Investigating the mechanism of action of hormones used in hormone replacement therapy via estrogen receptor subtypes and the influence of the progesterone receptor

by

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DECLARATION

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ABSTRACT

Estrogens and progestins used in conventional menopausal hormone therapy (HT) are associated with increased breast cancer risk. A diverse range of estrogens and progestins are available that mediate their effects primarily by binding to the estrogen receptor (ER) and progesterone receptor (PR), respectively. Although the link to breast cancer risk has not been shown for all estrogens and progestins, many women have turned to custom-compounded bioidentical hormone therapy (bHT) as it is claimed to not increase breast cancer risk. However, scientific evidence to support this claim is lacking. Estrogens and ERα are considered the main etiological factors driving breast cancer, while both ERα and the PR are required for progestin (medroxyprogesterone acetate (MPA)) effects on breast cancer cell proliferation. In this thesis, we investigated the activities of estrogens and progestins used in menopausal hormone therapies via the individual ER subtypes, and the role of ERα/PR crosstalk in mediating progestin-induced effects on gene expression, breast cancer cell proliferation and anchorage-independent growth. In the first part of the study, competitive whole cell bindings assays showed that bioidentical estradiol (bE_2) and estriol (bE_3) displayed similar binding affinities to the commercially available (natural) estradiol (E_2) and estriol (E_3) standards, while synthetic ethinylestradiol (EE) had a higher affinity for ERα, and natural E_1 a lower affinity for ERβ. Furthermore, the bioidentical estrogens mimicked their respective natural estrogens and synthetic EE on transactivation and transrepression of gene expression, proliferation and anchorage-independent growth of the estrogen-sensitive MCF-7 BUS human breast cancer cell line. These assays showed that E_3 and estrone (E_1) are efficacious estrogens that do not antagonize E_2. In the second part of this study, the estrogenic activities of selected progestins from different generations, MPA, norethisterone acetate (NET-A), levonorgestrel (LNG), gestodene (GES), nestorone (NES), nomegestrol acetate (NoMAC) and drospirenone (DRSP), were characterized relative to each other and natural progesterone (P_4). Competitive binding assays revealed that only NET-A, LNG and GES could bind to ERα, while no progestin bound ERβ. Both transactivation and transrepression transcriptional assays showed that NET-A, LNG and GES display estrogenic activity. In the third part of the study, the role of PR/ERα crosstalk in mediating the effects of MPA, NET and DRSP, relative to P_4, on breast cancer cell proliferation, anchorage-independent growth and the expression of the ER-regulated *trefoil factor 1* (*pS2*) and *cathepsin D* (*CTSD*) genes was investigated. All progestins could promote proliferation and anchorage-independent growth of MCF-7 BUS breast cancer cells to the same extent as P_4 and E_2 via a mechanism requiring both the PR and ERα, but DRSP was the least, and MPA the most potent for proliferation. Quantitative real-time RT-PCR (qPCR), chromatin immunoprecipitation (ChIP) and re-ChIP assays showed that only MPA and NET increased the expression of the *pS2* and/or *CTSD* genes via a mechanism requiring co-recruitment of the PR and ERα to the promoter regions of these genes. In contrast, P_4, MPA, NET and DRSP all caused recruitment of the PR/ERα complex to the PR-regulated oncogenes *cyclin D1* and *MYC*. Taken together, the findings of this study suggest that there is no advantage in choosing bHT above conventional HT, and that while it is unlikely that the progestins used in this study will exert biological effects via ERα or ERβ *in vivo*, some progestins may increase breast cancer risk via a mechanism involving interplay between the PR and ERα.

OPSOMMING

Die gebruik van estrogene en progestiene in konvensionele menopousale hormoonterapie (HT) word geassosieër met ‘n toename in die risiko van borskanker. ‘n Verskeidenheid van estrogene en progestiene, wat hul effekte hoofsaaklik uitvoer deur die estrogeenreseptor (ER) en progesteroonreseptor (PR) onderskeidelik, is beskikbaar. Alhoewel die toenemende risiko van borskanker nog nie vir al die estrogene en progestiene getoon is nie, maak baie vrouens eerder gebruik van persoonlike saamgestelde bioidentiese hormoonterapie (bHT) aangesien daar beweer word dat dit nie borskanker risiko verhoog nie. Wetenskaplike bewyse om hierdie bewering te ondersteun is egter nie beskikbaar nie. Estrogene en ERα word beskou as die hoof etiologiese faktore wat borskanker dryf, terwyl beide ERα en die PR vir die effekte van progestien (medroksieprogesteroonasetaat (MPA)) op borskankerselproliferasie benodig word. In hierdie tesis, het ons die aktiwiteite van estrogene en progestiene, gebruik in menopousale hormoonterapies, deur die individuele ER subtipes ondersoek, asook die rol van ERα/PR wisselwerking in progestien-geïnduseerde geenuitdrukking, borskankerselproliferasie en geankerde-onafhanklike groei. In die eerste deel van die studie het kompeterende heelsel bindingstoetse getoon dat bioidentiese estradiool (bE_2) en estriool (bE_3) dieselfde bindingsaffiniteite het as die komersieël beskikbare (natuurlike) estradiool (E_2) en estriool (E_3) standaarde, terwyl sintetiese etinielestradiool (EE) ‘n hoër affiniteit vir ERα, en natuurlike estroon (E_1) ‘n laer affiniteit vir ERβ het. Verder, boots die bioidentiese estrogene hul onderskeidelike natuurlike estrogene en sintetiese EE na in terme van transaktivering en transonderdrukking van geenuitdrukking, proliferasie en geankerde-onafhanklike groei van die estrogeen-sensitiewe MCF-7 BUS menslike borskankersellyn. Hierdie toetse het getoon dat E_3 and estroon (E_1) doeltreffende estrogene is wat nie E_2 antagoniseer nie. In die tweede deel van die studie was die estrogeniese aktiwiteite van geselekteerde progestiene van verskillende generasies, MPA, noretisteroonasetaat (NET-A), levonorgestrel (LNG), gestodeen (GES), nestoroon (NES), nomegestroolasetaat (NoMAC) en drospirenoon (DRSP), relatief tot mekaar en natuurlike progesteroon (P_4), gekarakteriseer. Kompeterende bindingstoetse het aan die lig gebring dat slegs NET-A, LNG en GES aan ERα kon bind, terwyl geen van die progestiene ERβ bind nie. Beide transaktiverings- en transonderdrukkingstoetse het gewys dat NET-A, LNG en GES estrogeniese aktiwiteite toon. In die derde deel van die studie was die rol wat PR/ERα wisselwerking speel in die uitvoering van MPA, NET en DRSP, relatief tot P_4, op borskankerselproliferasie, geankerde-onafhanklike groei en die uitdrukking van die ER-gereguleerde *trefoiël faktor 1* (*pS2*) en *katepsien D* (*CTSD*) gene ondersoek. Al die progestiene kon proliferasie en geankerde-onafhanklike groei van die MCF-7 BUS borskankerselle tot dieselfde mate as P_4 en E_2 bevorder deur ‘n meganisme wat beide die PR en ERα benodig, maar DRSP was die minste, en MPA die meeste potent vir proliferasie. Kwantitatiewe intydse RT-PKR, kromatienimmunopresipitasie (ChIP) en her-ChIP toetse het getoon dat slegs MPA en NET die uitdrukking van die *pS2* en/of *CTSD* gene verhoog deur ‘n meganisme wat die mede-werwing van die PR en ERα tot die promotor areas van hierdie gene vereis. In teendeel, P_4, MPA, NET en DRSP het almal die werwing van die PR/ERα kompleks tot die PR-gereguleerde onkogene *siklien D1 (CCND1)* en *MYC* veroorsaak. In samevatting, die bevindinge van hierdie studie stel voor dat daar geen voordeel is om bHT te kies bo konvensionele HT nie, en alhoewel dit onwaarskynlik is dat die progestiene wat gebruik is in hierdie studie biologiese effekte deur ERα of ERβ sal uitvoer *in vivo*, mag sommige progestiene wel borskanker risiko verhoog deur ‘n meganisme wat wisselwerking tussen die PR en ERα behels.

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ABBREVIATION LIST

3sFRET	three-color spectral Förster resonance energy transfer
AI's	aromatase inhibitors
ANOVA	analysis of variance
AP-1/2	Activator Protein-1/2
AR	androgen receptor
ARE	androgen response element
ATCC	American Type Culture Collection
bDHEA	bioidentical dehydroepiandrosterone
bE_1	bioidentical estrone
bE_2	bioidentical estradiol
bE_3	bioidentical estriol
bHT	bioidentical hormone therapy
bProg	bioidentical progesterone
BSA	bovine serum albumin
bT	bioidentical testosterone
bp	base-pair
CBG	corticosteroid-binding globulin
CCND1	cyclin D1
cdk1	cyclin-dependent kinase 1
cDNA	complementary DNA
CEE	conjugated equine estrogens
ChIP	chromatin immunoprecipitation
CMA	chlormadinone acetate
Co-IP	co-immunoprecipitation

CS-FCS	charcoal-stripped fetal calf serum
CPA	cyproterone acetate
cpm	counts per minute
CTSD	cathepsin D
DBD	DNA-binding domain
Dex	dexamethasone
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DRSP	drospirenone
DUSP1	dual specificity protein phosphatase 1
E_1	estrone
E_2	estradiol
E_3	estriol
EC_{50}	half maximal effective concentration
EE	ethinylestradiol
ELITE	Early Versus Late Intervention Trial
EMA	European Medicines Agency
EMAS	European Menopause and Andropause Society
EPHT	Estonian Postmenopausal Hormone Therapy
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element

ESPRIT	Estrogen in the Prevention of Re-infarction Trial
EtOH	ethanol
FCS	fetal calf serum
FDA	(United) States Food and Drug Administration
fwd	forward
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GES	gestodene
GPER	G-protein estrogen receptor
GPR30	G protein-coupled receptor 30
GR	glucocorticoid receptor
HERS	Heart and Estrogen/Progestin Replacement Study
HI-FCS	heat-inactivated fetal calf serum
HI-CS-FCS	heat-inactivated, charcoal-stripped fetal calf serum
HPLC	high-performance liquid chromatography
HRE(s)	hormone response element(s)
HRP	horseradish peroxidase
Hsp70/90	heat shock protein 70/90
HT	hormone therapy
ICI(-182,780)	fulvestrant
IGF(1)	insulin-like growth factor (1)
IgG	immunoglobulin G
IMS	International Menopause Society
IL-6	interleukin -6
KEEPS	Kronos Early Estrogen Prevention Study
K_d/K_i	equilibrium dissociation constant

LBD	ligand binding domain
LNG	levonorgestrol
Luc	luciferase
MIB	mibolerone
MR	mineralocorticoid receptor
MPA	medroxyprogesterone acetate
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MWS	Million Women Study
NAMS	North American Menopause Society
NES	nestorone
NET	norethisterone
NET-A	norethisterone acetate
NFκB	nuclear factor kappa-B
NGM	norgestimate
nHRE(s)	negative hormone response element(s)
NoMAC	nomegestrol acetate
NSC	non-silencing control
OHF	hydroxyflutamide
P_4	progesterone
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PELP-1	proline-, glutamic acid- and leucine-rich protein 1
PEPI	Postmenopausal Estrogen and Progestin Intervention
PgR	progesterone receptor gene
PMA	phorbol 12-myristate 13-acetate

PR	progesterone receptor
PRE	progesterone response element
pS2	trefoil factor 1
qPCR	quantitative polymerase chain reaction
R5020	promegestone
RANTES	Regulated-upon-Activation, normal T cell Expressed and Secreted
RBA	relative binding affinity
rev	reverse
RLU	relative light units
SERMS	selective estrogen receptor modulators
SB	specific binding
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SGK1	serine/threonine protein kinase 1
SHBG	sex hormone binding globulin
siRNA	short interfering ribonucleic acid
Sp1	specificity protein 1
T	testosterone
TFF1	trefoil factor 1
TGFβ3	transforming growth factor beta 3
TNFα	tumor necrosis factor-alpha
UPLC–MS	ultra-performance liquid chromatography-mass spectrometry
VEGF	vascular endothermal growth factor
Veh	vehicle
WHI	Women's Health Initiative

WHO	World Health Organization
WISDOM	Women's International Study of Long Duration Oestrogen after the Menopause

THESIS OUTLINE

This thesis consists of five chapters. Chapters 1 and 4 are written up in manuscript format and will soon be submitted for publication. Chapters 2 and 3 consist of recently published research articles and supplementary data. Chapter 5 is a general discussion and conclusion chapter. References are presented at the end of each individual chapter.

1. **Chapter 1: Hormone Therapy and Breast Cancer: Emerging Steroid Receptor Mechanisms.** This chapter is a detailed literature review discussing menopausal hormone therapy (HT) and its association with increased breast cancer risk, with specific focus on different types of HT, the role of steroid receptors in mediating increased breast cancer risk, and the influence of crosstalk between steroid receptors. This review was written by the candidate, with Dr. Renate Louw-du Toit and Prof. Donita Africander providing intellectual input, as well as proofreading and editing of the manuscript.
2. **Chapter 2: A comparative characterization of estrogens used in hormone therapy via estrogen receptor (ER)-α and -β.** This chapter is composed of an article published in *The Journal of Steroid Biochemistry and Molecular Biology* comparing the effects of estrogens used in conventional and bioidentical HT on gene regulation, as well as proliferation and anchorage-independent growth of the MCF-7 BUS human breast cancer cell line. All experiments and data analysis were performed by the candidate.
3. **Chapter 3: Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy.** This chapter is composed of an article published in *Biochemical and Biophysical Research Communications* investigating the off-target activities of selected progestins via the estrogen- and androgen receptors. The candidate performed and analyzed all experiments pertaining to the estrogen receptor experiments, while contributing equally to Dr. Renate Louw-du Toit in terms of the

writing, editing and intellectual input of the publication. The candidate thus shares first authorship with Dr. Renate Louw-du Toit.

4. **Chapter 4: Upregulation of estrogen receptor-regulated genes by first generation progestins requires both the progesterone receptor and estrogen receptor alpha.** This chapter contains the results of a study investigating the effects and potential underlying mechanism(s) of selected progestins used in HT on the proliferation and anchorage-independent growth of the MCF-7 BUS human breast cancer cell line, as well as the expression of ER-regulated target genes. All experiments and data analysis were performed by the candidate.
5. **Chapter 5: Conclusion and Future Studies.** This chapter discusses the overall results of the study and draws conclusions based on the new findings presented in Chapters 2-4, together with the existing information of estrogens and progestins used in HT at the physiological and cellular level. This chapter also provides perspectives for future studies.

Two appendices are presented at the back of the thesis. Appendix A contains additional experimental data, while Appendix B contains other outputs of the PhD study as well as other publications to which the candidate contributed.

Consistent with manuscript format, the collective term “we” and “our” is often used in this thesis. However, all the experimental work was performed by the candidate, with the exception of the experimental work for the AR conducted by Dr. Renate Louw-du Toit as disclosed at the beginning of Chapter 3. As each chapter of the thesis is presented as an individual publication, some overlap is found between the chapters.

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Chapter 1

Hormone Therapy and Breast Cancer: Emerging Steroid Receptor Mechanisms

Hormone Therapy and Breast Cancer: Emerging Steroid Receptor Mechanisms

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Abbreviations: AI's, aromatase inhibitors; AR, androgen receptor; bDHEA, bioidentical dehydroepiandrosterone; bE_1, bioidentical estrone; bE_2, bioidentical estradiol; bE_3, bioidentical estriol; bHT, bioidentical hormone therapy; CBG, corticosteroid-binding globulin; CEE, conjugated equine estrogens; CMA, chlormadinone acetate; CPA, cyproterone acetate; DHT, dihydrotestosterone; DRSP, drospirenone; E_1, estrone; E_2, estradiol; E_3, estriol; ELITE, Early Versus Late Intervention Trial; EMAS, European Menopause and Andropause Society; EPHT, Estonian Postmenopausal Hormone Therapy; ER, estrogen receptor; ESPRIT, Estrogen in the Prevention of Re-infarction trial; GES, gestodene; GPER, G-protein estrogen receptor; GPR30, G protein-coupled receptor 30; GR, glucocorticoid receptor; HERS, Heart and Estrogen/Progestin Replacement Study; IMS, International Menopause Society; HT, hormone therapy; KEEPS, Kronos Early Estrogen Prevention Study; LNG, levonorgestrel; MPA, medroxyprogesterone acetate; MR, mineralocorticoid receptor; MWS, Million Women Study; NAMS, North American Menopause Society; NES, nestorone; NET-A, norethisterone acetate; NGM, norgestimate; NoMAC, nomegestrol acetate; P_4, progesterone; PEPI, Postmenopausal Estrogen and Progestin Intervention; PR, progesterone receptor; SERMS, selective estrogen receptor modulators; SHBG, sex hormone-binding globulin; T, testosterone; WHI, Women's Health Initiative; WISDOM, The Women's international study of long-duration oestrogen after menopause

Abstract

Hormone therapy (HT) has been used by millions of women for several decades to relieve the symptoms of menopause. Although effective at relieving menopausal symptoms, HT has been associated with several severe side-effects such as coronary heart disease, stroke and increased invasive breast cancer risk. Interestingly, estrogen-progestin HT combinations have been associated with a greater breast cancer risk than estrogen only HT regimens. The highly publicized side-effects of HT have caused many women to seek alternatives to conventional HT, including the controversial custom-compounded bioidentical hormone therapy (bHT), suggested to not increase breast cancer risk. Considering that breast cancer is the most prevalent cancer among women worldwide, understanding the mechanism behind the increased breast cancer risk associated with HT is a priority. Although estrogens and the estrogen receptor were historically considered the principal factors promoting breast cancer development and progression, evidence has highlighted a role for other members of the steroid receptor family including the progesterone, androgen, glucocorticoid and mineralocorticoid receptors in breast cancer pathogenesis. Moreover, recent studies have revealed a role for crosstalk between steroid receptors and their signaling pathways in breast cancer. The implications of this crosstalk on the breast cancer risk associated with HT therefore requires investigation, especially since interactions between many different steroid receptors have been reported. In this review, we discuss examples of HT used for the relief of menopausal symptoms, highlighting the distinction between conventional HT and custom-compounded bHT. We also summarize the current knowledge regarding the role of steroid receptors in mediating the carcinogenic effects of hormones used in HT, with special emphasis on the influence of the interplay or crosstalk between steroid receptors. Unraveling the intertwined nature of steroid hormone receptor signaling pathways in breast cancer biology may reveal novel prevention or

treatment options and lead to the development of HT that does not cause increased breast cancer risk.

