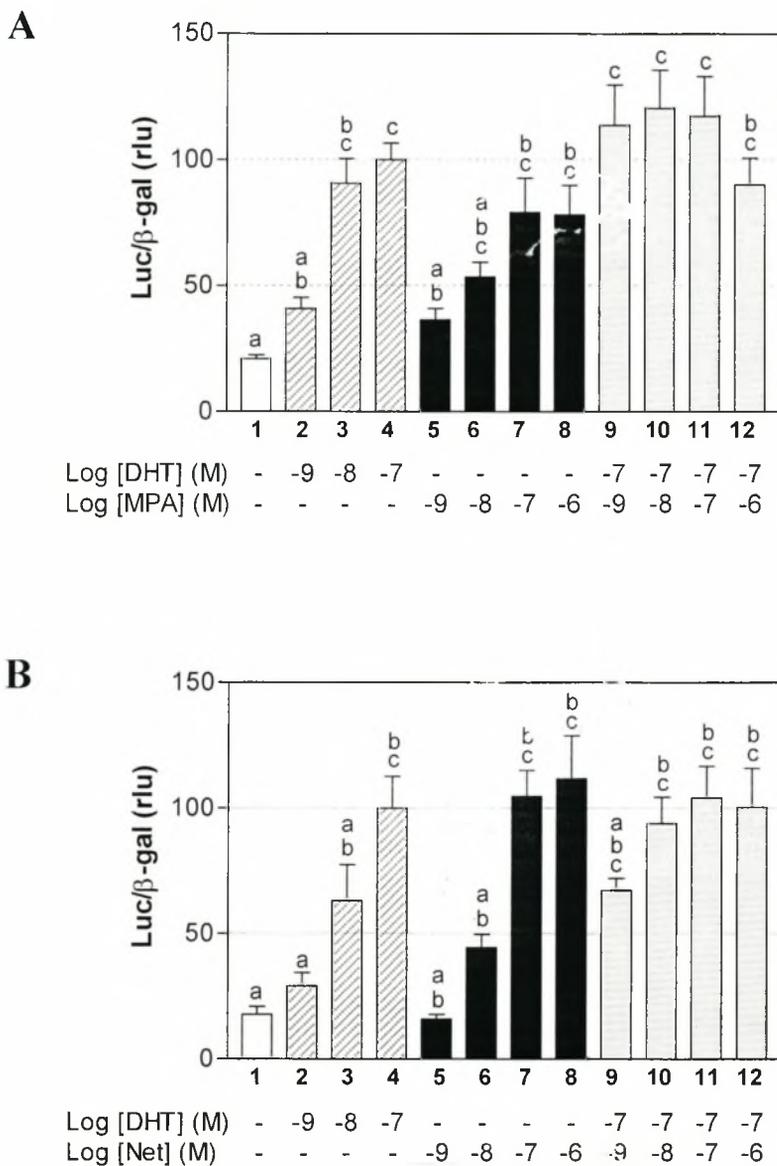


Figure 5: Similarly to DHT, MPA and NET-A are both able to induce the ligand-dependent interaction of the co-activator, SRC1, with the AF2 domain.

COS-7 cells were transiently transfected with the pTAT-GRE-E1b-luc reporter, the pSG5-hAR(DBD-LBD), the pSG5-SRC1 and the pCMV-β-galactosidase expression vectors. Subsequently, the cells were exposed to the indicated test compounds, (A) MPA or (B) NET-A, for 24 hours. Induction is expressed in relative light units (rlu). Results are averages of three independent experiments with each condition in triplicate (\pm SEM).

Discussion

The whole cell binding assays of the present study reveal that both MPA and NET-A compete for binding to the hAR with a similar, significantly lower, affinity than that of mibolerone (figure 2A). The trend observed in figure 2A indicates that MPA may have a slightly greater affinity for the hAR than NET-A. However, figure 2B shows that, in the context of this series of experiments, there is no significant difference in the relative binding affinities of MPA and NET-A for the hAR. This result is consistent with a previous report where it appears that, in intact MCF-7 cells, NET and MPA bind with a similar relative affinity to the endogenously expressed AR (Bergink *et al.*, 1983 and references therein). The data used in this report is, however, obtained from separate studies. The result reported in the present study may therefore better reflect the relative affinities of these compounds, as a direct comparison between the two compounds was made. The present study confirms that MPA and NET-A have similar relative binding affinities for the hAR.