1. Introduction

Menopause is characterized by the natural, age-related decrease in endogenous estrogen production in women, often leading to a variety of symptoms such as hot flashes, mood swings and night sweats [1–3]. Conventional United States Food and Drug Administration (FDA)-approved hormone therapy (HT) has been used for decades to alleviate these symptoms and is typically administered as estrogen alone to hysterectomized women, or an estrogen-progestin combination to women with a uterus [1–4]. While the estrogen component alleviates the symptoms of menopause by compensating for reduced endogenous estrogen production, the progestin constituent counteracts the proliferative effects of estrogens on the uterine epithelium [1]. Even though HT is effective in relieving menopausal symptoms, some HT regimens have been associated with several severe side-effects including coronary heart disease, stroke and increased invasive breast cancer risk [4–9]. Considering that breast cancer is the most prevalent cancer among women in developed counties [10–12], the association between HT and increased breast cancer risk is of significant concern.

The increased breast cancer risk linked to conventional HT has caused many women and medical professionals to seek various safer HT options, including the use of 'natural' alternatives such as custom-compounded bioidentical HT (bHT) [13,14]. Notably, some bioidentical hormones such as bioidentical estradiol (bE_2) or bioidentical progesterone (bP_4) are available in FDA-approved standard dose prescription medications [15,16]. However, unlike these FDA-approved HT products containing bioidentical hormones, custom-compounded bHT formulations are administered in personalized doses and are typically composed of a mixture of up to six hormones [17,18]. Uncertainty remains regarding the efficacy and safety of custom-compounded bHT, especially pertaining to these multiple-hormone combination therapies [17,18]. Although proponents of bHT claim that there is in fact evidence to support the efficacy and safety of custom-compounded bHT in terms of breast

cancer risk [19,20], these claims are unsubstantiated due to the lack of large-scale, double-blinded clinical trials investigating custom-compounded multiple-hormone bHT regimens at various doses.

Estrogen only and estrogen-progestin combination conventional HT have both been implicated in increased breast cancer risk, however, evidence suggests that the estrogen-progestin combination therapies are associated with a greater risk than the estrogen alone therapies [5,6,9,21,22]. Estrogens predominantly mediate their effects by binding to the estrogen receptor (ER), while progestins are synthetic progestogens (progesterone receptor (PR) ligands) that were designed to mimic the activity of the natural progestogen, progesterone (P_4), by binding to the PR. However, it is known that some progestins can bind to the glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and androgen receptor (AR) ([23–25], reviewed in [26–28]). Whether progestins bind to the ER is contradictory. While some studies suggest that medroxyprogesterone acetate (MPA) and norethisterone (NET) can bind to the ER and elicit estrogenic activity, others suggest that they do not (reviewed in [28]). Furthermore, it has been suggested that it is the progestin metabolites rather than the parent progestin itself that bind to the ER [29,30]. Although several studies have investigated effects of progestins via steroid receptors other than the PR [27,31], these studies seldom directly compare different progestins in parallel and often use cell lines that endogenously express other steroid receptors to which progestins can bind, which may result in inaccurate results in terms of binding affinities, as well as potencies and efficacies for gene expression. It is thus essential that the pharmacological properties of the progestins for each individual steroid receptor are determined in parallel in a model system expressing only the receptor of interest, as has been done for MPA and NET via the human GR, MR and AR [23–25].

The role of the ER, which exist as two subtypes transcribed from two distinct genes [32–34], ERα and ERβ [35], has been extensively studied in breast cancer cell biology. Traditionally, estrogens and ERα were thought to be the main etiological factors contributing to breast cancer pathogenesis, while the PR was considered only as an indicator of a functional ER in breast cancer tumors, implying that the cancer should be sensitive to endocrine targeting therapies [36–38]. However, recent studies have highlighted novel roles for the PR in breast cancer cell biology [39–42]. Two main PR isoforms have been identified, PR-A and PR-B [43], and have been shown to elicit differential effects [44–51]. A recent study has shown that unliganded PR-B enhances the effects of ER agonists on ERα-mediated breast cancer cell proliferation and gene expression by forming a complex with ERα [52]. Moreover, it has been shown that when PR-B is activated by P_4 or the synthetic PR agonist, promegestone (R5020), it is recruited to the ERα complex and redirects the complex to different target genes such that the new gene expression profile is associated with a good clinical outcome [40]. ERα has also been shown to be required for PR-mediated increased cell proliferation and the expression of PR-regulated genes induced by the progestin MPA [41]. Interestingly, the presence and critical roles of other steroid receptors in breast cancer cell biology has been highlighted in recent studies. For example, the AR is expressed in 90% of breast cancer tumors (reviewed in [53]) and its expression is associated with either a good or poor prognosis depending on the absence or presence of the ER [54]. Similarly, GR expression has been associated with a good outcome in ER-positive cancers, but is associated with a poor outcome in ER-negative cancers [55,56].

Steroid receptor signaling pathways have often been studied in isolation, however, it is becoming increasingly clear that these pathways are intertwined. The ability of some steroid hormones, such as progestins, to activate multiple steroid receptors, coupled with the complexity of steroid receptor crosstalk, highlights the intricacies of the mechanisms through which hormones used in HT may increase breast cancer risk and promote breast cancer

pathogenesis. In order to elucidate the involvement of steroid receptor crosstalk in the mechanism behind HT and increased breast cancer risk, additional comparative studies of hormones used in HT are need at the cellular level. The aim of this review is to highlight differences between conventional HT and custom-compounded bHT and to discuss known mechanisms behind conventional HT-induced increased breast cancer risk with an emphasis on the role of steroid receptor crosstalk.

2. Menopause and hormone therapy

Menopausal transition typically occurs in women between the ages of 40 and 60 years and is characterized by the natural age-related loss of ovarian follicular function leading to decreasing endogenous estrogen, P_4 and testosterone (T) levels (Table 1) [1,2]. There are three main endogenous human estrogens, namely E_2, estriol (E_3) and estrone (E_1), the latter being the most abundant circulating estrogen in post-menopausal women (Table 1). However, E_1 is not present in sufficient levels to prevent the symptoms of menopause, such as amenorrhea, hot flushes, night sweats, vaginal atrophy and mood fluctuations [1,2,20]. HT was first administered in the 1930s to alleviate these menopausal symptoms [4], but also to prevent the medical implications of decreased endogenous estrogen levels including osteoporosis, Alzheimer's disease, arthritis, coronary heart disease and cataract formation [1,2]. Today, a large variety of HT regimens are commercially available and can be broadly divided into conventional HT and custom-compounded bHT.

Table 1. Serum estradiol (E_2), estriol (E_3), estrone (E_1), progesterone (P_4) and testosterone (T) levels in pre- and post-menopausal women [57–75]**.**

	E_2	E_3	E_1	P_4	T
Pre-menopausal (pg/ml)	7 - 400	8 - 2 408	12 - 144	566 - 15 700	217 - 2 200
Post-menopausal (pg/ml)	1 - 20	< 10	7 - 44	39 - 700	461 - 1050

2.1. Conventional hormone therapy

The term conventional HT can be interpreted in many ways due to the fast-evolving nature of drug discovery, however, for the purposes of this review, conventional HT will refer to all FDA-approved HT regimens available in the United States of America (USA). Conventional HT regimens are marketed under different brand names and contain either natural, synthetic or bioidentical hormones which are available in standardized doses and various routes of administration. Depending on the HT, it can be administered either orally, subcutaneously, transdermally, intravaginally or by intramuscular injection [1,3].

HT preparations are composed of various hormones that can be ascribed to a specific class. Class A steroids include hormones that are naturally occurring and administered without chemical modification. For example, conjugated equine estrogens (CEE) containing estrogens such as equilin are extracted from pregnant mare's urine [76,77]. Although, these steroids are naturally occurring, they are not endogenous to the human body [76,77]. Class B steroids are often referred to as natural or bioidentical, however these hormones are chemically synthesized from a natural steroidal precursor using numerous chemical reactions [78] and are thus semi-synthetic [58,77,79,80]. Class C steroids differ from class B steroids in that they are synthesized from non-steroidal, rather than steroidal, precursors in a process called total synthesis [77]. The shortcoming of class B and C steroids is that various isomers are produced during the synthesis process, with only one of these isomers structurally identical to the endogenous human hormone [14,77]. For example, during total E_1 synthesis, eight racemates (differentiated by the left- and right-handed enantiomers of a chiral molecule) are produced, resulting in 16 isomers of which only one is structurally and biochemically identical to endogenous human E_1 [77]. The remaining isomers have different structures and varying degrees of estrogenicity, while some are even completely inactive [77]. Lastly, class D steroids are man-made steroidal compounds synthesized either from the same steroidal plant precursors

as class B hormones by semi-synthesis, or from nonsteroidal starting material by total synthesis [77]. Examples of class D steroids include estrogens such as estropipate, esterified estrogens and ethinylestradiol (EE), as well as the progestins MPA, NET acetate (NET-A), levonorgestrel (LNG) and norgestimate (NGM).

Premarin is an example of a HT containing natural CEE that has been effective in relieving menopausal symptoms from as early as 1942 [4]. Various other estrogens have subsequently become available for use in conventional HT and include synthetic, rather than natural CEE, bE_2, as well as the less commonly used esterified estrogens, estropipate and synthetic E_2 derivatives including EE, E_2 valerate, E_2 cypionate and E_2 acetate (Table 2). Although estrogen only HT is effective at relieving menopausal symptoms, studies in the 1960's reported increased incidence of endometrial cancer in Premarin users [4,81,82]. This necessitated the addition of a progestin to CEE regimens for women with a uterus, to prevent estrogen-induced endometrial hyperplasia [4]. Progestins are used to mimic the activity of P_4, but have a longer half-life and a higher bioavailability [31,77]. Products such as Prempro (CEE-MPA) and Provera (MPA) (Table 3) thus became commercially available as early as 1965 [83]. Although Provera is produced as a progestin-only HT, it is administered in combination with an estrogen only HT [84]. Various generations of progestins have subsequently been developed, derived either from P_4, T or the MR antagonist spironolactone, where each new generation is designed to have a greater affinity for the PR and elicit biological effects more like P_4 than progestins from the earlier generations [28,85,86]. Note that P_4-derivatives can be either 17-hydroxy-P_4 derivatives or 19-Nor-P_4 derivatives.

Progestins currently used in FDA-approved HT include the first-generation progestins MPA and NET-A, second-generation progestin LNG, third-generation progestin NGM and the fourth-generation progestin, drospirenone (DRSP) (Fig.1). Not all progestins are used clinically in the USA. For example, nomegestrol acetate (NoMAC) is used in HT in Europe

[22], but not in the USA. Interestingly, NoMAC and progestins such as nestorone (NES), and gestodene (GES) (Fig. 1) are currently being investigated in clinical trials for use in contraception in the USA [86,87].

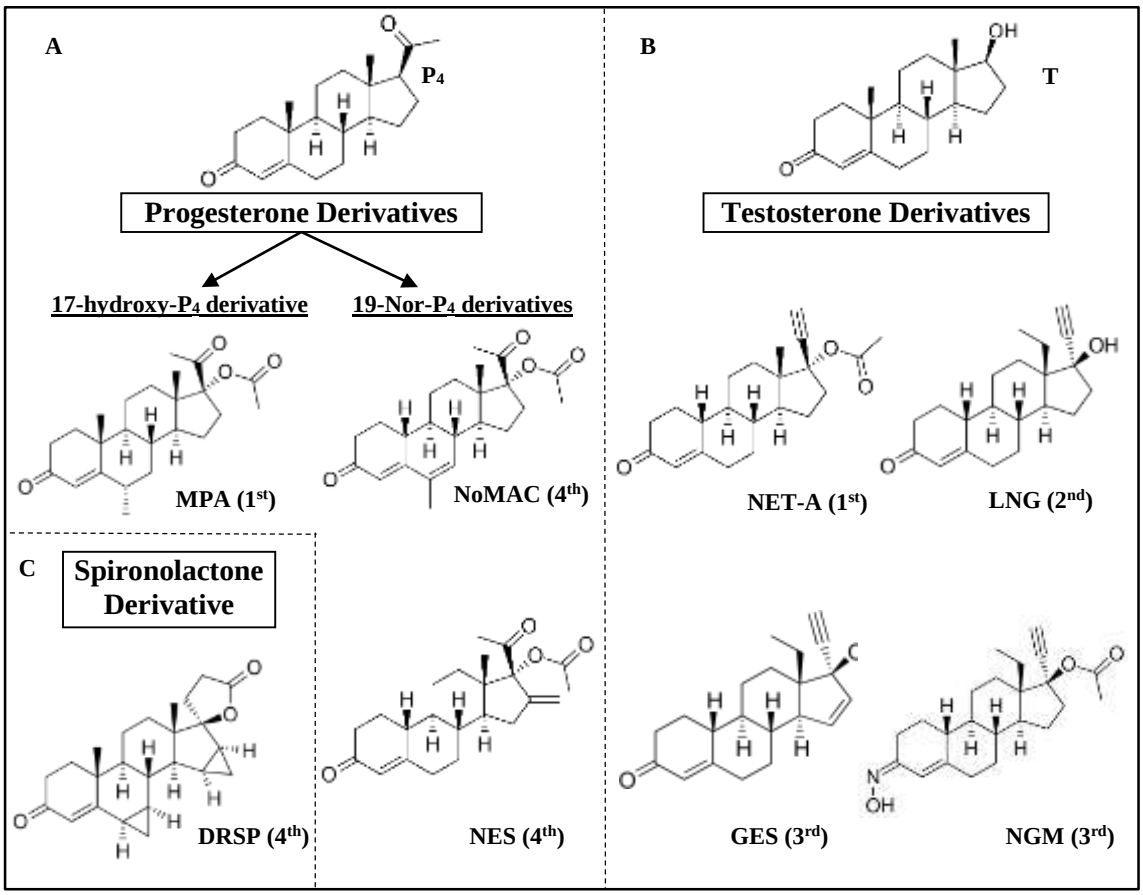

Figure 1. Structures of various first (1st)-, second (2nd)-, third (3rd)- and fourth (4th)-generation progestins. Progestins structurally related to **(A)** progesterone (P_4) include the first-generation, 17-hydroxy-P_4 derivative, medroxyprogesterone acetate (MPA) and the fourth-generation, 19-Nor-P_4 derivatives, nomegestrol acetate (NoMAC) and nestorone (NES), while progestins structurally related to **(B)** testosterone (T) include the first-generation progestin, norethisterone acetate (NET-A), the second-generation progestin, levonorgestrel (LNG), and the third-generation progestins, gestodene (GES) and norgestimate (NGM). **(C)** The fourth-generation progestin drospirenone (DRSP) is currently the only progestin structurally related to spironolactone, a MR antagonist. Structures were obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov).

The continual evolution of HT is thought to be aimed at designing estrogens and progestogens that effectively manage menopausal symptoms without eliciting unwanted side-effects. More

recent advances in HT evolution saw the introduction of bioidentical hormones. FDA-approved bE_2 only products [88,89] are available in standardized doses in products such as Alora, Vivelle-Dot, Divigel, Elestrin, Estrogel and Estrace (Table 2), or in combination with progestins such as NET-A (Activella, Mimvey, Combipatch), DRSP (Angeliq), NGM (Prefest) or LNG (Climara Pro) (Table 3). Interestingly, although FDA-approved bP_4 is available as Prometrium in the form of a cream or as a pill that is administered together with an estrogen only HT, there are no standardized FDA-approved bE_2-bP_4 combination formulations available. Notably, bP_4 is administered in a micronized form, referring to the fact that the particle size has been decreased to generate finer powders that are more readily absorbed, and thus have an increased bioavailability to compensate for the short half-life of the natural hormone [19,90]. However, both oral and transdermal micronized bP_4 formulations are dissolved in peanut oil and consequently cannot be used by women with nut allergies [91–95]. Another alternative HT regimen involves the use of SERMs (selective estrogen receptor modulators). SERMs elicit tissue-selective estrogenic activity by acting as ER agonists in bone tissue, increasing bone mineral density and bone strength, but as ER antagonists in the breast and endometrium to prevent breast and endometrial cancer [96]. FDA-approval was recently granted to Duavee, a CEE-SERM (bazedoxifene) combination to be used for the relief of menopausal symptoms as well as to prevent post-menopausal osteoporosis [96–100].

Estrogen only and progestogen-containing FDA-approved HT products are outlined in Tables 2 and 3, respectively. These tables indicate the routes of administration and dose range of the various HTs and, where available, the resulting serum concentrations of E_2, E_1, EE, the progestogens or bazedoxifene. Although these conventional HT regimens are still widely used and have proved efficient at relieving menopausal symptoms [101], the reported side-effects associated with conventional HT have caused many women to seek alternate menopausal relief in the form of custom-compounded bHT [77].

Table 2. FDA-approved estrogen only HT products [102–104] (a-ad).

Composition		Products (™)	Route of Administration	Administered Doses (min - max) (mg)	Serum E_2 (pg/ml)	Serum E_1 (pg/ml)
Natural CEE		Premarin	Pill Vaginal Cream	0.3 - 1.25 0.625*	NA NA	87 - 4 500 42 - 600
Synthetic CEE		Cenestin, Enjuvia	Pill	0.3 -1.25	NA	20 - 85
bE_2		Alora, Climara, Esclim, Estraderm, Estradot, Menostar, Minivelle, Vivelle-Dot	Patch	0.025 - 0.1	6 - 174	9 - 65
		Divigel, Elestrin, Estrogel	Topical Gel	0.025 - 2.0*	9 - 67	33 - 66
		Estrace, Gynodiol	Pill	0.5 - 2.0	203 - 355	ND
		Estrace	Vaginal Cream	0.1*	ND	ND
		Estrasorb	Topical Cream	2.5*	59 - 70	ND
		Estring	Vaginal Insert	2.0	8 - 63	44 - 66
		Evamist	Topical Spray	1.53#	11 - 57	NA
		Vagifem	Vaginal Tablet	0.01 - 0.025	6 -21	17 - 28
E_2 derivatives	**E_2 valerate**	Delestrogen	IM Injection	10 - 40	ND	ND
	E_2 cypionate	Depo-Estradiol	IM Injection	1.0 - 5.0	ND	ND
	E_2 acetate	Femring	Vaginal Ring	0.05 - 0.10	41 - 76	36 - 46
		Femtrace	Pill	0.45 - 1.8	57 - 177	155 - 680
Esterified estrogen		Menest	Pill	0.3 - 2.5	ND	ND
		Estragyn	Vaginal Cream	1.0*	ND	ND
Estropipate		Ogen	Pill	0.75 - 3.0	ND	ND
			Vaginal Cream	1.5*	ND	ND

* - mg hormone per g cream # - mg hormone per single HT spray **NA** - not applicable as these HT products do not contain the relevant hormone
ND - serum concentrations have not been reported **a-ad** - package inserts can be found at the end of the reference list **IM** - intramuscular injection

Table 3. FDA-approved progestogen or SERM-containing HT products [105,106] (ae-ao).

Composition	Products	Route of Administration	Administered Doses (mg)		Serum E_2 (pg/ml)	Serum E_1 (pg/ml)	Serum Progestogen (pg/ml)
Progestogen only							
Micronized bP_4[#]	Prometrium	Pill	100 - 300		NA	NA	310 - 60 600
MPA[#]	Provera	Pill	2.5 - 10		NA	NA	710 - 1 010
Estrogen-Progestin Combinations							
			Estrogen	***Progestin***			
CEE + MPA	Prempro	Pill	0.3 - 0.625	1.5 - 5.0	NA	79-175	1 200 - 4 800
EE + NET-A	FemHRT	Pill	0.0025 - 0.01	0.5 - 1.0	34 - 38 (EE)	ND	4 000 - 10 700
E_2 + NET-A	Activella, Mimvey	Pill	0.5 - 1.0	0.1 - 0.5	26 - 28	196 - 200	2 375 - 5 250
	Combipatch	Patch	0.05	0.14 - 0.25	27 - 71	49 - 78	386 - 1 060
E_2 + DRSP	Angeliq	Pill	0.5 - 1.0	0.25 - 0.5	30 - 64	166 - 362	2 000- 85 000
E_2 + NGM	Prefest	Pill	1.0	0.09	39 - 50	285 - 325	515 - 643
E_2 + LNG	Climara Pro	Patch	0.045	0.015	27 - 54	33 - 82	110 - 194
Estrogen-SERM Combinations							
CEE + bazedoxifene	Duavee	Pill	0.45 + 20		NA	≤ 2 600	≤ 6 900 (SERM)

[#] - progestogen-only products that are generally prescribed in combination with an estrogen only product

NA - not applicable as these HT products do not contain the relevant hormone

ND - serum concentrations have not been reported

ae-ao - package inserts can be found at the end of the reference list

2.2. Custom-compounded bioidentical hormone therapy

Custom-compounded bHT refers to the constitution of personalized bHT regimens containing class B steroids by compounding pharmacies, and can include any number of bioidentical hormones including bioidentical estrone (bE_1), estriol (bE_3), testosterone (bT), dehydroepiandrosterone (bDHEA), bE_2 and/or bP_4 [77,80]. Unlike FDA-approved HT which is available in standardized doses, a customized dose of bHT is prescribed based on a saliva test that estimates serum hormone levels [19,95]. However, this method contradicts a global consensus that the lowest possible dose of HT that effectively relieves menopausal symptoms should be prescribed [20,89,107]. Moreover, numerous studies have shown a poor correlation between hormone levels found in saliva and serum, due to saliva hormone levels fluctuating based on time of day, diet and other variables [19,20,58,80,95,108].

The safety and efficacy of custom-compounded bHT is controversial and proponents of bioidentical hormones claim that these hormones are natural and identical in structure to endogenous human hormones, hence they are safer than conventional HT products [80]. This is despite the fact that the proposed 'natural' hormones used in custom-compounded bHT are in fact semi-synthetic and synthesized in a similar manner to the bioidentical hormones used in FDA-approved HT [20,58,80,108]. However, because of this 'natural' classification, custom-compounding pharmacies may legally dispense products containing bioidentical hormones without obtaining FDA-approval for each product [80,108]. This means that personalized hormone preparations are dispensed without the rigorous quality control checks that FDA-approved drugs are subjected to. Furthermore, unlike conventional HT products, custom-compounded bHT products lack black-box warnings of the potential adverse effects of HT [17,77,108]. A major concern raised by randomized FDA-checks is the fact that custom-compounded preparations frequently result in accidental under- or overdosing, possibly due to variations in purity and/or human error associated with personalized combination constitution

[17,58,77,92,108,109]. Moreover, compounded bHT patches have been shown to yield lower serum estrogen levels than bioequivalent standard-dose E_2 patches, emphasizing that the pharmacodynamics of compounded bHT requires further research and/or regulation [92].

Clinical trials investigating the safety and efficacy of bP_4 creams over a 12-week period have revealed that custom-compounded micronized bP_4 creams do not relieve vasomotor symptoms, nor inhibit the proliferative effects of E_2 on the endometrium, nor improve mood swings and libido [72,75,110–113]. It has been suggested that this may be due to insufficient bP_4 absorption and thus low bioavailability [69,70,113–116]. However, the Postmenopausal Estrogen and Progestin Intervention (PEPI) trial revealed that oral micronized bP_4 effectively relieves vasomotor symptoms [117–119], suggesting that oral micronized bP_4 may be more effective at relieving vasomotor symptoms than localized micronized bP_4 creams.

Custom-compounded bHT often contains bE_2 in combination with bE_3 and/or bE_1 [17,20,79,80]. Biest or triest combination regimens can be obtained from compounding pharmacies, where a biest is composed of bE_2 and bE_3 in a 20:80 ratio and a triest is composed of bE_2, bE_3 and bE_1 in a 10:80:10 ratio [13,19,108,120]. Proponents of bHT claim that bE_3 and bE_1 are weaker, safer estrogens than bE_2 [80,120,121] and that bE_3 antagonizes the potent estrogenic activity of bE_2 [19,122]. To the best of our knowledge, however, a detailed comparison of the agonist and antagonist properties of these bioidentical hormones for transactivation and transrepression via the ER subtypes has not been previously reported. In fact, the incorporation of E_3 into bHT products appears to be based on murine work conducted by Lemon, more than 30 years ago [123,124], showing that E_3 was more protective against carcinogen-induced neoplasms than E_2 or E_1 (reviewed in [79]). However, these claims have not been validated in human models [122] and large-scale, double-blinded, placebo-controlled clinical trials evaluating the safety and efficacy of E_3 or bE_3 are lacking. However, some preliminary small-scale trials have suggested that E_3 sometimes relieves vasomotor symptoms

but does not protect against bone loss [125,126], while others have provided evidence that E_3 can protect against bone loss [127–131], highlighting the uncertainties regarding E_3 use. Interestingly, although there are no FDA-approved E_3-containing HT products [19], bE_3 is used in regulated HT products in parts of Europe and Asia [58,132], where it is usually referred to as E_3 rather than bE_3 [133,134].

Androgens such as bT and bDHEA, are also often used in personalized bHT formulations in combination with estrogens and/or progestogens to relieve the symptoms of menopause [19,109,120,135]. However, observational studies have reported adverse effects of androgen-containing HT such as endometrial cancer, hair loss, acne, hirsutism, and deepening of the voice [2,109]. In fact, cases of endometrial cancer have been reported in users of oral bHT products containing combinations of bE_2, bP_4, bT and bDHEA or bE_1, bE_2, bE_3, bP_4, bT and bDHEA [109]. Interestingly, various androgens, including the T precursor, methyltestosterone, are approved for HT use in Europe [136], while there is no FDA-approved androgen-containing female HT [14,17]. Moreover, although there is a lack of clinical trials examining the effectiveness and possible side-effects of androgen use in HT, bT and bDHEA are distributed by compounding pharmacies in both the USA [19,58,137] and South Africa [138]. In terms of breast cancer risk, the inclusion of bT is especially concerning as T can be aromatized to E_2 within breast tissue [53] and endogenous T levels are only marginally decreased after menopause (Table 1). This suggests that the incorporation of bT into an estrogen-containing bHT may thus result in greater estrogen exposure than intended, which may increase risk of breast cancer development.

Overall, the lack of large-scale clinical trials investigating the safety and efficacy of custom-compounded bHT such as biest and triest regimens [20,58], together with the absence of black-box warnings, lack of thorough regulatory bodies and uncertainties regarding salivary testing, has resulted in a consensus between several organizations including the North American

Menopause Society (NAMS), The International Menopause Society (IMS), The Endocrine Society and The European Menopause and Andropause Society (EMAS) recommending against the use of custom-compounded bHT [107].

3. Hormone therapy and breast cancer risk

Numerous clinical trials and observational studies have associated conventional HT with multiple side-effects such as elevated risk of developing breast-, ovarian- and endometrial cancers, as well as cardiovascular disease and stroke [1,2,5,6,9,15,83,117,139–152]. Considering that breast cancer is the most common cancer in women worldwide and the leading cause of cancer-related deaths in women in developed countries [11,12], the association between HT and breast cancer risk is alarming. Although several studies reported adverse effects associated with HT prior to 2002 [83,117,146,151], it was the highly publicized findings of the Women's Health Initiative (WHI) [6] that caused alarm and confusion about the safety of HT. The WHI study was a large-scale randomized, controlled clinical trial that evaluated the benefits and risks of CEE alone in hysterectomized postmenopausal women, or CEE in combination with MPA in postmenopausal women with a uterus [6]. The results of the trial suggested that CEE-MPA combinations, but not CEE alone, were associated with increased invasive breast cancer risk [6]. In contrast, the Million Women Study (MWS), a cohort study comprising over one million postmenopausal women from across the United Kingdom, found that the use of estrogen alone or estrogen-progestin combinations were both associated with increased invasive breast cancer risk [5]. Interestingly, this study found increased breast cancer risk with all HT preparations investigated, and no difference in risk between specific estrogens (CEE and EE) or progestins (MPA, NET and LNG) [5].

Many additional studies investigating breast cancer risk associated with HT use have been conducted [8,9,21,83,142–145,149–156], often with contradictory results. For example, while

the Women's international study of long-duration oestrogen after menopause (WISDOM) clinical trial also found increased breast cancer risk associated with CEE-MPA use [8], the Heart and Estrogen/Progestin Replacement Study (HERS) I, HERS II [143] and the Estonian postmenopausal hormone therapy (EPHT) clinical trial [144] amongst others [83,145,154,155], found no increased breast cancer risk associated with CEE or CEE-MPA. It is thus evident that results from the above-mentioned studies are often contradictory and have resulted in much confusion regarding the safety of these HT regimens. However, it is noteworthy that the studies showing no increased breast cancer risk had significantly less participants [83,143–145,154,155] than the large-scale WHI, MWS and WISDOM studies [5,6,8].

Notably, although most clinical and observational studies investigating the association between HT and increased breast cancer risk examined the effects of CEE and MPA, a few studies have in fact investigated other estrogens and progestogens. For example, three clinical trials have reported no increased breast cancer risk associated with the use of oral E_2 alone or in combination with NET-A [149,151,152], the latter of which was previously shown to increase breast cancer risk when used in combination with CEE in the MWS [5]. Similarly, at least one other study reported no increased risk with the use of an E_2 only patch [150], while the Estrogen in the Prevention of Re-infarction Trial (ESPRIT) found no increased risk with the use of E_2 valerate alone [156]. However, the Kronos Early Estrogen Prevention Study (KEEPS) found increased breast cancer risk associated with the use of E_2 patches in combination with oral micronized P_4, while the Early Versus Late Intervention Trial (ELITE) also found increased risk with oral E_2 used in combination with a P_4 vaginal gel (reviewed in [9]). In contrast to the above-mentioned studies showing increased breast cancer risk with the inclusion of P_4, the PEPI trial reported no increased breast cancer risk in women administered CEE plus oral micronized P_4 or CEE plus MPA [117]. Similarly, the French E3N cohort study found that estrogen (CEE or bE_2) alone or in combination with oral P_4 or dydrogesterone (a P_4 isomer not

used clinically in the USA) was not associated with increased breast cancer risk. Interestingly, results from the same study showed that other estrogen-progestin combinations containing the progestins MPA, NET-A, medrogestone, chlormadinone acetate (CMA), cyproterone acetate (CPA), promegestone (R5020) or NoMAC, were associated with increased breast cancer risk [21,142]. This French cohort study also suggested that administration of oral versus transdermal E_2 does not influence the degree of breast cancer risk [21,142]. Interestingly, a recent Cochrane review examining the adverse side-effects of HT compiled the results of 22 clinical studies, including most of the above-mentioned studies, and suggested that estrogen-progestin HT combinations increased breast cancer risk, while use of estrogen-only HT did not [9]. It is clear from the above that more clinical studies investigating the association between different hormones used in HT and breast cancer are needed.

Taken together, the evidence in the literature investigating an association between specific hormones used in HT and increased breast cancer risk is contradictory. However, there are many other hormones used in FDA-approved HT products such as esterified estrogens, E_2 acetate, trimegestone and DRSP, or custom-compounded bHT products such as bE_3, bE_1 and bT, that have not been investigated in large-scale clinical trials or cohort studies, and thus it is not known whether these steroid hormones are linked to increased breast cancer risk. At the molecular level, steroid hormones predominantly elicit their effects by binding to steroid receptors which are ligand-activated transcription factors belonging to the nuclear receptor superfamily [157,158].

4. Steroid receptors as mediators of hormone activity and carcinogenesis

When steroid hormones enter the bloodstream, they bind to various serum binding proteins such as sex-hormone-binding globulin (SHBG), corticosteroid-binding globulin (CBG) and/or albumin (Table 4). SHBG predominantly binds estrogen and T, while CBG binds cortisol and

P_4 [159]. The hormones bound to SHBG or CBG are considered unavailable to tissues, while the free, unbound hormones and those bound to albumin are considered biologically available to enter cells of target tissues and elicit a response by binding to a steroid receptor [160–162]. As indicated in Table 4, some estrogens and progestogens bind to SHBG and/or CBG, while others do not, resulting in large differences in the availability of estrogens and progestogens used in HT. For example, while approximately 37% of serum E_2 can bind to SHBG, 16% of E_1 binds, only 1% of E_3 binds and EE does not bind at all. As a result, E_3, E_1 and EE are mostly available to enter cells of target tissues (Table 4) suggesting that these estrogens may be more abundant than E_2 in target tissues, and therefore may compete with E_2 for binding to the ER. In the same context, progestins also differentially bind to SHBG as shown by 35.5% of NET and between 47.5 - 73.6% of LNG binding, while MPA and DRSP do not bind at all (Table 4). Considering that the data in Table 4 indicates that progestogens are mostly available, it is plausible that even when administered at low concentrations, they may be more abundant in target tissues than endogenous steroid hormones, and thus may compete with these hormones for binding to their cognate steroid receptors.

Table 4. Binding of hormones to transport proteins [26,31,63,162–166]**.**

	SHBG (%)	CBG (%)	Albumin (%)	Free (%)	Available (%)
Estrogens					
E_2	37	0	61	2	63
E_3	1	0	91	8	99
E_1	16	0	80	4	84
EE	0	0	99	1	100
Progestogens					
P_4	0.6	17.7 - 36	79.3 - 90.3	0 - 2.4	81.7 - 92.7
MPA	0	0	88	12	100
NET	35.5	0	60.8	3.7	64.5
LNG	47.5 - 73.6	0	25.5 - 50	0 - 2.5	26.4 - 52.5
DRSP	0	0	95 - 97	3 - 5	100

Steroid hormones can permeate the cell membrane and elicit effects by binding to steroid receptors, such as the GR and MR, as well as the sex-steroid hormone receptors, the ER, PR and AR [157]. A high degree of homology exists between the steroid receptor family, and the receptors are organized into four evolutionary-conserved domains (Fig. 2), namely the N-terminal domain containing the ligand-independent activation function 1 (AF-1) region, the highly conserved DNA-binding domain (DBD), a hinge region and a relatively conserved ligand-binding domain (LBD) containing an additional ligand-dependent activation function (AF-2) region [157,158,167–170]. Generally, unliganded AR, GR and MR are found in the cytoplasm, the ER and PR-A predominantly in the nucleus [159,171], while PR-B is distributed between the cytoplasm and the nucleus [157,171]. Unliganded steroid receptors are associated with chaperone proteins such as heat shock protein (Hsp)90 and Hsp70 [172], but dissociate from the chaperone proteins upon ligand binding, as the steroid receptors undergo a conformational change [157]. The ligand-bound cytoplasmic steroid receptor can then enter the nucleus [157], where it generally binds as a dimer to semi-palindromic DNA sequences known as hormone response elements (HREs) to activate target gene expression (transactivation) [158,167], or as a monomer to negative-HREs (nHREs) or other DNA-bound transcription factors such as nuclear factor kappa B (NFκB), to repress target gene expression (transrepression) (reviewed in [167]). Steroid hormones can also elicit non-genomic effects either by binding to membrane-bound receptors to activate signaling cascades which ultimately result in the downstream regulation of gene expression, or by interacting with membrane kinases to activate rapid signaling pathways [167,173–179].