Our results show for the first time, by direct comparison, that MPA and NET-A have similar androgenic activity (figure 3). Kemppainen *et al.*, 1999, showed that 100-fold more MPA is required to achieve the same agonist activity as DHT, mibolerone and R1881. This AR agonist activity was determined in CV-1 cells transiently transfected with a mouse mammary tumor virus (MMTV)-luciferase reporter and full-length hAR expression vectors. In a separate study, the relative agonistic activity of NET for the AR was found to be only about 1% of that of DHT (Deckers *et al.*, 2000). In these studies CHO cells stably transfected with the MMTV-luciferase reporter and hAR expression vectors were used. In these two aforementioned studies, MPA and NET are both required

at concentrations 100-fold greater than DHT to achieve the same agonist activity as this androgen, and as a consequence MPA is described as a weak AR agonist whereas NET is said to have low androgenic activity. In the present study we demonstrate that although MPA and NET-A exhibit similar and potent AR agonist activity, they both have a lower efficacy of about 50% of that of DHT. They can thus be described as partial AR agonists in the context of the present study (figure 3). Although both MPA and NET-A have a lower efficacy in comparison to DHT, the efficacy of these compounds in our study is much higher than that reported in the studies mentioned above. The discrepancies between the results, reported in the present as compared to previous studies, could be attributed to the different systems in which the agonist activities were measured. If this is the case, then contributing factors could be the cell-line used and/or the sequence or context of the hormone response element used in the reporter construct.

Amongst the factors contributing to the potency of a ligand are its affinity for the receptor, as well as its influences on dissociation of heat-shock proteins, nuclear translocation, receptor conformation and stabilisation, the recruitment of essential co-factors and transcription machinery, as well as DNA binding and rate of ligand dissociation. Having addressed the issue of ligand binding the next step was to study the influence of MPA and NET-A on some of the other parameters. Therefore, the issue of receptor conformation and stabilisation was investigated. In this regard we focused on the characteristic N/C-interaction of the hAR. It was found that MPA, although it can compete for binding to and is an agonist for the AR, does not induce the N/C-interaction (figure 4A). These results are consistent with those previously reported by Kemppainen *et al.*, 1999. The ability of a compound to bind to the AR but not induce the N/C-interaction has been reported for the anti-androgens hydroxyflutamide (Langley *et al.*, 1995; Kemppainen

et al., 1999) and cyproterone acetate (Kemppainen *et al.*, 1999). These results suggest that the conformation of the AR resulting from the N/C-interaction is not imperative to render the receptor transcriptionally active, since MPA still has the ability to activate an androgen-responsive reporter. In contrast, NET-A, which is demonstrated to be an agonist that competes for binding to the AR, was indeed able to induce the N/C-interaction (figure 4B). An explanation for this difference could be the fact that MPA is a 21-carbon series steroid and therefore a true progestin, whereas NET-A is a 19-carbon series steroid and therefore structurally more closely related to the androgens. This difference in the structures could therefore account for the more typical androgenic profile of NET-A.

Lastly, we determined whether or not these two compounds in any way influenced the ligand-dependent recruitment of co-activator proteins. Here we studied the interaction between the AF2 domain of the AR and the p160 co-activator protein, SRC1. Both MPA and NET-A were able to induce the ligand-dependent interaction between SRC1 and the AF2 domain with similar efficacy to androgen. The structural relationship of the test compound to androgens does not appear to influence this ligand-dependent interaction.