The ER, PR, AR and GR are expressed in most breast cancers, and it is therefore not surprising that they all play functional roles in breast cancer cell biology (reviewed in [180]). Moreover, emerging evidence suggests that their signaling pathways are not always distinct, but are in fact extensively intertwined. This too is not surprising considering the high degree of homology

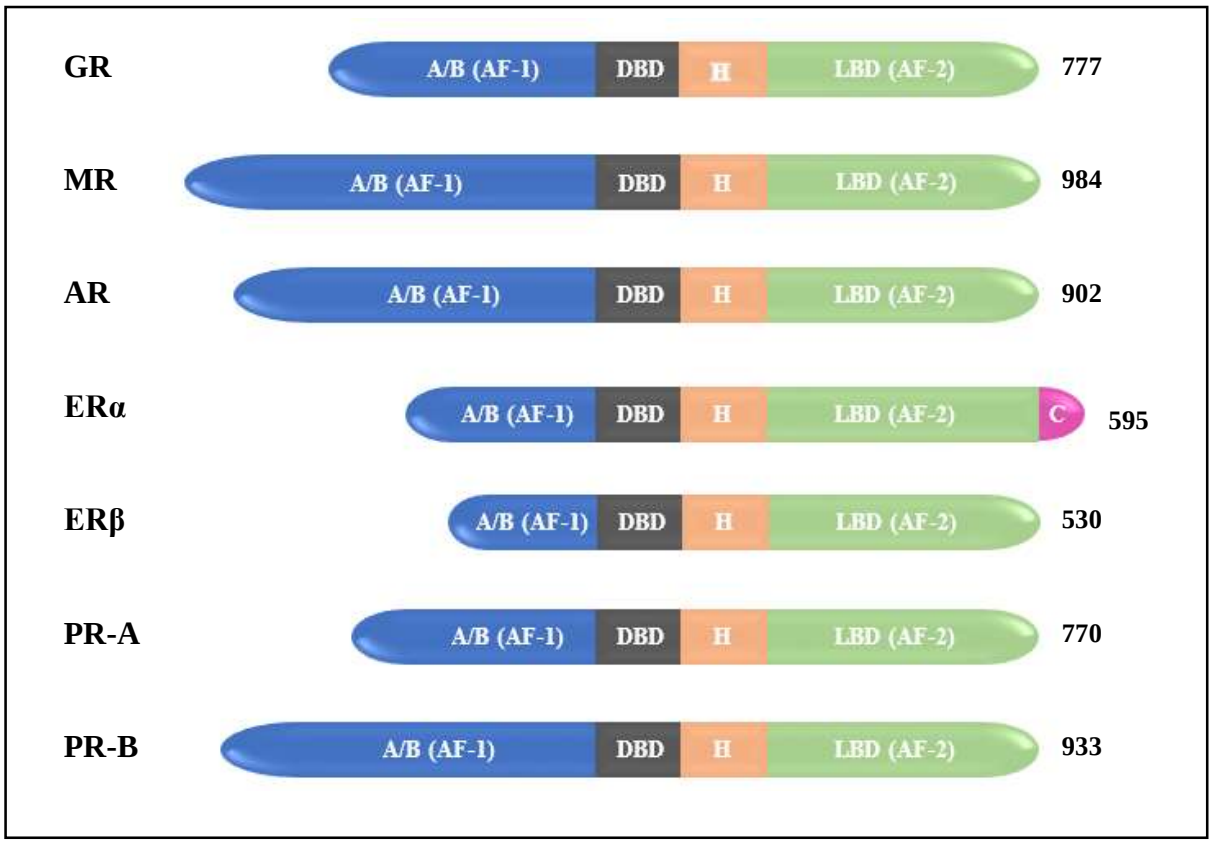

Figure 2. A simplified representation of the structure of steroid hormone receptors. These receptors contain a variable N-terminal domain (A/B) containing the ligand-independent activation function 1 (AF-1) region, a highly-conserved DNA-binding domain (DBD), a hinge region (H) enabling flexibility, and a relatively conserved ligand-binding domain (LBD) containing the ligand-dependent activation function (AF-2) region. ERα contains an additional C-terminal domain (C) of which the function is not known. The values indicated on the right represent the number of amino acids constituting each steroid receptor. Figure adapted from [157].

between the steroid hormone receptors and their cognate DNA-binding sites [157,158,167–170,181]. In the following sections, the role of steroid receptors and their interplay in breast cancer is summarized, with a focus on the known effects of estrogens and progestogens via the ER and PR, respectively.

4.1. Estrogens and the ER

The first association between estrogen signaling and breast cancer can be traced back to 1896 [182] and since then, copious *in vivo* and *in vitro* studies have shown that estrogens promote

breast cancer development and progression [183–195]. Although the precise mechanisms whereby estrogens promote breast cancer is still an area of ongoing research, it is well-established that ERα is crucial for E_2-induced breast cancer cell growth [196,197]. This critical role for ERα was highlighted by a study showing that E_2 exposure did not cause breast cancer tumor formation in ERα knockout mice (reviewed in [196,197]). Considering that the ER is expressed in approximately 75% of breast cancers, current therapies target ER activity or the synthesis of endogenous estrogen [38,49,198]. For example, tamoxifen or fulvestrant are used to antagonize ER signaling by blocking or degrading the ER respectively [38,49,198–200], while aromatase inhibitors (AIs) are used to decrease the production of endogenous estrogens by inhibiting the metabolism of T and androstenedione to E_2 and E_1 respectively.

Estrogens and ER signaling lead to the development and progression of breast cancer largely through the regulation of gene expression [184,192,201–205]. For example, E_2 treatment of the MCF-7 breast cancer cell line results in the upregulation of genes encoding growth factors such as *insulin-like growth factor* (*IGF*)*-binding proteins* and *vascular endothelial growth factor* (*VEGF*) [184,201,206–209]. Furthermore, genes regulating the cell cycle such as *cyclin D1*, *cyclin A2* and *cyclin-dependent kinase 1* (*cdk1*) are also upregulated by E_2 in MCF-7 cells, as are genes promoting proliferation such as *Ki67* [184,210,211]. In contrast, E_2 has previously been shown to downregulate the expression of genes inhibiting proliferation such as *transforming growth factor beta 3* (*TGFβ3*) [184,210], and genes promoting apoptosis such as *caspase 9* [184]. ChIP-seq analysis mapped ER-binding sites to the promoter regions of the *cyclin D1*, *cyclin A2*, *cdk1*, *Ki67* and *TGFβ3* genes in response to E_2 treatment [210], highlighting the role of the ER in mediating the tumor-promoting effects of E_2. However, the ER subtypes, ERα and ERβ, are known to play different roles in breast cancer [34,212–221]. For example, ERα has been shown to promote breast cancer pathogenesis by upregulating the expression of *cyclin D1*, while ERβ inhibited its expression [222]. Interestingly, the role of

ERβ is dependent on whether ERα is expressed or not. In the presence of ERα, ERβ can inhibit ERα-driven proliferation, while in the absence of ERα, ERβ promotes proliferation [34,212–221]. The differential action of the ER subtypes may in part be due to the regulation of subtype-specific target genes [219,220,223–227], possibly due to differences in the N- and C-terminals of ERα and ERβ [228]. In addition, ERβ has been shown to downregulate the transcriptional activity of ERα by modulating the recruitment of transcription factors required by the ERα transcription complex and by increasing ERα degradation [229].

In addition to the full-length ER subtypes, several ER splice variants have been identified in various cell lines, however, it is not clear whether all these variants are also expressed in tissue and whether they are functional proteins (reviewed in [230]). An ERα46 splice variant which lacks part of the N-terminal domain has in fact been detected in breast tumor tissue, but its function is still unknown (reviewed in [228]). In contrast, several ERβ splice variants are expressed in breast tissue and have been shown to differentially regulate estrogen signaling [231–235]. For example, the ERβcx splice variant contains a unique sequence in its LBD and although it cannot bind ligand, it forms heterodimers with ERα, preventing ERα from activating gene expression [236]. In contrast, a second ERβ splice variant which also cannot bind ligand, forms dimers with either ERα or ERβ, blocking their activity [237].

From the above, it is clear that the role of E_2 and the ER subtypes in breast cancer is complex. An added complexity is the fact that many different estrogens are used in HT and it is not clear whether these estrogens will elicit similar effects to E_2 in the breast context. Studies directly comparing the effects of these estrogens on hallmarks of breast cancer such as cell proliferation, migration, invasion and apoptosis are scarce. The limited studies that are available suggest that while both CEE and E_2 increase proliferation, E_2 increased proliferation to a greater extent [187,238]. Furthermore, at least three studies have directly compared the proliferative effects

of E_1, E_2 and E_3 and although all these studies showed that these estrogens increase breast cancer cell proliferation, differences were observed [185,186,189]. For example, Gutendorf and co-workers [185] reported potencies in the picomolar range and showed that E_2 was more potent than E_3, while E_1 was the least potent. In contrast, Lippman and co-workers [186] reported potencies in the nanomolar range and showed that E_2 and E_1 were equipotent, and more potent than E_3. From these studies, it is evident that E_2 is the most potent estrogen, yet it is not clear how the proliferative effects of E_1 and E_3 compare to E_2. Further comparative studies are thus required to clarify this ambiguity. Furthermore, although Lippert and co-workers [189] did not determine potencies, they showed no significant difference in the proliferative effects of 10 nM or 100 nM E_1, E_2 and E_3, and showed that while E_3 also stimulated proliferation at 1 µM and 10 µM, E_2 inhibited proliferation and E_1 had no effect. Interestingly, only Gutendorf and co-workers [185] investigated the proliferative effects of the synthetic estrogen, EE, and showed that it was as potent as E_2. To the best of our knowledge, studies investigating the effects of estrogens on migration, invasion and apoptosis only focused on E_2 and showed increased migration [239,240] and invasion [239–245], as well as decreased apoptosis [245–249], in the ER-positive MCF-7 and T47D breast cancer cell lines.

In terms of gene expression, two studies have directly compared the EC_{50} values of E_2, E_3, E_1 and EE for both ERα- and ERβ-mediated transactivation [29,250], however the EC_{50} values of these studies differed by up to 750-fold for some estrogens. Although both studies found that E_2 and EE were the most potent estrogens, E_3 was the least potent estrogen in one study [250], while E_1 was the least potent in the other [29]. These discrepancies may be due to the different model systems or promoter-reporter constructs used, as the first study used yeast cells and a p406-CYC1 yeast expression vector containing two EREs [250], while the latter study used a cell line derived from HeLa cells stably expressing a plasmid containing one ERE (HELN cells) [29]. In addition to transactivation of gene expression, the estrogen-bound ER can also

transrepress gene expression, however there is a paucity of studies characterizing this mechanism of action. At least two studies have investigated the efficacy and potency of E_2 and/or EE for transrepression of gene expression via ERα [251,252], however, not much is known for other estrogens used in HT or for the transrepressive activities via ERβ. Considering the lack of comparative studies and the fact that the effects of bioidentical hormones have not been compared to the endogenous human hormones or synthetic estrogens such as EE, it is imperative that such molecular studies are conducted. This is critical considering that some estrogens used in HT and bHT have not been tested in large-scale, double-blinded clinical trials and their safety and efficacy is unknown.

4.2. Progestogens and the PR

The role of progestogens including natural P_4 and progestins in breast cancer is not straightforward, as some progestins have been associated with increased breast cancer risk [5,6,9,21], while others and P_4 have not [21,83,143–145,149,151,152,154,155]. In addition, results from studies investigating the effects of progestogens on breast cancer cell proliferation are also contradictory. For example, while some studies have shown that P_4 [253], R5020 [254,255], MPA [256,257] and NET-A [256–258] promote proliferation of the MCF-7, ZR75 or T47D breast cancer cell lines, others have shown that these progestogens are anti-proliferative in the T47D cell line [259–263]. Some studies have even suggested that P_4 and the progestin ORG 2058 are proliferative for one cell cycle, after which they exert anti-proliferative and pro-apoptotic effects [262,263]. Interestingly, the effects of progestogens on proliferation also seem to be dependent on the absence or presence of estrogen. For example, while progestogens such as P_4, MPA, NET, LNG, GES and R5020, have been shown to promote proliferation of the MCF-7 cell line, they exerted anti-proliferative effects in the presence of E_2 [258]. Investigations into the effects of the progestogens on other hallmarks of

breast cancer such as apoptosis, migration and invasion are scarce, and results from the limited studies are ambiguous. For example, some studies suggest that P_4 [256,257], MPA and NET increase apoptosis [257], while others suggest that these progestogens inhibit apoptosis [256,264]. While it has also been shown that P_4, MPA, NES and DRSP can promote migration [265–267] and invasion [266–268] in the T47D [266,267] and ZR75 [265,268] breast cancer cell lines, MPA was found to promote migration and invasion of the T47D cell line to a greater extent than P_4, NES and DRSP [267]. Interestingly, P_4, NES and DRSP, unlike MPA, reduced E_2-induced invasion but not migration [267]. In light of the above, it is clear that more molecular studies, particularly studies directly comparing the effects of the progestogens on hallmarks of breast cancer in the same model system, are needed.

It is well-known that P_4 elicits its biological effects primarily by binding to the PR [269], and that progestins were designed to mimic the actions of P_4 by also binding to the PR [85,86]. However, it is known that some progestins can also bind to and elicit biological effects via steroid receptors other than the PR, such as the AR [25] and GR [24]. Thus, whether the observed effects of the progestogens on proliferation and other hallmarks of breast cancer are mediated by the PR, or any of the other steroid receptors, is still an area of ongoing research. However, at least one study has provided evidence that PR knockdown using siRNA [270], abrogates MPA-induced breast cancer cell proliferation.

Three PR isoforms, PR-A, PR-B and PR-C, transcribed from different promoters of a single gene, have been identified [43,179]. However, only PR-A and PR-B are functional as PR-C lacks a DBD, and thus cannot bind to DNA to activate gene transcription [179,271]. Interestingly, approximately 65% of P_4-regulated genes are regulated only by PR-B, while approximately 4% are regulated only by PR-A and 25% are regulated by both PR isoforms [45,49]. These somewhat unique gene sets result in different biological roles for PR-A and PR-

B, where PR-B mediates P_4-induced normal breast cell proliferation [50,51], while P_4-bound PR-A is implicated in maintaining ovarian and uterine functions [49–51]. The transcriptional activity of the individual isoforms is also cell-specific and is extensively regulated by post-translational modifications including phosphorylation, sumoylation, acetylation and ubiquitination (reviewed in [179]). However, PR-A is generally more transcriptionally active than PR-B in the absence of ligand [47], while PR-B is more transcriptionally active in the presence of agonist [48,49]. The latter may be due to the additional AF-3 region in the N-terminal domain of PR-B, which enables the binding of cofactors to PR-B that cannot bind PR-A [272].

The PR is a well-known ER-target gene and is thus expressed in most ER-positive breast tumors [43,273,274]. Although traditionally thought of only as an indicator of active ER signaling pathways in breast cancer tumors, the role of the PR in breast cancer is quite complex and dependent on multiple factors such as the relative ratio of PR-A to PR-B [179]. PR-A and PR-B are generally expressed at equimolar ratios in the normal mammary gland [275], resulting in the formation of PR-A/B heterodimers that regulate a specific gene set (reviewed in [49]). In contrast, PR-A and/or PR-B expression is often increased in atypical breast lesions, dysregulating the ratio of the PR isoforms [276]. This dysregulation disrupts normal PR signaling due to the predominance of one PR isoform and the subsequent formation of homodimers that regulate a unique gene set (reviewed in [49]). Interestingly, PR-A is upregulated in most ductal carcinomas *in situ* and invasive breast cancers [275–277] and is thought to be more stable than PR-B [48]. Although the exact mechanism behind this has not been fully elucidated [48], it may be due to the fact that PR-B contains six more phosphorylation sites in its N-terminal domain than PR-A, and the increased kinase activity in pre- or early-malignant breast tissue drives PR-B phosphorylation resulting in both PR-B hyperactivity and degradation [278,279]. Moreover, the activity of PR-B, as well as the ER,

AR, GR and MR, can also be repressed by PR-A under normal cellular conditions [157,171,280–286], suggesting crosstalk between steroid receptors. Interestingly, steroid receptor crosstalk mechanisms have been described in the breast cancer context.

4.3. Interplay between ERα and the PR

Recent evidence in the literature suggests that crosstalk between ERα and the PR plays an important role in breast cancer pathogenesis [40–42,52]. For example, it has been suggested that the potent PR agonist, R5020, promotes breast cancer progression by activating kinase cascades via a mechanism requiring both ERα and the PR [287,288]. Furthermore, Giulianelli and co-workers [41] provided evidence of an interaction between ERα and the PR in breast cancer tissue and cell lines, and showed that this interaction is required for MPA-induced gene expression and cell proliferation in the T47D breast cancer cell line. Subsequent studies have revealed that the PR can modulate the transcriptional activity and chromatin localization of ERα through the formation of these ERα/PR complexes [40–42,52]. For example, Daniel and co-workers [52] showed that the unliganded PR can act as a molecular scaffold, resulting in PR, ERα, PELP-1 (proline-, glutamic acid- and leucine-rich protein 1) and IGF1 complexes that alter ERα gene regulation leading to a more aggressive proliferative response upon E_2 stimulation of the MCF-7 cell line. A second study by Mohammed and co-workers [40] showed that P_4- or R5020-bound PR is recruited to the ERα complex in both MCF-7 and T47D cells, and redirects E_2-activated ERα chromatin binding such that the gene expression profile is similar to that of PR alone. A similar mechanism was shown by Singhal and co-workers [42] in primary ER- and PR-positive human tumors, and is reported to lead to decreased proliferation and an improved clinical outcome [40]. The above-mentioned studies suggest that an interaction between ERα and the PR can be associated with either poor or good prognosis in breast cancer, and that the outcome is determined by the absence or presence of PR ligands.

Whether this is true for all PR ligands is not known. This is particularly important for progestins used in HT as some progestins from the earlier generations have been implicated in increasing breast cancer risk, while clinical trials implicating newer generation progestins that have a greater affinity for the PR and elicit biological effects more like P_4 than progestins from the earlier generations [28,85,86], are mostly lacking. Further studies are thus required to elucidate the role of ERα/PR crosstalk in breast cancer pathogenesis in response to different progestins. An added complexity in delineating the role of ER/PR crosstalk in response to progestins, is the fact that although a few studies report that some progestins and/or their metabolites may bind to the ER [29,30,289,290], conflicting results are often reported, and most of these studies fail to differentiate between the ER subtypes. Interestingly, it has previously been shown that ERα is required at least for MPA-induced breast cancer cell proliferation [41], thus studies investigating the estrogenic activity of progestins used in HT and whether ERα is required for the effects of all progestins on proliferation are needed. Furthermore, considering that the ERβ subtype is also expressed in breast cancers, investigations are required to determine whether a similar interaction occurs between ERβ and the PR and what the implications of such an interaction would be. Current molecular studies investigating the role of ERβ in breast cancer cell biology are however limited, likely due to the lack of an effective commercial antibody for this ER subtype [291]. A better understanding of the role of ER/PR crosstalk, and whether it is involved in the increased breast cancer risk associated with some progestins, may help with the design of a progestin that can be used in HT without breast cancer risk.

4.4. Interplay between the ER and AR

Many studies have suggested that androgens and the AR play a critical role in breast cancer biology (reviewed in [53,54,292–294]) and considering that the AR is expressed in approximately 90% of primary breast tumors (reviewed in [53]), it is not surprising that AR-

targeted treatment for breast cancer is actively being investigated. However, the precise role of the AR in breast cancer is dependent on whether ERα is present. While the AR generally plays an anti-proliferative role in ER-positive breast tumors by inhibiting the activity of ERα [295–300], the AR can also mimic the role of ERα in ER-negative breast cancers and promote breast cancer development (reviewed in [292]). As a result, clinical trials are currently evaluating the use of selective AR modulators in ER-positive breast cancer therapies, and anti-androgens for use in ER-negative breast cancer therapies [292]. One suggested mechanism whereby the AR can attenuate the activity of ERα is by displacing ERα from ER binding sites, either via binding to AREs in close proximity to ER binding sites in estrogen target genes or by competing with ERα for binding directly to EREs in target genes [181,301]. Another mechanism may be the reported AR-mediated increase in ERβ expression, as it is known that ERβ can inhibit the activity of ERα, and that ERβ expression is increased in the presence of natural dihydrotestosterone (DHT) or the synthetic androgen mibolerone in MCF-7 and ZR75 breast cancer cell lines [302].

Interestingly, it has been shown that the first-generation progestin MPA is a potent AR agonist [25], and that like DHT, it inhibited the transcriptional activity of ERα in MDA-MB-231 breast cancer cells overexpressing ERα and the AR [301]. However, AR-mediated effects in breast cancer appear to be a double-edged sword. For example, MPA treatment has effectively been used to treat breast cancer via a mechanism that potentially promotes AR-induced apoptosis [303]. In contrast, MPA used in HT has been associated with increased breast cancer risk [5,6,21] by a mechanism possibly involving the disruption of normal AR signaling [37,304]. As some progestins can bind to the AR and elicit androgenic effects, while others elicit anti-androgenic effects, it is important to determine the progestin agonist and antagonist properties for AR-mediated transactivation and transrepression of target genes in the same model system. We have previously performed these experiments for MPA and NET-A and showed that these

progestins display potent AR agonist activity, similar to that of DHT [25]. Considering that some progestins that have been reported to increase breast cancer risk can elicit androgenic activity, studies are required to determine if ERα/AR crosstalk plays a role in the mechanism whereby some progestins increase breast cancer risk.

4.5. Interplay between the ER and the GR or MR

Recent studies have also highlighted roles for the GR, MR and their cognate ligands in breast cancer cell biology. In terms of the GR and its ligands (glucocorticoids), both are involved in mammary gland development during puberty and pregnancy (reviewed in [305,306]). Glucocorticoids have also been shown to regulate breast cancer cell proliferation [305,307,308], invasiveness, motility and adhesiveness via the GR-mediated upregulation of oncogenes and downregulation of metastasis suppressor genes (reviewed in [309]). Interestingly, although the GR is expressed in approximately 60% of breast cancers [310], no definitive correlation has been found between GR expression and prognosis of breast cancers (reviewed in [305]). Some studies, however, suggest that its role may be context-dependent as high GR expression has been associated with a good outcome in ER-positive cancers, while it is associated with a poor outcome in ER-negative cancers [56]. For example, in ER-negative MDA-MB-231 cells, GR activation by dexamethasone (Dex) has been shown to increase the expression of genes involved in cell survival, such as *serine/threonine protein kinase 1* (*SGK1*) and *dual specificity protein phosphatase 1* (*DUSP1*) [56], while in the mouse xenograft model of MDA-MB-231 cells, apoptosis induced by the chemopreventive agent, paclitaxel, was inhibited by Dex [56]. In the presence of the ER, however, ERα/GR complexes were formed when MCF-7 cells were treated with E_2 and Dex, [311,312] resulting in the reprogramming of ERα and GR binding sites [312–314], and the subsequent activation of genes associated with a more favorable breast cancer outcome [312]. The reprogramming involved an assisted loading mechanism which entailed the GR altering the chromatin landscape to expose novel

binding sites to which ERα then binds [313,314]. Moreover, Dex has been shown to antagonize the proliferative effects of E_2 [315], while also inactivating estrogens by sulfation due to the activation of estrogen sulfotransferase [305,316]. However, the converse is also true as E_2 has been shown to decrease GR expression and dephosphorylate the GR, thereby inhibiting glucocorticoid action [305,317]. Taken together, although it is clear that the interplay between the ER and GR in breast cancer is complex, targeting of the GR in potential novel breast cancer therapies should not be excluded.

The MR has been shown to compensate for the absent GR during specific stages of mammary gland development [318], suggesting that the MR may play a similar role to the GR in breast cancer cell biology [180]. Although the MR is expressed in most breast cancer tumors [319–321], little is known about its role in breast cancer pathogenesis. Nevertheless, the MR ligand, aldosterone, has been shown to increase breast cancer cell proliferation and migration via a mechanism requiring the MR and the G-protein estrogen receptor (GPER), also known as the G protein-coupled receptor 30 (GPR30) [322]. This suggests that the MR is also involved in crosstalk mechanisms in breast cancer. Interestingly, at least one study has shown that the MR and ERα can form a complex in HEK293 cells transiently transfected with cDNA expression vectors for the MR and ERα [323], thus it is likely that a similar complex formation may be observed in breast cancer cells. Implications of this putative MR and ERα crosstalk is not clear. Although beyond the scope of this review, both the GR and MR have also been implicated in crosstalk with the PR [324], emphasizing the importance of future studies investigating the cellular mechanisms of the GR and MR in breast cancer, as well as the extensive interactions between different members of the steroid receptor family.

5. Conclusion

Despite the efficacy of FDA-approved conventional HT in relieving the symptoms of menopause, its association with increased breast risk is a major concern. Although both

estrogen only and estrogen-progestin HT regimens have been associated with increased breast cancer risk, findings from various clinical trials indicate that estrogen-progestin combinations are associated with a higher risk than estrogen only HT, suggesting that the progestin component is responsible for the increased risk. These studies, however, only compared a few progestins and considering that many different progestins known to elicit differential effects [25,31,325] are available, it cannot be assumed that all progestins would increase breast cancer risk. However, the alarm surrounding the associated breast cancer risk has caused some women to turn to custom-compounded bHT as an alleged safer, natural alternative. Evidence to support the safety and efficacy of these bHT regimens is however lacking and has resulted in many associations including the NAMS, IMS, EMAS and Endocrine Society recommending against the use of custom-compounded bHT [107]. Considering the large variety of hormones used in HT and bHT, and the confusion as to whether any of these regimens are safe in terms of breast cancer risk, it is clear that more clinical and molecular studies directly comparing their mechanism of action are needed. Molecular studies should include the determination of binding affinities, relative agonist and antagonist efficacies and potencies for transactivation and transrepression of various estrogens and progestins for individual steroid receptors. Moreover, since estrogen-progestin combination regimens are associated with a higher risk than estrogen only regimens and that progestins elicit differential effects via steroid receptors, it is possible that signaling via multiple steroid receptors may contribute to the observed increased breast cancer risk. Furthermore, recent studies have revealed roles for interplay between many of the steroid receptors in breast cancer cell biology. If the extensively intertwined nature of steroid hormone receptor signaling pathways can be unraveled, it may be possible to elucidate the mechanism behind progestin-induced breast cancer and design novel progestins that do not increase breast cancer risk.

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Package Inserts:

[a] Premarin tablets package insert, Wyeth Pharmaceuticals Inc.; [b] Premarin vaginal cream package insert, Wyeth Pharmaceuticals Inc.; [c] Cenestin package insert, Teva Pharmaceuticals USA, Inc.; [d] Enjuvia package insert, Barr Pharmaceuticals, Inc.; [e] Alora package insert, Watson Pharmaceuticals, Inc.; [f] Climara package insert, Bayer HealthCare Pharmaceuticals Inc.; [g] Esclim package insert, Serono Laboratories, Inc.; [h] Estraderm, Novartis Pharmaceuticals Corporation; [i] Estradot package insert, Novartis Pharmaceuticals Australia Pty Ltd; [j] Menostar package insert, Bayer HealthCare Pharmaceuticals Inc.; [k] Minivelle package insert, Novartis Pharmaceuticals Corporation; [l] Vivelle-dot package insert, Novartis Pharmaceuticals Corporation; [m] Divigel package insert, Upsher-Smith Laboratories Inc.; [n] Elestrin package insert, Bradley Pharmaceuticals Inc.; [o] Estrogel package insert, ASCEND Therapeutics Inc.; [p] Estrace tablets package insert, Warner Chilcott Inc.; [q] Gynodiol package insert, Barr Pharmaceuticals Inc.;[r] Estrace vaginal cream package insert, Allergan USA, Inc.; [s] Estrasorb package insert, Novavax, Inc.; [t] Estring package insert, Pfizer Inc.; [u] Evamist package insert, Ther-Rx Corporation; [v] Vagifem package insert, Novo Nordisk Inc.; [w] Delestrogen package insert, Monarch Pharmaceuticals Inc.; [x] Depo-Estradiol package insert, Pfizer Inc.; [y] Femring package insert, Warner Chilcott Inc.; [z] Femtrace package insert, Warner Chilcott Inc.; [aa] Menest package insert, Monarch Pharmaceuticals Inc.; [ab] Estragyn package insert, Searchlight Pharma Inc.; [ac] Ogen tablet package insert, Pfizer Inc.; [ad] Ogen vaginal cream package insert, Pfizer Inc.; [ae] Prometrium package insert, Solvay Pharmaceuticals, Inc.; [af] Provera package insert, Pfizer Inc.; [ag] Prempro package insert, Wyeth Pharmaceuticals Inc.; [ah] FeHRT package insert, Warner Chilcott Inc.; [ai] Activella package insert, Novo Nordisk Inc.; [aj] Mimvey package insert, Teva Pharmaceuticals Inc.; [ak] Combipatch package insert, Novartis Pharmaceuticals Corporation; [al] Angeliq package insert, Bayer HealthCare Pharmaceuticals Inc.; [am] Prefest package insert, Teva Pharmaceuticals Inc.; [an] Climara Pro package insert, Berlex; [ao] Duavee package insert, Pfizer Inc.

HYPOTHESIS AND AIMS

It is evident from the literature that whether and how estrogens and progestogens used in conventional HT increase breast cancer risk remains an enigma. This problem is confounded by the fact that many different estrogens and progestins differing in structure and biological activity are used in HT. Although proponents of bHT claim that bioidentical hormones used in bHT do not increase breast cancer risk, the literature clearly lacks scientific evidence to support this claim. In light of this, the primary hypothesis of this thesis was that natural and bioidentical estrogens used in HT and bHT, respectively, would elicit similar biological effects to one another, but different to a synthetic estrogen via human ERα and ERβ. It was also hypothesized that progestins used in HT would elicit differential biological effects to one another and P_4 via the individual ER subtypes, and on ER-mediated gene regulation and hallmarks of breast cancer. Considering that crosstalk between ERα and the PR has been shown to play a critical role in breast cancer, we also hypothesized that interplay between these receptors may be an underlying mechanism mediating the reputed oncogenic effects of some progestins.

The aims of this project were three-fold:

Firstly, the activities of bioidentical E_2 (bE_2) and E_3 (bE_3) and synthetic EE were directly compared to each other and commercially available E_2, E_3 and E_1 standards. Accurate equilibrium dissociation constants (K_d/K_i values) of these estrogens for human ERα and ERβ overexpressed in the COS-1 cell line were determined. Relative efficacies and potencies of the estrogens for ERα- and ERβ-mediated transcriptional activation and repression were also determined in the HEK293 cell line. Using the MCF-7 BUS estrogen-sensitive breast cancer cell line that endogenously expresses both ER subtypes, effects on anchorage-independent growth were determined as well as the relative efficacies and potencies of the estrogens for proliferation and the transactivation and transrepression of two markers of breast cancer, the

trefoil factor 1 (*pS2*) and *interleukin-6* (*IL-6*) genes. Moreover, we investigated the claims that E_3 and E_1 are weak estrogens that possibly antagonize the activity of E_2 by evaluating their antagonistic effects on gene expression, proliferation and anchorage-independent growth.

Secondly, the mechanism of action of selected progestins from all four generations, namely MPA, NET-A, LNG, GES, NES, NoMAC and DRSP, were characterized relative to each other and natural P_4, via overexpressed ERα and ERβ in the COS-1 and HEK293 cell lines. We aimed to determine precise equilibrium dissociation constants (K_d/K_i values) for the progestins that could bind the ER. Furthermore, the relative efficacies and potencies of the estrogenic progestins for transcriptional activation and repression were determined in the HEK293 cell line.

Thirdly, using the MCF-7 BUS breast cancer cell line, the effects of MPA, NET and DRSP, relative to P_4, were assessed on breast cancer cell proliferation, anchorage-independent growth and the expression of the ER target genes *pS2* and *cathepsin D* (*CTSD*), in the absence and presence of E_2. Relative efficacies and potencies of the selected progestins for breast cancer cell proliferation were determined and compared to each other and natural P_4. To investigate the hypothesis that crosstalk between the PR and ER plays a critical role in mediating the oncogenic effects of some progestins, we re-evaluated the effects of the progestogens on gene expression, proliferation and anchorage-independent growth in the presence of PR and ER antagonists, or siRNA targeting these receptors. Moreover, the recruitment of the PR/ERα complex to the promoters of ER and PR target genes implicated in breast cancer progression in response to the progestins and P_4 was investigated in the absence and presence of E_2.

Chapter 2

A comparative characterization of estrogens used in hormone therapy via estrogen receptor (ER)-α and -β

The article in this chapter was published in *The Journal of Steroid Biochemistry and Molecular Biology*, Volume 174, November 2017, pages 27-39, and is presented as it was published in combination with the supplementary data to form part of this thesis. Data that is referred to as 'data not shown' in the publication can be found in Appendix B.

The candidate is the first author and planned and conducted all experimental work, analyzed data and wrote the publication. Dr. Renate Louw-du Toit is the co-supervisor of the study and contributed to the critical evaluation of the study and editing of the publication. Prof. Donita Africander is the supervisor of the PhD study, the corresponding author, and was involved with critical evaluation of the study, and editing of the publication.

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A comparative characterization of estrogens used in hormone therapy via estrogen receptor (ER)-α and -β

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ABSTRACT

Conventional hormone therapy (HT) containing estrogens such as ethinylestradiol (EE) have been associated with an increased risk of breast cancer and cardiovascular disease resulting in women seeking safer alternatives that are claimed to have fewer health risks. One such alternative gaining popularity, is custom-compounded bioidentical (b)HT formulations containing bioidentical estradiol (bE_2) and estriol (bE_3). However, the preparation of these custom-compounded estrogens is not regulated, and depending on the route of synthesis, steroid mixtures with differing activities may be produced. Thus, an investigation into the activities of estrogens prepared by custom-compounded pharmacies is warranted. The aim of this study was therefore to directly compare the pharmacological properties of bE_2 and bE_3 of unknown purity relative to commercially available, pure E_2, E_3 and estrone (E_1) standards as well as synthetic EE used in conventional HT via the human estrogen receptor (ER)-α and −β. We determined precise equilibrium dissociation constants (K_d or K_i values) and showed that bE_2 and bE_3 display similar binding affinities to the E_2 and E_3 standards, while EE had a higher affinity for ERα, and E_1 a lower affinity for ERβ. Furthermore, all the estrogens display similar agonist efficacies, but not potencies, for transactivation on a minimal ERE-containing promoter via the individual ER subtypes. Although E_2 and E_3 were equally efficacious and potent on the endogenous ERE-containing pS2 promoter in the MCF-7 BUS breast cancer cell line co-expressing ERα and ERβ, E_1 was less efficacious and potent than E_2. This study is the first to demonstrate that the bioidentical estrogens, commercially available estrogen standards and synthetic EE are full agonists for transrepression on both minimal and endogenous NFκB-containing promoters. Moreover, we showed that these estrogens all increase proliferation and anchorage-independent growth of MCF-7 BUS cells to a similar extent, suggesting that custom-compounded bHT may in fact not be a safer alternative to conventional HT. Furthermore, our results showing that E_3 and E_1 are not weak estrogens, and that E_3 does not antagonize the activity of E_2, suggest that the rationale behind the use of E_3 and E_1 in custom-compounded bHT formulations should be readdressed. Taken together, the results indicating that there is mostly no difference between the custom-compounded bioidentical estrogens, commercially available estrogen standards and synthetic EE, at concentrations reflecting serum levels in women using estrogen-containing HT, suggest that there is no clear advantage in choosing bHT above conventional HT.

1. Introduction

Menopause is characterized by the natural cessation of endogenous estrogen production in aging women, resulting in symptoms such as hot flashes, mood swings, bone loss and urogenital atrophy [1–3]. To relieve these symptoms and improve quality of life, women have been using hormone therapy (HT) for decades [1–4]. Conventional HT is administered as either estrogen alone to hysterectomized women or as an estrogen-progestin combination to women with a uterus. Progestins, such as medroxyprogesterone acetate (MPA) [5] and norethisterone-acetate [6], are used to counteract the estrogen-induced proliferation of the uterine epithelium. Estrogens used in conventional HT regimens include chemically synthesized estrogens such as estradiol (E_2) [1,2] and ethinylestradiol (EE) [7], or conjugated equine estrogens (CEE) containing naturally occurring estrogens [8]. Despite the effective relief of menopausal symptoms with conventional HT use, several side-effects have been reported [4,9–14]. For example, the Women's Health Initiative study investigating the benefits and risks of CEE alone or in

Abbreviations: AR, androgen receptor; bE_2, bioidentical estradiol; bE_3, bioidentical estriol; bHT, bioidentical hormone therapy; CEE, conjugated equine estrogens; CTSD, cathepsin D; E_1, estrone; E_2, estradiol; E_3, estriol; EE, ethinylestradiol; ER, estrogen receptor; ERE, estrogen response element; HT, hormone therapy; IL-6, interleukin-6; MPA, medroxyprogesterone acetate; pS2, trefoil factor 1; RANTES, regulated upon activation normal T-cell expressed and secreted; RBA, relative binding affinity

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combination with MPA, revealed increased incidence of coronary heart disease, stroke and breast cancer amongst postmenopausal women [12], while the Million Women Study investigating the effects of different HT regimens found that estrogen alone (CEE or EE) and estrogen-progestin combinations increased breast cancer risk [13]. Interestingly, a recent Cochrane review analyzing 22 HT studies [15], including the WHI postintervention study [14], concluded that while the use of estrogen alone increased the risk of stroke, it did not increase the risk of coronary events and moreover, decreased breast cancer risk.