The inability of MPA to induce the ligand-dependent N/C-interaction suggests that MPA and NET-A activate the AR by different mechanisms. Crystal structure analysis of the retinoic acid receptor- γ (Renaud *et al.*, 1995), thyroid hormone receptor (Wagner *et al.*, 1995) and estrogen receptor (Brzozowski *et al.*, 1997) show that ligand binding causes helix 12 of the C-terminal domain to undergo a conformational change that closes the ligand binding pocket. This change in conformation allows the receptor to 'hold' the bound ligand. Such closure of the ligand binding pocket by helix 12 has also been proposed for the AR. For the AR closure of the pocket by helix 12 is thought to slow the

rate of ligand dissociation (Zhou *et al.*, 1995). Furthermore, this change in conformation forms the interface for the N/C-interaction, in that the AF2 domain is activated (Zhou *et al.*, 1995; Kempainen *et al.*, 1992). Ligand-dependent activation of the AF2 domain is also required for the recruitment of co-activators to this domain (Alen *et al.*, 1999). Therefore, although MPA does not induce the N/C-interaction, the ability of MPA to induce recruitment of SRC1 to the AF2 domain implies that this domain is still activated by the closure of the ligand-binding pocket by helix 12.

Our finding that NET-A shows similar efficacy to MPA in inducing transcription via the AR, but has a different effect on the N/C-interaction, suggests that the effect that a compound has on the N/C-interaction is not the main determinant for agonist activity. Other factors such as the dissociation of heat-shock proteins, nuclear translocation, DNA binding affinity and rate of ligand dissociation may also play a role. The results presented are not consistent with the finding that the degree of agonist potency at low physiological concentrations parallels the ability of a compound to induce the N/C-interaction (Kempainen *et al.*, 1999). The results in this study clearly show MPA to be a potent AR agonist, at low concentrations, even though it does not induce the N/C-interaction.

At present women are exposed to an array of contraceptive choices. Elucidating the mechanisms by which contraceptive agents exert their effects, will provide women and their clinicians with more information to facilitate the selection of method of contraception. These insights into mechanisms of action could also facilitate improved drug design for contraceptive agents and be helpful in unravelling molecular mechanisms by which these compounds exert their side effects. This need to better our understanding of these mechanisms is extended to the concern that breast-fed infants will be exposed to

significant quantities of progestins, if the mother is receiving contraceptive doses of the intramuscularly administered progestins (Fotherby *et al.*, 1983). Evidence that neo-natal exposure to progestin via milk can exert an effect on the subsequent generation has been provided in rat experiments (Holzhausen *et al.*, 1984). In this study female pups were exposed to progestins via maternal milk. When they reached adulthood the effects of these progestins on cyclicity in these rats were measured. It was found that MPA, but not NET-EN, significantly reduced the pro-oestrus luteinizing hormone (LH) peak as well as the total amount of LH secreted during pro-oestrus, and that neither MPA nor NET-EN had an affect on pro-oestrus progesterone secretion.

The progestins used in this study have clinical applications other than being used as female contraceptives. Firstly, both MPA and NET are used in the treatment of breast cancer, and have a number of other therapeutic uses, at doses that are higher than those used for contraception. Secondly, NET-EN is going through clinical trials to be used in combination with the long-acting testosterone ester, testosterone undecanoate (TU) as a male contraceptive (Kamischke *et al.*, 2000a; 2000b; 2002). These applications once again highlight the importance of defining the mechanisms of action by which these compounds function and potentially exert their side effects. More importantly, there is a need to directly compare the mechanisms by which these compounds act. The results of this study emphasise this point as it is demonstrated that compounds used for the same applications can have different effects at the molecular level. The different effects that MPA and NET-A have on the ligand-dependent N/C-interaction of the AR are just a single example, the physiological significance of which remains unknown. However, this ability of different compounds to exert similar effects via different mechanisms raises the concern that such

compounds could then also exhibit very different effects via steroid receptors in some target tissues, depending on the promoter and repertoire of co-factors.

Although all steroid hormones are structurally similar, relatively minor differences can cause profound alterations in their biochemical activity. Taken together our results provide evidence for such differences in activity at the molecular level, once again emphasising the importance of defining the mechanisms of action of each of these compounds.