Despite reports from follow-up studies indicating possible favorable outcomes with the use of estrogen alone, the highly-publicized initial reports of risks of conventional HT prompted postmenopausal women to seek safer HT alternatives like custom-compounded bioidentical HT (bHT) [17]. Advocates of bHT claim that bioidentical hormones are more effective and safer than conventional HT as they are natural and structurally identical to hormones endogenous to the human body [18,19]. In fact, it has been claimed that bHT does not increase the risk of breast cancer or cardiovascular disease [19,20]. It should be noted that bioidentical hormones are chemically synthesized from naturally occurring plant sterols, and are thus semi-synthetic and not natural [17,18]. In fact, most synthetic estrogens and progestogens used in conventional HT are synthesized in this manner [21]. Notably, steroids can be chemically produced by either semisynthesis or total synthesis, with the possibility of the latter yielding isomers that are not natural and which may differ from the naturally occurring steroid in terms of activity [17,22]. Although this may also be true for hormones used in FDA-approved HT, the production of conventional HT products is highly regulated whereas custom-compounded preparations are not subjected to thorough regulatory evaluation [17]. A variety of different bioidentical estrogens are available for postmenopausal bHT and include E_2 (bE_2), estriol (bE_3) and estrone (bE_1) [18–20,23–25]. These estrogens are prescribed as either a bE_2 monotherapy or as a combination of estrogens. Biest (bE_2 and bE_3 in a 20:80 ratio) and triest (bE_2, bE_3 and bE_1 in a 10:80:10 ratio) formulations [26–28] are based on suggestions that E_3 and E_1 are weaker and safer estrogens than E_2 [19–21,25]. Moreover, although it is claimed that E_3 can antagonize the potent estrogenic activity of E_2 and thereby protect against the deleterious effects of E_2 [21,29], evidence to support these claims is lacking.

Estrogens elicit their biological effects primarily by binding to the estrogen receptor (ER), a ligand-activated transcription factor that exists as two subtypes, ERα and ERβ [30–32]. These subtypes play opposing roles in breast cancer cell proliferation, with ERα promoting breast cancer cell proliferation and ERβ inhibiting ERα-mediated cell proliferation [30,33–42]. It has been proposed that these opposing effects on cellular proliferation may be due to the differential regulation of subtype-specific target genes [33,37,43–47]. The ER subtypes are activated upon estrogen binding and can subsequently bind to estrogen response elements (EREs) to activate target gene expression (transactivation) [33,48–52] or tether to DNA-bound transcription factors, such as nuclear factor kappa B (NFκB), to repress target gene expression (transrepression) [53–59]. Considering the importance of estrogens and ER signaling in breast cancer biology, it is thus crucial to pharmacologically characterize estrogens used in conventional and bioidentical HT via the human ER subtypes. Surprisingly, studies directly comparing the mechanism of action of various estrogens used in endocrine therapies to commercially available estrogen standards, via the ER, are lacking.

In light of the above, this study aimed to provide a comparative biochemical profile of synthetic EE, bE_2 and bE_3 relative to commercially available estrogen standards via both ERα and ERβ. We determined accurate equilibrium dissociation constants (K_d or K_i values) of these estrogens for overexpressed human ERα or ERβ, while transcriptional activation and repression were evaluated via the overexpressed human ER subtypes in HEK293 cells, or in the human MCF-7 BUS breast cancer cell line endogenously expressing both ERα and ERβ. Moreover, we compared the potential of these estrogens to modulate proliferation and anchorage-independent growth of the MCF-7 BUS cells. Briefly, we showed that the bioidentical estrogens mimic the activity of their respective commercially available estrogen standards, and often, but not always, mimic synthetic EE and the E_1 standard. Most importantly, we demonstrated that E_3 and E_1, like E_2, are full ER agonists in most assays, and do not antagonize the activity of E_2. These findings suggest that the use of E_3 and E_1 in custom-compounded bHT formulations should be re-evaluated.

2. Materials and Methods

2.1. Inducing compounds

Commercially available estrogen standards; E_1, E_2, and E_3 as well as synthetic EE were obtained from Sigma-Aldrich, South Africa, with an HPLC profile and an FTNMR (Fourier transform nuclear magnetic resonance) spectra confirming purity of at least 98%. [^{3}H]-E_2 (100 Ci/mmol) was purchased from AEC-Amersham, South Africa. Compounded bioidentical E_2 (bE_2) and E_3 (bE_3) were received from the Compounding Pharmacy of South Africa and human tumor necrosis factor alpha (TNFα) was obtained from Celtic Diagnostics, South Africa. We did not include bE_1 in this study as it was not accessible to us.

2.2. Cell Culture

The HEK293 human embryonic kidney cell line and the COS-1 monkey kidney cell line were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/ml glucose (Sigma-Aldrich, South Africa), 10% fetal calf serum (FCS) (The Scientific Group, South Africa), 100 IU/ml penicillin and 100 µg/ml streptomycin (1% penicillin-streptomycin) (Sigma-Aldrich, South Africa) as previously described [60,61]. The human MCF-7 BUS breast cancer cell line, received from Prof. Ana Soto (Tufts University, Boston), was maintained in DMEM containing 4.5 g/ml glucose, 5% (v/v) heat-inactivated (HI)-FCS (The Scientific Group, South Africa) and penicillin-streptomycin as previously described [62]. All experiments were conducted within the first 35 passages since thawed from storage. Hoechst staining was routinely conducted to test for mycoplasma infection [63] and only mycoplasma-negative cell lines were used.

2.3. Plasmids

Human ERα and ERβ encoding cDNA expression vectors (pSG5-hERα and pSG5-hERβ) [64] were received from Prof. Frank Gannon (European Molecular Biology Laboratory, Germany). The pGL3-2xERE-pS2-luciferase promoter-reporter construct [65], driven by the pS2 (trefoil factor 1) promoter containing two copies of the ERE and a luciferase reporter, was a gift from Prof. Borja Belandia (Institute for Biomedical Research, Spain), while the p(IL6κB)$_3$50hu. IL6P-luciferase promoter-reporter construct, driven by the interleukin (IL)-6 promoter containing three copies of the NFκB binding site and a luciferase reporter [66] was received from Prof. Guy Haegeman (Ghent University, Belgium). The pGL2basic cDNA expression vector (Promega, Madison, USA), containing no eukaryotic promoter or enhancer sequences, was used as a filler plasmid.

2.4. Whole cell binding assay

Competitive whole cell binding assays were performed essentially as described by Bamberger et al. [67]. Briefly, COS-1 cells were seeded into 10 cm^2 dishes at a density of 2×10^6 cells and washed 24 h later with pre-warmed phosphate buffered saline (PBS) prior to the addition of phenol red-free DMEM supplemented with 10% charcoal-stripped (CS)-FCS and 1% penicillin-streptomycin. The cells were then transiently transfected with either 6000 ng of the pSG5-hERα or pSG5-hERβ

cDNA expression vector using XtremeGene HP (Roche Molecular Biochemicals) according to the manufacturer's instructions, and replated into 24-well plates at a density of 5×10^4 cells per well after 24 h. Two days later, the cells were washed with pre-warmed PBS and incubated for 4 h with 10 nM or 20 nM [^{3}H]-E_2 in the absence or presence of increasing concentrations of unlabeled E_2, bE_2, E_3, bE_3, E_1 and EE. Cells were subsequently washed and lysed as previously described [60,68]. Total binding ([^{3}H]-E_2 only) was determined by scintillation counting and set as 100%, while binding of competitor ligands was set relative to this. Protein concentration was determined using the Bradford protein assay method [69] to correct for plating differences. The equilibrium dissociation constant (K_d) of E_2 for each ER subtype was determined from homologous displacement curves using a global fitting model [70], whilst the K_i values for the competing ligands were determined from heterologous displacement curves using the EC_{50} value, K_d determined for E_2 and the concentration of [^{3}H]-E_2, according to the equation by Cheng and Prusoff [71].

2.5. Luciferase reporter assays

HEK293 cells were seeded into 10 cm^2 dishes at a density of 2×10^6 cells and after 24 h, the medium was replaced as described in Section 2.4. For transactivation assays, the cells were transiently transfected with 150 ng of the pSG5-hERα or pSG5-hERβ cDNA expression vector and 6 000 ng (for ERα) or 3 000 ng (for ERβ) of the pGL3-2xERE-pS2-luciferase promoter-reporter construct. To ensure that the total amount of transfected DNA remained constant, 3000 ng of the pGL2basic cDNA expression vector was co-transfected for ERβ assays. After 24 h, cells were replated into 96-well plates at a density of 1×10^4 cells per well and allowed to settle. The next day, cells were treated with increasing concentrations of the test compounds (agonist mode) or 1 nM E_2 in the absence and presence of varying concentrations of E_3 and/or E_1 (antagonist mode) for 24 h. For transrepression assays, cells were transiently transfected with 150 ng of either the pSG5-hERα or pSG5-hERβ cDNA expression vector and 1500 ng of the p$(IL6\kappa B)_3$50huIL6P-luciferase promoter-reporter construct for 24 h. Cells were subsequently replated into 96-well plates and following 24 h, treated with 0.02 μg/ml TNFα in the absence and presence of the test compounds described above for agonist and antagonist modes. For both transactivation and transrepression assays, cells were lysed and analyzed as previously described [61,72]. The efficacies (maximal responses) and potencies (EC_{50}: concentration at which half maximal response is elicited) were determined.

2.6. Quantitative real-time PCR (qPCR)

The MCF-7 BUS breast cancer cell line was seeded into 12-well plates at a density of 1×10^5 cells per well. The next day the medium was replaced with phenol red-free DMEM supplemented with 5% HI-CS-FCS and 1% penicillin-streptomycin to estrogen starve the cells. The cells were treated for 24 h (transactivation) or 6 h (transrepression) as described in Section 2.5. Total RNA was isolated using Tri-reagent (Sigma-Aldrich, South Africa) and reversed transcribed using the Im-Prom-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. Real-time qPCR was performed using the KAPA SYBR FAST qPCR master mix and the LightCycler® 96. The mRNA expression of pS2, CTSD (cathepsin D), IL-6, RANTES (regulated upon activation, normal T-cell expressed and secreted) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was measured using primer sets obtained from Roche Molecular Biochemicals (South Africa) (Table 1). The PCR efficiency was more than 90% for all primer sets. Relative transcript levels were normalized to the relative transcript levels of GAPDH calculated using the method described by Pfaffl [73]. Duration of hormone treatment was based on time course studies showing maximum E_2-induced pS2 mRNA expression at 24 h, while maximum E_2-induced repression of TNFα-stimulated IL-6 mRNA expression was observed at 6 h (data not shown).

Table 1
Forward (fwd) and reverse (rev) primer sequences.

Gene	Primer sequence (5′-3′)	Ref.
pS2	ATACCATCGACGTCCCTCCA (fwd) AAGCGTGTCTGAGGTGTCCG (rev)	[74]
CTSD	GCGAGTACATGATCCCCTGT (fwd) CTCTGGGGACAGCTTGTAGC (rev)	[75]
IL-6	TCTCCACAAGCGCCTTCG (fwd) CTCAGGGCTGAGATGCCG (rev)	[76]
RANTES	TACCATGAAGGTCTCCGC (fwd) GACAAAGACGACTGCTGG (rev)	[77]
GAPDH	TGAACGGGAAGCTCACTGG (fwd) TCCACCACCCTGTTGCTGTA (rev)	[78]

2.7. Cell viability assays

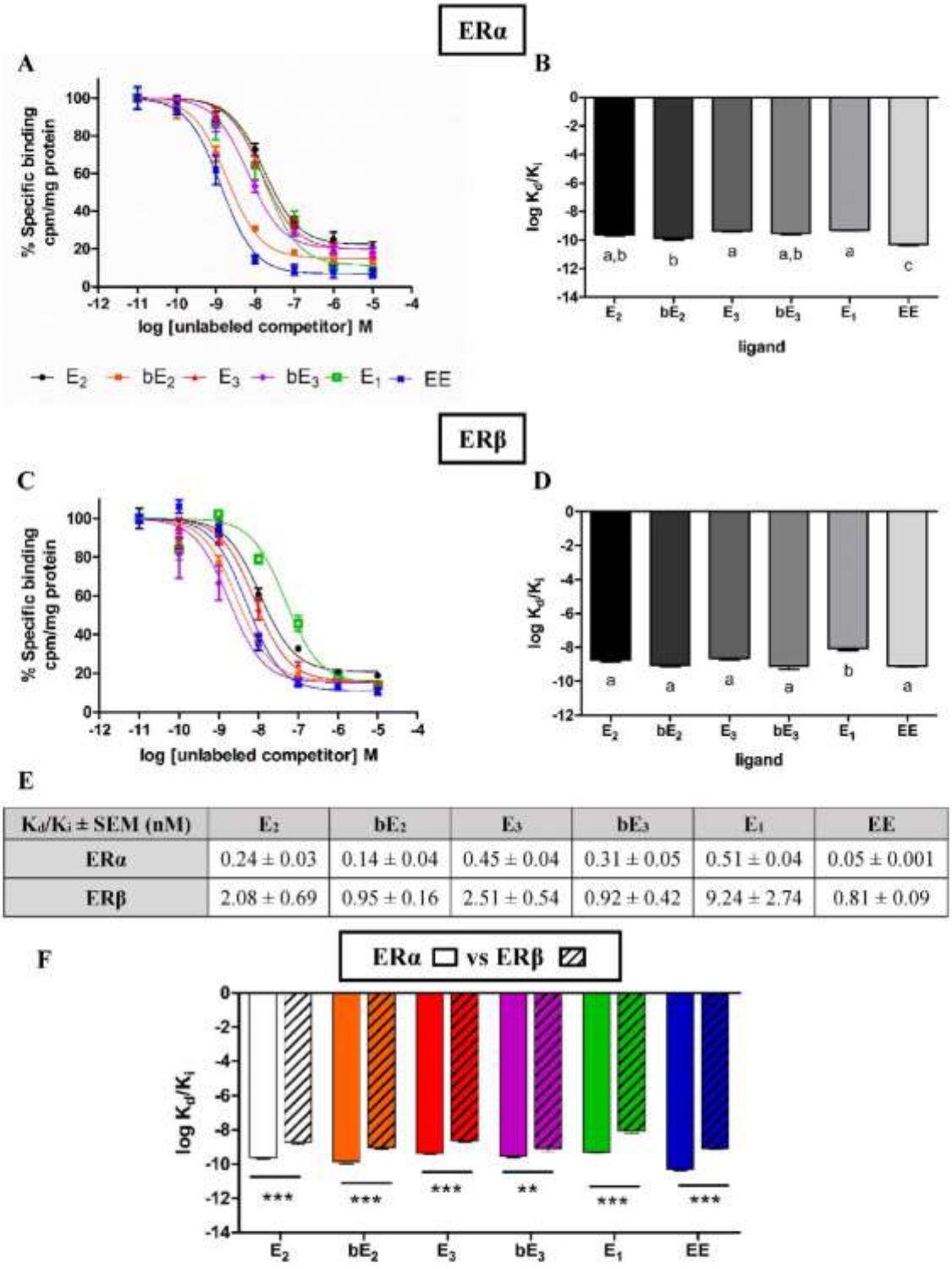
MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell viability assays were conducted essentially as described by Verhoog et al. [79] in order to evaluate effects on the proliferation of the MCF-7 BUS cell line. Briefly, MCF-7 BUS cells were seeded into 96-well plates at a density of 1×10^4 cells per well in phenol red-free DMEM. The next day, cells were treated with increasing concentrations of the test compounds for 48 h and subsequently incubated with 1.25 mg/ml pre-warmed MTT solution for 4 h. The medium was removed and 200 μl dimethyl sulfoxide (DMSO) was added to each well prior to an absorbance measurement at 550 nm.

2.8. Anchorage-independent Growth

Soft agar assays were conducted as previously described, with a few modifications [80]. Briefly, 600 μl phenol red-free DMEM containing 5% HI-CS-FCS, 1% penicillin-streptomycin and 0.5% agar was pipetted into 12-well plates and incubated for 1 h at room temperature to solidify. Thereafter, a second layer of medium containing 0.3% agar and 1×10^3 MCF-7 BUS cells was added. The cells were then treated with 1 nM test compound (agonist mode) or 1 nM E_2 in the absence and presence of equimolar concentrations of either E_3, E_1, or a combination of E_3 and E_1 (antagonist mode). The cells were incubated for 21 days and the colonies subsequently fixed with 37% formaldehyde and stained with 0.005% crystal violet. Colonies were counted using ImageJ software (Version 1.49).

2.9. Data manipulation and statistical analysis

Data manipulation, graphical presentations and statistical analysis were performed using GraphPad Prism® version 5 (GraphPad Software). Non-linear regression analysis was used to determine K_d (competitive binding: one-site homologous) and K_i (competitive binding: one site heterologous) values, as well as the efficacies and potencies for gene expression and proliferation. One-way ANOVA analysis of variance with Newman-Keuls (compares all pairs of columns) post-test, or two-way ANOVA analysis of variance with Bonferroni's (compares all pairs of columns) post-test was used to determine statistical significance of results. Statistically significant differences are indicated by *, ** or ***, to indicate $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, whereas ns indicates no statistical significance ($p > 0.05$). Alternatively, the letters 'a', 'b', 'c', etc., were used to indicate statistically significant differences (significantly different values are assigned a different letter). Figures are representative of at least two independent experiments and error bars represent the standard error of the mean (SEM).