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3.2 Comments and suggestions

One of the main concerns about the data, presented in the current version of the manuscript, is that the whole cell binding assay was performed using mibolerone as reference androgen, whereas the rest of the assays were performed using DHT as reference androgen. It is well established that the relative binding affinities of these two androgens for the AR differ. Mibolerone has a greater affinity than DHT for the AR. This makes it difficult to directly compare and correlate the data obtained from the different assays performed in the present study. It is thus proposed that at least two of the assays be repeated. Firstly, the whole cell binding assay will be repeated. Once again [³H]mibolerone will be used and homologous displacement by mibolerone as well as heterologous displacement by DHT, MPA and NET-A, will be performed. Secondly, the transactivation assay will be repeated and dose response curves obtained for mibolerone, DHT, MPA and NET-A.

In repeating the whole cell binding assay, a few other parameters could be optimised to yield data from which more information can be obtained. In the assay presented in the manuscript, 1 nM [³H]mibolerone was used. The EC₅₀-value obtained from the homologous displacement curve of mibolerone is 0.653 nM (figure 2B). The EC₅₀-value of mibolerone is lower than the concentration of [³H]mibolerone used and therefore it is not possible to obtain an accurate K_d for mibolerone from this data. Thus, when repeating this series of experiments, lower concentrations of [³H]mibolerone (e.g. 0.1 nM) will be tested, to obtain an EC₅₀-value about 2 to 10 times higher than the concentration of [³H] mibolerone used, in order to calculate an accurate K_d for mibolerone. Once the K_d for mibolerone has

been established in this system, the relative K_i values for DHT, MPA and NET-A can also be determined, which will better reflect the relative binding affinities of these compounds for the AR.

In repeating the transactivation assay, again there are some parameters that could be altered to obtain more information from the results. Together with including incubation with mibolerone, it is further suggested that incubations with additional concentrations for DHT be included. It is therefore suggested that for all the compounds, DHT, mibolerone, MPA and NET-A, a concentration range of at least 10^{-16} M to 10^{-5} M be used. For DHT, and possibly also mibolerone, even higher concentrations may be necessary to determine the true maximum of induction. The current data may not necessarily reflect this for DHT, because there is only one concentration (10^{-5} M) at which this maximum is attained, and at least two consecutive concentration points that are not significantly different from one another are required. The result obtained from this suggested experiment would therefore also allow a better comparison of the relative efficacies of the different compounds. Furthermore, the new data could also be plotted as dose-response curves for which the EC50-values could be determined. This would allow a more accurate quantification of the relative potencies of the compounds. Taken together, repeating this experiment will most likely still show MPA and NET-A to be partial AR agonists, but this interpretation will be based on better statistical evidence.

In the present study it is suggested that both MPA and NET-A facilitate the recruitment of SRC1 to the AF2 domain of the hAR in a mammalian two hybrid assay. However, the assay can not exclude the possibility that another co-activator

besides SRC1 is facilitating transcription and therefore the data do not show conclusively that there is a direct interaction between the AF2 domain and SRC1. To definitively show direct interaction a more direct assay such as GST pull-down assays could be performed.

Lastly, all of the assays used were based on the overexpression of the AR in the COS-7 cell-line and thus do not reflect the effects of the test compounds on physiological AR. It is therefore also proposed that the actions of MPA and NET-A via endogenously expressed AR be tested. Two further experiments in this regard are suggested. The ability of MPA and NET-A to activate transcription of an androgen-specific responsive (ARE-) reporter construct transfected into a cell-line that expresses endogenous AR could be studied. Finally, the effect of MPA and NET-A on the expression of androgen-dependent, endogenously expressed protein (i.e. the PSA proteins in the LNCaP cell-line) could also be investigated. In both of these experiments additional incubations with MPA/NET-A in the presence of an AR-specific antagonist (e.g. hydroxyflutamide) will be included. This will determine whether the AR is involved in MPA-/NET-A- induced transactivation of the ARE-reporter as well as in the expression of PSA.

Discussion and Concluding Remarks

4.1 Summary and discussion of the results presented in this thesis

The results of the two studies presented in this thesis have been summarised in Table I. This table will be referred to extensively in this discussion, to highlight the issues raised as well as the insights gained from these studies.