E

K_d/K_i ± SEM (nM)	E_2	bE_2	E_3	bE_3	E_1	EE
ERα	0.24 ± 0.03	0.14 ± 0.04	0.45 ± 0.04	0.31 ± 0.05	0.51 ± 0.04	0.05 ± 0.001
ERβ	2.08 ± 0.69	0.95 ± 0.16	2.51 ± 0.54	0.92 ± 0.42	9.24 ± 2.74	0.81 ± 0.09

Fig. 1. Custom-compounded bE_2 and bE_3 have similar binding affinities to the respective E_2 and E_3 standards for the individual ER subtypes. The COS-1 cell line transiently transfected with a human **(A)** ERα or **(C)** ERβ expression vector was incubated with 10 nM [^{3}H]-E_2 in the absence and presence of increasing concentrations of either unlabeled E_2 (●), bE_2 (■), E_3 (▲), bE_3 (♦), E_1 (□) or EE (×) for 4 h. Counts per minute (cpm) were measured and normalized to protein concentration determined using the Bradford method [69]. Competition for binding is illustrated by the percentage of [^{3}H]-E_2 bound to the ER subtypes, where total binding ([^{3}H]-E_2 only) was set as 100% and the binding of unlabeled competitor estrogens set relative to this. Results shown are representative of at least three independent experiments with each condition performed in triplicate (± SEM). **(B)** Log K_d/K_i values of the ligands for ERα and **(D)** ERβ were plotted, and one-way ANOVA analysis of variance with Newman-Keuls post-test was performed to determine statistical differences. **(E)** The K_d and K_i values ± SEM of the estrogens for ERα and ERβ are tabulated. **(F)** Log K_d/K_i values of the estrogens for the ER subtypes (from Fig. 1B and D) were plotted and two-way ANOVA analysis of variance with Bonferroni's post-test was conducted to determine statistical differences for ERα versus ERβ.

3. Results

3.1. bE_2, E_3 and bE_3 have similar binding affinities to E_2 for both ERα and ERβ

Competitive whole cell binding assays were performed in COS-1 cells transiently transfected with either a human ERα or ERβ expression vector to determine accurate K_d and K_i values for the estrogens. Homologous competitive binding curves were fitted using a global fitting model [70] (Supplementary Fig. 1), whereas heterologous competitive binding curves were fitted using the equation described by Cheng and Prusoff [71] (Fig. 1A and C). The curves for each competing ligand indicate competitive binding to the same site as E_2, and the K_d values of E_2 and K_i values of bE_2, E_3, bE_3, E_1 and EE are summarized in Fig. 1E. Results in Fig. 1A–D show that bE_2 and bE_3 bind to both ER subtypes with similar affinities to E_2 and E_3. In fact, most of the estrogens have similar binding affinities to E_2 for the individual ER subtypes, with the exception that EE has a higher affinity for ERα (Fig. 1B), while E_1 has a lower affinity for ERβ (Fig. 1D). In agreement with others [81–83], we showed that E_2, E_3, E_1 and EE have higher affinities for ERα than ERβ (Fig. 1F).

3.2. All estrogens are full agonists for transactivation on a synthetic promoter-reporter gene, while E_1 is a partial agonist on the endogenous pS2 gene

Having shown that bE_2 and bE_3 have similar binding affinities to the E_2 and E_3 standards for both ERα and ERβ, while EE and the E_1 standard display differential binding affinities relative to E_2, we next investigated whether the estrogens activate the individual ER subtypes to the same extent. We determined the agonist efficacies and potencies of the estrogens for transactivation on the pS2-ERE-luciferase promoter-reporter construct in the HEK293 cell line overexpressing either human ERα (Fig. 2A–C) or ERβ (Fig. 2D–F). The cells were treated with increasing concentrations of the estrogens for 24 h and dose response curves were fitted using non-linear regression analysis. Results in Fig. 2B, C, E and F show that bE_2 and bE_3 had similar efficacies and potencies for transactivation to E_2 and E_3 respectively. In fact, all the estrogens had similar efficacies via the ER subtypes individually expressed in HEK293 cells (Fig. 2B and E), while differences in potencies were observed (Fig. 2C and F). Although E_3 had a similar binding affinity to E_2 for ERα and ERβ respectively (Fig. 1B and D), E_3 was less potent than E_2 via ERα (Fig. 2C). Interestingly, even though E_1 had a similar affinity to E_2 for ERα, and a lower affinity for ERβ, E_1 displayed a significantly weaker agonist potency than E_2 via both ERα and ERβ (Fig. 2C and F). Furthermore, we show that although EE had a higher affinity than E_2 for ERα (Fig. 1B), EE was less potent than E_2 via ERα. In contrast, EE and E_2 had a similar affinity for ERβ and were equipotent ERβ agonists. Moreover, although no differences were observed in the efficacies of the estrogens for ERα versus ERβ (Fig. 2G), most estrogens, except E_3 and bE_3, displayed higher potencies via ERα (Fig. 2H). Considering that the ER subtypes are often co-expressed in breast cancer tissues, we next investigated the agonist efficacies and potencies of the estrogens on the endogenous ERE-containing pS2 gene in the estrogen-responsive MCF-7 BUS breast cancer cell line endogenously expressing both ERα and ERβ. Cells were treated as above and results in Fig. 2I show that all the estrogens, except E_1, were full agonists for pS2 mRNA expression. E_1 displayed partial agonist activity, and was the only estrogen less potent than E_2 (Fig. 2J and K). The maximal response and EC_{50} values determined from Fig. 2A, D and I are indicated in Fig. 2L.

3.3. EE, bE_2 and bE_3 display similar efficacies, but not potencies, to the estrogen standards for ER-mediated transrepression

Steroid receptors elicit their effects through both transactivation and transrepression of gene expression [84]. Although the transrepression mechanism is well-studied for glucocorticoids and the glucocorticoid receptor [85–87], such studies for estrogens and the ER are limited. We therefore investigated the efficacies and potencies of the estrogens for transrepression on a synthetic NFκB-luciferase promoter-reporter construct in HEK293 cells overexpressing either human ERα (Fig. 3A) or ERβ (Fig. 3D), as well as on an endogenous NFκB-containing promoter in MCF-7 BUS cells expressing both ER subtypes (Fig. 3I). Cells were incubated with 0.02 μg/ml TNFα in the absence and presence of increasing concentrations of the estrogens for either 24 h (IL6-NFκB-luciferase promoter-reporter construct) or 6 h (endogenous IL-6 mRNA expression). The fold induction obtained in the presence of 0.02 μg/ml TNFα and the repression in the presence of 100 nM E_2 are depicted in Supplementary Fig. 2. Dose response curves were subsequently fitted using non-linear regression analysis and agonist efficacies and potencies were determined. Fig. 3B and E indicate that all the estrogens displayed similar agonist efficacies via both ER subtypes. While E_1 displayed a higher potency than all other estrogens via ERα (Fig. 3C), and EE a higher potency than E_2 and bE_2 via ERβ (Fig. 3F), most of the estrogens had similar, relatively strong, agonist potencies compared to each other and E_2 via ERα and ERβ. Furthermore, results mostly showed no difference in the efficacies and potencies of the estrogens for ERα versus ERβ (Fig. 3G and H), except for E_3 and bE_3 which had higher potencies via ERβ (Fig. 3H). In MCF-7 BUS cells expressing both ER subtypes, the apparent lower maximal repression displayed by E_2 and E_1 on the endogenous IL-6 promoter (Fig. 3I), was not statistically significant ($p > 0.05$) (Fig. 3J). Surprisingly, E_3, bE_3 and EE displayed relatively weaker agonist potencies (352.3 pM, 63.7 pM and 30.7 pM, respectively) compared to E_2 and E_1 (2.6 pM and 4.1 pM, respectively) (Fig. 3K). The maximal repression and EC_{50} values for repression of TNFα-induced gene expression by the estrogens via ERα or ERβ on the synthetic NFκB promoter-reporter construct (Fig. 3A and D), and via endogenously expressed ERα and ERβ on the endogenous NFκB-containing IL-6 promoter (Fig. 3I), are summarized in Fig. 3L.

3.4. The estrogens are all full agonists, but display differential potencies, for breast cancer cell proliferation

Uncontrolled breast epithelial cell proliferation is a recognized physiological phenotype of breast cancer [88], and it is known to be promoted by estrogens acting via the ER [33,89,90]. Having established that the estrogens are all ER agonists for both transactivation and transrepression of gene expression, we next investigated the effects of the estrogens on breast cancer cell proliferation, a response of the collective effects of transactivation and transrepression of gene expression [72]. The MCF-7 BUS breast cancer cell line was incubated with increasing concentrations of estrogens for 48 h, and cell proliferation quantified using the MTT cell viability assay. This cell line was selected as it is highly proliferative in response to estrogen stimulation [91]. Interestingly, results showed no significant difference between the efficacies of the estrogens for breast cancer cell proliferation (Fig. 4A and B). This was similar to the observation that the estrogens had similar efficacies for both transactivation (Fig. 2) and transrepression (Fig. 3) of gene expression, except for E_1, which was less efficacious than E_2 in terms of transactivation of the endogenous pS2 gene (Fig. 2J). Despite these similarities in efficacies, significant differences in potencies were observed (Fig. 4C). E_3, bE_3 and EE were approximately 10- to 20-fold less potent than E_2 and bE_2, while E_1 was approximately 500-fold less potent. The maximal responses and EC_{50} values are summarized in Fig. 4D.

3.5. Anchorage-independent growth of MCF-7 BUS cells is promoted to the same extent by all estrogens

The ability of a breast cancer cell to grow without being anchored to

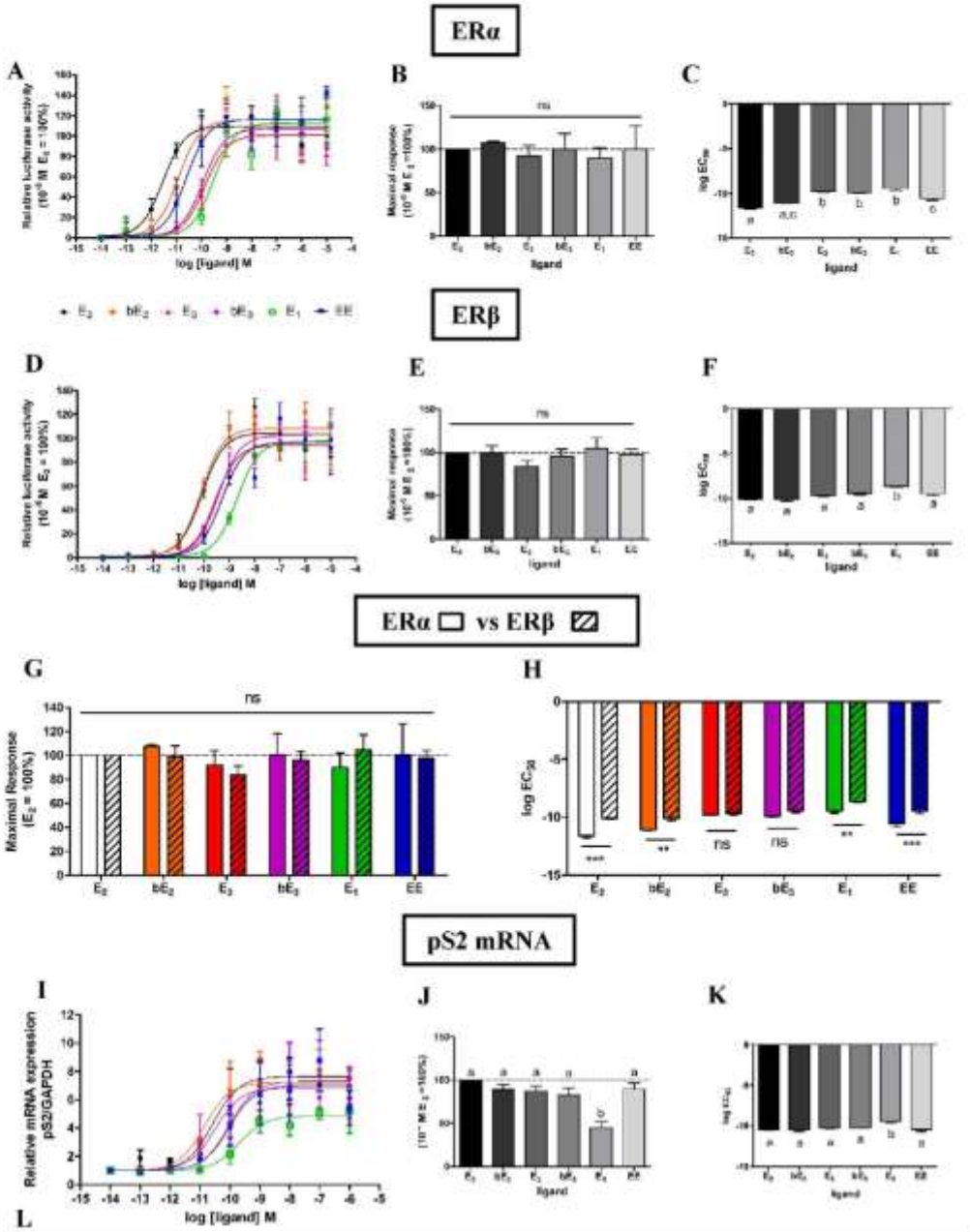


Fig. 2. With the exception of E_1, all the estrogens are full agonists for transactivation on both a minimal and endogenous promoter. The HEK293 cell line transiently transfected with a human **(A)** ERα or **(D)** ERβ expression vector and the pS2-ERE-luciferase promoter-reporter construct was incubated with increasing concentrations of E_2 (●), bE_2 (■), E_3 (▲), bE_3 (♦), E_1 (□) and EE (×) for 24 h. Luciferase activity was measured in relative light units and normalized to protein concentration determined using the Bradford method [69]. Results are shown as relative luciferase activity where induction with 10^{-5} M E_2 was set as 100% and all other responses set relative to this. Plots are shown for the maximal response and log EC_{50} values of the estrogens for transactivation on an ERE-containing synthetic promoter via overexpressed **(B, C)** ERα and **(E, F)** ERβ. The **(G)** maximal response and **(H)** log EC_{50} values for ERα versus ERβ were plotted, and two-way ANOVA analysis of variance with Bonferroni's post-test was performed to determine statistical differences. **(I)** The MCF-7 BUS cell line was treated as described above and real-time qPCR was performed to determine the mRNA expression levels of the ERE-containing pS2 gene, using GAPDH as the internal standard. Relative pS2 mRNA expression of treated samples was calculated relative to the vehicle control (EtOH), which was set as 1. Plots are shown for the **(J)** maximal response and **(K)** log EC_{50} values of the estrogens for transactivation on the endogenous pS2 gene. One-way ANOVA analysis of variance with Newman-Keuls post-test was performed to determine statistical differences of maximal response and EC_{50} values reported in **(L)**. Data should depict accurate efficacies and potencies as the fractional occupancy of the ER subtypes by 100 nM, 1 μM and 10 μM ligand is near 100% (Supplementary Table 1).

L

Maximal Response ± SEM (%)						
	E_2	bE_2	E_3	bE_3	E_1	EE
ERα + ERE-luc	100	107 ± 1.6	91 ± 12	100 ± 18	100 ± 26	90 ± 12
ERβ + ERE-luc	100	99 ± 9.2	84 ± 7.5	96 ± 7.7	105 ± 12	98 ± 6.5
pS2 mRNA (ERα+ERβ)	100	89.2 ± 5.3	87.1 ± 6.1	82.6 ± 7.8	44.5 ± 7.3	89.6 ± 6.8
EC_{50} ± SEM (pM)						
ERα + ERE-luc	3.09 ± 1.07	8.53 ± 0.86	170 ± 18.0	131 ± 30.0	408 ± 167	29.2 ± 12.3
ERβ + ERE-luc	86.3 ± 17.9	108 ± 58.6	236 ± 62.5	418 ± 167	2 320 ± 333	394 ± 186
pS2 mRNA (ERα+ERβ)	53.7 ± 17.1	42.9 ± 20.4	66.0 ± 18.6	60.4 ± 6.9	463.8 ± 163.9	48.9 ± 19.2

an epithelial layer (in vivo) or a plate surface (in vitro) is indicative of a transformed cell with the potential to metastasize [92]. We thus next evaluated the effects of the estrogens on the anchorage-independent growth of the MCF-7 BUS cell line by means of a soft agar assay [92]. The cells were incubated with 1 nM of the estrogens for 21 days and the results in Fig. 5 show that all the estrogens induced the formation of a similar number of colonies.