The anti-androgenic actions of CpdA are summarised in Table I. Although CpdA does not activate transcription via the AR it can, at 10 μ M, repress R1881-induced activation of both non-specific and specific androgen response elements (AREs) (Chapter 2; figure 2). In contrast to CpdA, MPA and NET-A display partial androgen receptor agonist activity in transactivation assays (Chapter 3; figure 3). The whole cell binding assays revealed that CpdA does not compete (Chapter 2; figure 4), whereas MPA and NET-A are able to compete (Chapter 3; figure 2) with [3 H]mibolerone for binding to the AR. These results are consistent with the actions of CpdA, MPA and NET-A observed in the transactivation assays with respect to agonist activity. The inability of CpdA to compete for binding was unexpected, since anti-androgens normally compete with androgens for binding. In this respect, CpdA appears to be unique in its mechanism of eliciting its anti-androgenic actions. However, this ability of a compound to modify the action of a steroid receptor without directly binding to the receptor is not unique, since it has been demonstrated that ursodeoxycholic acid can activate the GR into a DNA binding species without direct binding to the GR (Tanaka *et al.*, 1996).

| Table I: Summary of Results | | | |
|--|----------------------|------------|--------------|
| Assay | Test compound | | |
| | CpdA | MPA | NET-A |
| Steroidal or non-steroidal compound | Non-steroidal | Steroidal | Steroidal |
| Transactivation of GRE-responsive reporter via the AR | No | Yes | Yes |
| Repression of androgen-induced activation of GRE-responsive reporter | Yes | <i>ND</i> | <i>ND</i> |
| Repression of androgen-induced activation of androgen-specific responsive reporter | Yes | <i>ND</i> | <i>ND</i> |
| Repression of ligand-induced activation via other SRs | Yes, the PR | <i>ND</i> | <i>ND</i> |
| Competitive binding for the AR | No | Yes | Yes |
| Induction of N/C-interaction | No | No | Yes |
| Repression of ligand-induced induction of N/C-interaction | Yes | Yes | No |
| Induction of SRC1 recruitment to AF2 domain | No | Yes | Yes |
| Repression of ligand induced recruitment of SRC1 to AF2 domain | Yes, slightly | <i>ND</i> | <i>ND</i> |
| Effect on SRC1 interaction with the NTD | No | <i>ND</i> | <i>ND</i> |
| Effect on constitutive activity of NTD | No | <i>ND</i> | <i>ND</i> |

Where *ND* stands for 'not determined'.

To further define the mechanism(s) by which these compounds exert their effects, their interactions with the LBD of the AR were investigated. Here two assays that rely on the binding of the androgen to the LBD with the subsequent activation of the AF2 domain were used.

The first assay measured the N/C-interaction. Here it was shown that CpdA itself is unable to induce the ligand-dependent N/C-interaction (Chapter 2; figure 5A) and that, similarly to other anti-androgenic compounds (Langley *et al.*, 1995; Kempainen *et al.*, 1999), CpdA represses the DHT-induced interaction of these domains. An intriguing result was observed when this assay was performed for the two progestins. Although MPA displayed androgenic actions in the transactivation experiments, it was unable to induce the N/C-interaction. DHT and NET-A, on the other hand, were able to induce this interaction, as was expected considering they both exert androgenic effects in the transactivation experiments. More surprising was the fact that MPA repressed the DHT-induced N/C-interaction similarly to anti-androgenic compounds such as hydroxyflutamide, cyproterone acetate and CpdA. These results imply that MPA activates the AR by a mechanism that differs from conventional agonist and NET-A action. Furthermore, the results suggest that MPA, but not NET-A, displays anti-androgen activity.

CpdA did not displace agonist from the AR nor did it prevent agonist binding to the AR, yet it was able to inhibit transactivation as well as the N/C-interaction induced by the agonist. MPA, on the other hand, could displace agonist from the AR and could transactivate via the AR, and yet, like CpdA, did not induce the N/C-interaction and also inhibited agonist induced N/C-interaction. It was therefore postulated that the lack of N/C-interaction and inhibition of agonist-induced N/C-interaction by CpdA and MPA might extend to an additional feature of the activation of the LBD, namely recruitment of co-activator proteins to the AF2 domain. A second assay was therefore performed, to determine whether closure of the ligand binding pocket and thus activation of the AF2 domain takes place in the presence of CpdA,

MPA and NET-A. Here, the effect of these compounds on the ligand-dependent recruitment of SRC1 to the AF2 domain was studied. It was demonstrated that CpdA is capable of repressing this interaction (Chapter 2; figure 5B), although to a lesser degree than the repression observed for the N/C-interaction. Because the interaction between the AF2 domain and SRC1 is not completely lost, it is unlikely that CpdA prevents the structural changes of the LBD that occur as a result of ligand binding. In contrast, MPA does not repress this interaction but in fact induces it similarly to NET-A and DHT (Chapter 3; figure 5). The regions of the AF2 domain involved in the N/C-interaction and the interaction with SRC1 overlap, but are not identical (Thompson *et al.*, 2001). Therefore, it is possible that CpdA may interact with or modify amino acid residues that are essential for the N/C-interaction and are important, but not essential, for the interaction between SRC1 and the AF2 domain. MPA, on the other hand, may induce a conformation that compromises amino acid residues that are essential for the N/C-interaction but that are not of importance for the interaction between SRC1 and the AF2 domain. This suggests that CpdA and MPA may exert slightly different effects at the LBD of the AR.

The repressive effect of CpdA on AR activity prompted an additional investigation. The potential of CpdA to interact with the NTD of the AR was studied, as it is this domain of the AR that has been shown to possess most of the transcriptional activity. The effect of CpdA on the ligand-independent interaction between the NTD and SRC1 (Chapter 2; figure 5C), as well as its effect on the constitutive activity of this domain (Chapter 2; figure 5D), were addressed. It was found that CpdA in no way impairs the recruitment of SRC1 to the NTD, and neither does it affect the transcriptional activity of the NTD. These results imply that CpdA in

no way affects the establishment of or interactions of the NTD with the basal transcription machinery. These results do not however, exclude the possibility that CpdA may interact with region(s) in the NTD that are essential for the N/C-interaction but that are not of importance for SRC1 recruitment or the constitutive activity of this domain.

Another point to take note of is the fact that CpdA can cyclize to an aziridine at physiological pH. Aziridines are highly reactive, unstable alkylating compounds. Their more stable precursors are often used clinically in cancer treatment (Calabresi and Chabner, 1990). These precursors undergo cyclization to the highly reactive aziridine. It is the aziridine ring and its protonated form, that react with nucleophiles to form alkylated, ring-opened products (Dermer and Ham, 1969). One possible explanation for the anti-androgenic activities of CpdA may be that CpdA is cyclized to the aziridine, which could then react with nucleophilic amino acid residues. Such modifications to specific amino acid residues could account for the effects that CpdA has on, for example, the N/C-interaction. However, if the aziridine is the active species then it would be expected that incubations with CpdA would be toxic for the various cell-lines used, since aziridines alkylate DNA as well as other biological molecules (e.g. proteins) resulting in general cytotoxicity (Calabresi and Chabner, 1990). CpdA, however, had no effect on SRC1 recruitment to the NTD nor did it affect the constitutive activity of this domain. In addition, CpdA had no effect on AR or CK18 levels (Chapter 2, figure 6) nor was it generally cytotoxic as evidenced by the fact that anti-proliferative effects were seen only with LNCaP cells but not with COS-7 cells (Chapter 2, figure II). This indicates that there are no cytotoxic effects

and therefore suggests that it is CpdA rather than the aziridine that is the active species in the transfection experiments performed in this study.

The following conclusions can also be drawn from the data presented in this thesis. Firstly, CpdA slightly impairs the recruitment of SRC1 to the AF2 domain but has no effect on SRC1 recruitment to the AF1 domain. This result indicates that the AF2 domain is more important than the AF1 domain for transactivation via the AR in the systems and cell-lines tested in this study. This is contrary to literature reports stating that the AF2 domain of the AR displays weak transcriptional activity whereas the AF1 domain is the major activating region of the AR (Moilanen *et al.*, 1997). Secondly, the different mechanisms of MPA and NET-A action are most likely explained by the structural differences between the two compounds. The classical mechanism of androgen agonist action displayed by NET-A can be attributed to its similarity to testosterone. On the other hand, MPA is more closely related to progesterone, which may account for its effects in the N/C-interaction assay, as it has previously been shown that progesterone represses the DHT-induced N/C-interaction (Kemppainen *et al.*, 1999). Although MPA has a similar effect to CpdA in this assay, the mechanisms involved are clearly different as MPA can compete for binding to the receptor and transactivate via the receptor. However, the principles may be similar, in that in both cases the compound may induce a conformation in the LBD that compromises the alignment of amino acid residues of the AF2 domain. This altered arrangement may then hinder the amino acid residues that are essential for the N/C-interaction.

4.2 Implications and future perspectives

4.2.1 Compound A

Anti-androgens are designed for and used in the treatment of a number of androgen-dependent diseases. To date, chemical anti-androgen therapy has been based on the use of compounds that compete with androgens for binding to the AR and as a result inhibit androgen action. In chapter 2 the potential of CpdA to inhibit androgen action by a mechanism that does not involve competition for binding is described. However, the precise mechanism of action by which CpdA exerts its anti-androgenic effects is not yet known. A thorough investigation to further elucidate these mechanisms would be of great value. An understanding of CpdA's actions together with knowledge of how other anti-androgenic compounds elicit their effects would be extremely useful in aiding the design of therapeutic agents for the treatment of diseases such as prostate cancer. Such investigations would also facilitate the unravelling of the mechanisms involved in AR action.

With regards to future studies to elucidate the mechanism(s), by which CpdA exerts its actions, there are numerous aspects of AR action that could be investigated. Firstly, it is suggested that CpdA may directly interact with a region of the AR, resulting in inhibition of the N/C-interaction. Whether CpdA binds to a region of the AR other than the LBD could be investigated using custom synthesized [^3H]CpdA in whole cell binding experiments. Secondly, because it is known that androgen still binds the AR in the presence of CpdA, and that CpdA represses the N/C-interaction, an investigation of the potential effect of CpdA on the rate at which ligand dissociates from the receptor could be performed. The ligand dissociation rate is influenced by

the N/C-interaction, and has been suggested as a measure of agonist activity (Kemppainen *et al.*, 1999).

Furthermore, the influence of CpdA on the conformation of the LBD adopted by the ligand-bound receptor protein could be examined. Limited proteolytic digestion of *in vitro* produced AR protein that has been incubated with androgen in the absence and presence of CpdA would be a possible strategy. In the presence of androgen, the receptor would be expected to generate a protected fragment of about 29 kDa in size (Kuil *et al.*, 1994). In the presence of both androgen and CpdA, one would expect to observe a protected fragment of a different size or no protection at all if CpdA alters the androgen-induced conformation of the LBD. However, due the unpredictable nature of this assay, as discussed in chapter 1, seeing no effect in the presence of CpdA would not necessarily mean that CpdA has no effect on the conformation of the LBD.

Finally, as it is still unclear as to how CpdA exerts its effect, the influence of CpdA on other steps in AR functioning could also be investigated. In this respect the effects of CpdA on AR dimer formation, DNA binding, dissociation of associated proteins (i.e. heat-shock proteins), as well as cellular localisation could be studied.

4.2.2 Medroxyprogesterone acetate and norethindrone acetate

It is assumed that the contraceptive agents, DMPA and NET-EN, exert their contraceptive action predominantly via the PR as these compounds are both synthetic progestins. However, both of these compounds have been shown to have androgenic agonist activity. It was the purpose of this study to define the androgenic properties of these two compounds, as it is possible that some of the side-effects observed when using these contraceptives could be attributed to their androgenicity. More

importantly, the aim was to make a direct comparison between the two compounds, as to date only some of these aspects have been investigated, and these have not involved direct comparisons. The results presented demonstrate how compounds that may appear to be structurally similar and possess similar mechanisms of action via one SR, can then also exhibit different effects via another SR. An important insight into steroid action was therefore highlighted, namely, that different steroid hormones have the capacity to exhibit different effects, via different SRs, in various target tissues. Therefore, when considering the number of applications of these two compounds, it is clear that it is of importance to assess and define their actions under various conditions, which in turn could assist clinicians in choosing the most appropriate method of treatment.

With regards to future studies to further elucidate the mechanisms of MPA and NET-A action via the AR, there are a few elements of AR action that could be investigated. Firstly, knowing that both MPA and NET-A bind the AR but that unlike NET-A, MPA does not induce the N/C-interaction, it is suggested that the effect of these compounds on AR stability as well as the rate at which they dissociate from the receptor be investigated. Both ligand dissociation rate and receptor stability are influenced by the N/C-interaction, and have been suggested as measures of agonist activity (Kemppainen *et al.*, 1999). It would thus be expected that MPA would dissociate from the AR at a quicker rate than NET-A. Since there does not appear to be a difference in the potency and efficacy of these two compounds with respect to transcriptional activation, it would be interesting if there was indeed a difference in their dissociation rates. Secondly, the ability of MPA to repress the androgen-induced N/C-interaction confers an anti-androgenic property on MPA. It would therefore be of interest to investigate the potential for MPA to repress androgen-induced activation of

a GRE-responsive reporter. Furthermore, as it is possible that MPA may induce a slightly different conformation in the LBD as opposed to NET-A, a comparative study of the effects of MPA and NET-A on dimer formation as well as DNA binding affinity could be performed. These studies would be appropriate as both stabilization of dimer formation and dissociation of hsp90 involve regions of the LBD.

4.3 Concluding Remarks

Taken together, the experiments presented in this thesis illustrate the importance of defining the precise mechanism by which individual compounds exert their effects. Although the basic molecular mechanism of steroid hormone action via the SRs has been defined, our results demonstrate how both apparently major and minor differences in the structures of different compounds can alter the mechanism by which a SR acts.

Firstly, with regard to major structural differences we show how non-steroidal (CpdA) and steroidal (MPA and NET-A) compounds can affect AR functioning in both the same and different ways. Even though CpdA does not compete with androgens for binding to the AR, whereas MPA does, it is shown that both of these compounds are unable to induce the ligand-dependent N/C-interaction but are able to repress the DHT-induced N/C-interaction (refer to Chapter 2; figure 5A and Chapter 3; figure 4A). Furthermore, CpdA impairs the recruitment of SRC1 to the AF2 domain whereas MPA has no effect on this interaction (refer to Chapter 2; figure 5B and Chapter 3; figure 5A). Secondly, by directly comparing the androgenic activities

of MPA and NET-A, we have shown how minor structural differences between steroidal agents can have an effect on their respective mechanisms of action. Although both compounds bind the AR and transactivate via the AR, MPA does not induce the N/C-interaction whereas NET-A does.

Two important aspects of steroid hormone action were also highlighted. Firstly, binding of a compound to the agonist-binding site of the receptor, a basic step in AR function, may not be a prerequisite for such a compound to alter the actions of the receptor. Secondly, the importance of directly comparing the actions of different compounds at the molecular level was demonstrated. This is not only of importance to elucidate the mechanisms by which the compounds may act, but also in identifying and defining the exact regions of the interacting protein, in this case the receptor protein, that are imperative for the optimal functioning of the respective compound.

Finally, combining the knowledge available on AR function (including various mechanisms of androgenic and anti-androgenic action) with the results presented here, can contribute to an improved understanding of AR functioning. Furthermore, such information could also facilitate the development of new anti-androgenic compounds for diseases such as prostate cancer, as well as aid the improved design of progestins for contraceptive purposes that would be more specific for the PR and thereby possibly exert fewer side-effects.

4.4 References

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