3.6. E_3 and E_1 do not antagonize E_2-induced transcriptional activity or proliferation

Proponents of custom-compounded bHT claim that E_3 and E_1 are weak estrogens and that E_3 can antagonize the activity of E_2 [23,24]. Thus, it has been suggested that estrogen therapies containing E_3 or E_1 such as biest and triest formulations do not increase breast cancer risk

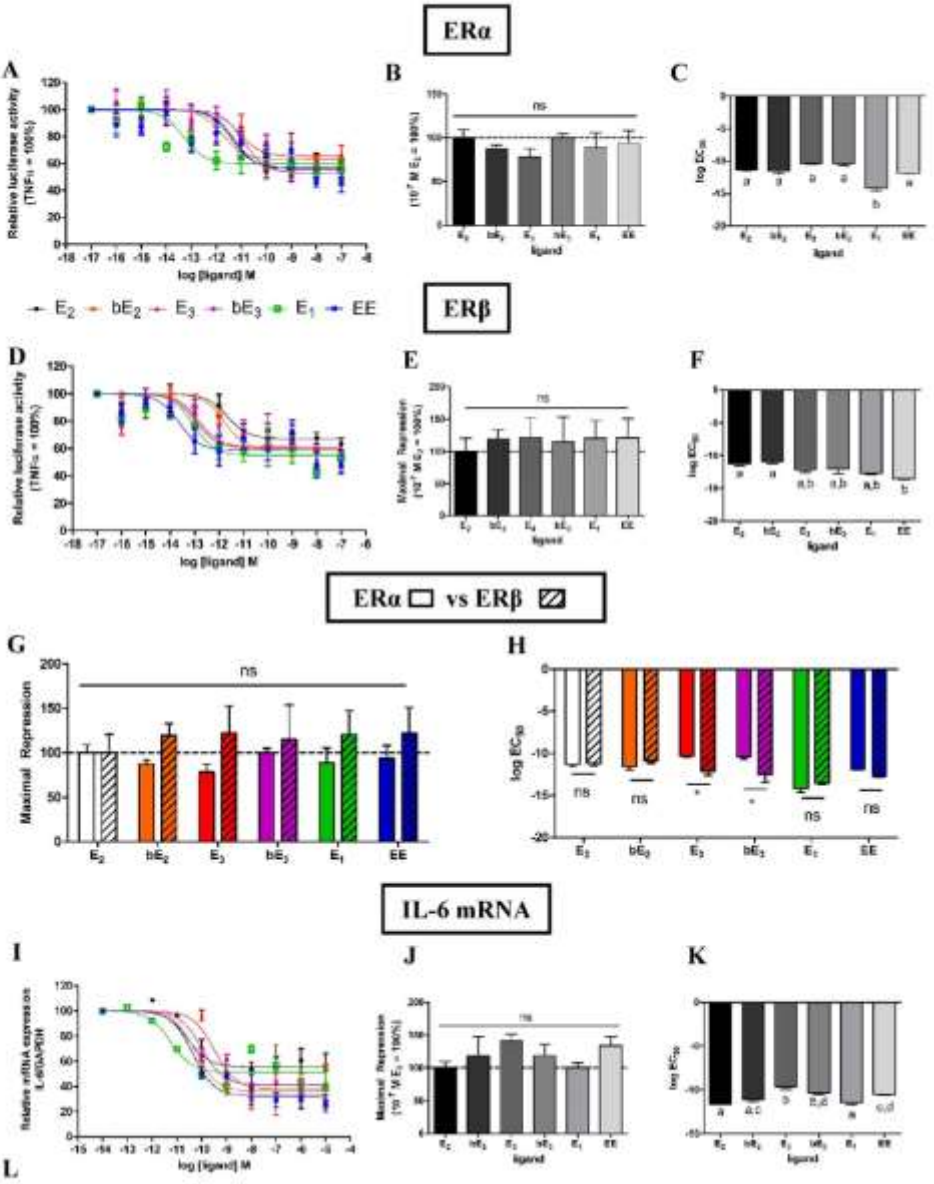
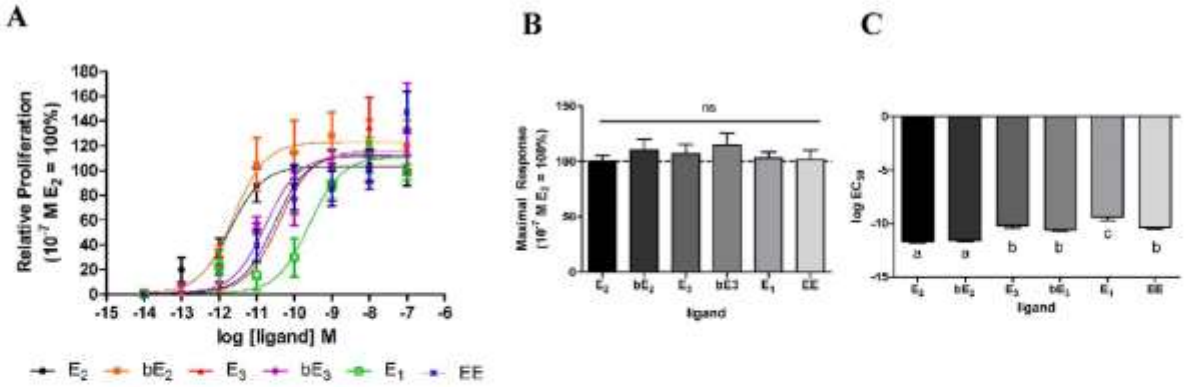


Fig. 3. All the estrogens are full agonists for transrepression. The HEK293 cell line was transiently transfected with either a human **(A)** ERα or **(D)** ERβ expression vector and the synthetic IL6-NFκB-luciferase promoter-reporter construct and incubated with 0.02 μg/ml TNFα in the absence and presence of increasing concentrations of E_2 (●), bE_2 (■), E_3 (▲), bE_3 (♦), E_1 (□) and EE (×) for 24 h. Luciferase activity was measured in relative light units and normalized to protein concentration determined using the Bradford method [69]. Plots are shown for the maximal repression and log EC_{50} values of the estrogens for transrepression on an NFκB-containing synthetic promoter via overexpressed **(B, C)** ERα or **(E, F)** ERβ. The **(G)** maximal repression and **(H)** log EC_{50} values for ERα versus ERβ were plotted and two-way ANOVA analysis of variance with Bonferroni's post-test was performed to determine statistical differences. **(I)** The MCF-7 BUS cell line was treated as described above for 6 h and real-time qPCR was performed to determine the mRNA expression levels of the NFκB-containing IL-6 gene, using GAPDH as the internal standard. Relative gene expression of treated samples was calculated relative to TNFα alone, which was set as 100% and all other responses set relative to this. Plots are shown for **(J)** the maximal repression and **(K)** log EC_{50} values of the estrogens for transrepression on the endogenous IL-6 gene. One-way ANOVA analysis of variance with Newman-Keuls post-test was performed to determine statistically significant differences of maximal response and EC_{50} values reported in **(L)**.

Maximal Repression ± SEM (%)						
	E_2	bE_2	E_3	bE_3	E_1	EE
ERα + NFκB-luc	100	87 ± 5	78 ± 9	100 ± 5	89 ± 17	94 ± 15
ERβ + NFκB-luc	100	120 ± 14	122 ± 31	115 ± 39	121 ± 27	122 ± 29
IL-6-mRNA (ERα+ERβ)	100	118 ± 29	142 ± 10	119 ± 17	100 ± 7	134 ± 13
EC_{50} ± SEM (pM)						
ERα + NFκB-luc	15.3 ± 7.0	2.2 ± 1.7	52.3 ± 21.6	50.4 ± 29.8	0.02 ± 0.01	1.3 ± 0.09
ERβ + NFκB-luc	11.0 ± 5.1	39.9 ± 17.9	2.1 ± 1.7	3.5 ± 2.3	0.2 ± 0.01	0.03 ± 0.01
IL-6-mRNA (ERα+ERβ)	2.59 ± 0.32	10.5 ± 4.1	352.3 ± 162.7	63.7 ± 32.7	4.1 ± 2.3	30.7 ± 7.4

[19,20]. Given that the individual components of these formulations increase breast cancer cell proliferation (Fig. 4) and anchorage-independent cell growth (Fig. 5) to the same extent in our study, we next evaluated whether E_3, and possibly E_1, could antagonize E_2-induced transcriptional activation or repression, or proliferation and anchorage-independent growth of MCF-7 BUS cells. Here, we used the commercial E_2 and E_3 standards, as the custom-compounded bioidentical estrogens mimicked these estrogens in all previous assays (Figs. 1–5), while UPLC–MS accurate mass determinations also indicated that the molecular weights of bE_2 and bE_3 were identical to the E_2 and E_3 standards, respectively (Supplementary Fig. 4). Using equimolar concentrations of the estrogens, we showed that neither E_3 nor E_1 antagonized E_2-induced activity. Furthermore, we showed that the lack of antagonism of E_2-induced transcriptional activation by E_3 and E_1 was not promoter-specific, as similar effects were observed on the ERE-containing pS2 (Fig. 6C) and cathepsin D (CTSD) genes (Fig. 6D). A similar lack of antagonism was observed for E_2-induced transcriptional repression of the NFκB-containing IL-6 (Fig. 6G) and RANTES (regulated upon activation, normal T-cell expressed and secreted) genes (Fig. 6H). Likewise, when the cells were treated with 1 nM E_2 in the absence and presence of



D

	E_2	bE_2	E_3	bE_3	E_1	EE
Maximal response ± SEM	100	111 ± 10	107 ± 8	115 ± 10	103 ± 5	101 ± 8
EC_{50} ± SEM (pM)	2.3 ± 0.5	2.91 ± 0.70	57.2 ± 17.7	25.5 ± 8.04	1135 ± 759	52.1 ± 16.1

Fig. 4. The estrogens have similar efficacies, but not potencies, for breast cancer cell proliferation. **(A)** The MCF-7 BUS cell line was incubated with increasing concentrations of E_2 (●), bE_2 (■), E_3 (▲), bE_3 (♦), E_1 (□) and EE (×) for 48 h and cell proliferation quantified using the colorimetric MTT cell viability assay. Results are shown as relative proliferation (%) with the response obtained in the presence of 10^{-7} M E_2 set as 100% and all other responses set relative to this. **(B)** Plots are shown for the maximal response and **(C)** log EC_{50} values of the estrogens obtained for proliferation (from Fig. 4A). One-way ANOVA analysis of variance with Newman-Keuls post-test was performed to determine statistical differences of maximal response and EC_{50} values reported in **(D)**.

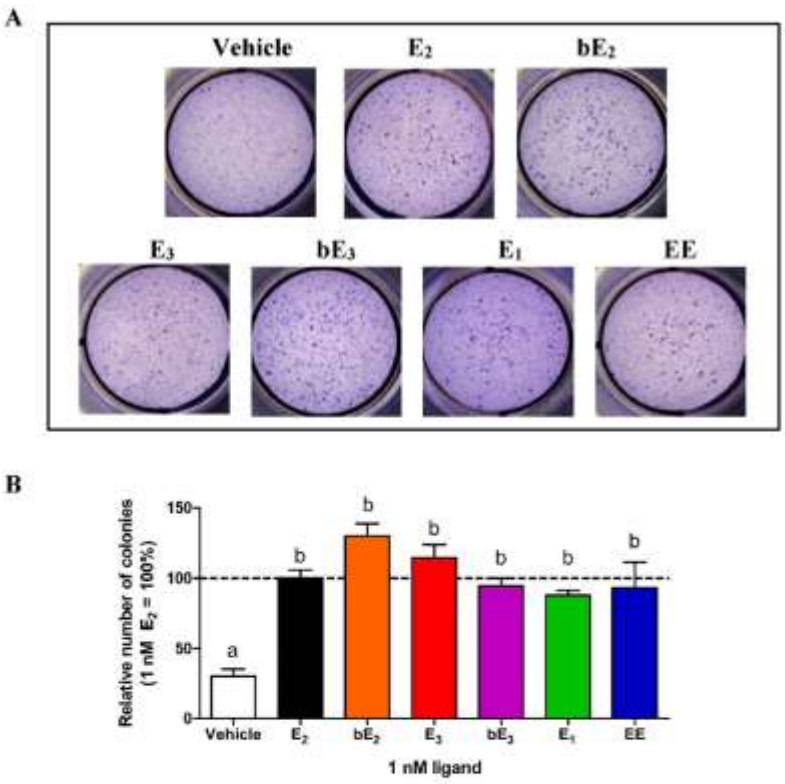


Fig. 5. Anchorage-independent growth of MCF-7 BUS cells was promoted to the same extent by all estrogens. **(A)** The MCF-7 BUS cell line was incubated with the estrogens for 21 days, followed by staining of colonies with 0.005% crystal violet and **(B)** quantification using ImageJ software (Version 1.49). Results shown in (A) are representative of at least three independent experiments, with the number of colonies formed in the presence of 1 nM E_2 set as 100%, and all other responses, including the vehicle control, set relative to this (± SEM). One-way ANOVA analysis of variance with Newman-Keuls post-test was performed to determine statistical differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4 nM E_3 (representing the 20:80 ratio used in biest formulations), or 1 nM E_2 in the absence and the presence of both 8 nM E_3 and 1 nM E_1, (representing the 10:80:10 ratio used in triest formulations), no antagonism was observed (Fig. 6A–I).

4. Discussion

Estrogens are used in conventional HT to alleviate the side-effects of menopausal transition [1,2] and can be divided into three categories; namely natural, synthetic and bioidentical [17,23,24]. Considering that estrogens acting via ER signaling pathways contribute to breast cancer pathogenesis [93], this study aimed to comparatively characterize the

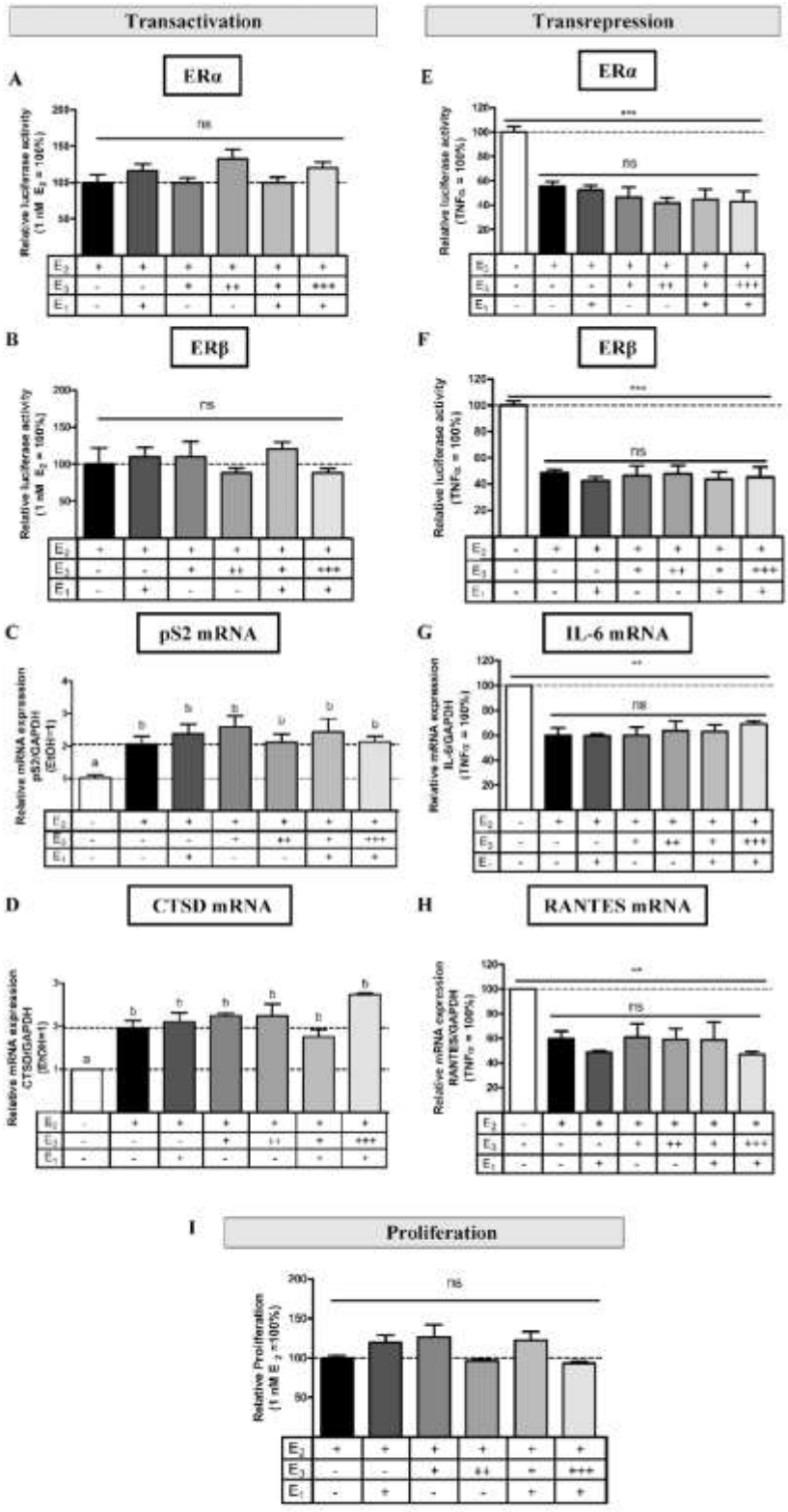


Fig. 6. E_3 and E_1 do not antagonize the activity of E_2. **(A–D)** For transactivation assays, the HEK293 cell line overexpressing either ERα or ERβ and the pS2-ERE-luciferase promoter-reporter construct, or the MCF-7 BUS cell line endogenously expressing both ER subtypes, was incubated with 1 nM E_2 in the absence and presence of 1 nM (+) E_1 and/or 1 nM (+), 4 nM (++) or 8 nM (+++) E_3 for 24 h. **(E–H)** For transrepression assays, the HEK293 cell line overexpressing either ERα or ERβ and the IL6-NFκB-luciferase promoter-reporter construct, or the MCF-7 BUS cell line endogenously expressing both ER subtypes, was incubated with 0.02 μg/ml TNFα in the absence and presence of the estrogens for 24 (promoter-reporter assays) or 6 (mRNA expression) hours. Results for transactivation via **(A)** ERα or **(B)** ERβ and transrepression via **(E)** ERα or **(F)** ERβ are shown as relative luciferase activity where induction with 1 nM E_2 (transactivation assays) or 0.02 μg/ml TNFα (transrepression assays) was set as 100%, and all other responses set relative to this. Relative mRNA expression of **(C)** pS2, **(D)** CTSD, **(G)** IL-6 or **(H)** RANTES normalized to GAPDH is shown. **(I)** MCF-7 BUS cells were treated for 48 h as described above for transactivation assays, and cell proliferation quantified using the MTT cell viability assay. Results are shown as relative proliferation with 1 nM E_2 set as 100%. One-way ANOVA analysis of variance with Newman-Keuls post-test was performed to identify statistical differences.

estrogenic properties of custom-compounded bioidentical E_2 and E_3 and synthetic EE relative to commercially available E_2, E_3 and E_1 standards (used as the natural controls in numerous studies [81,82,94–99]). While EE and E_2 are commonly used in both contraception [100–102] and conventional HT [7,103,104], bE_2, bE_3 and bE_1 are used in bHT [19,21,25]. This is the first study to compare the activities of EE, bE_2 and bE_3 relative to commercially available standards in parallel, in terms of binding affinity, gene expression, breast cancer cell proliferation and anchorage-independent growth. Previous studies investigating the binding affinities of estrogens for the ER often do not differentiate between the ER subtypes, and mostly report EC_{50} values or relative binding affinities (RBAs) [81,83,105–108] rather than equilibrium

dissociation constants (K_d/K_i values). Considering that EC_{50}/RBA values often differ between experimental systems, whereas K_d/K_i values remain constant, we determined precise K_d/K_i values of the estrogens for ERα and ERβ in the same model system. The K_d value of E_2 for ERα determined in our study (0.24 nM), is similar to K_d values reported by Matthews et al. [97] (0.4 nM), Kuiper et al. [98] (0.13 nM) and Stoica et al. [96] (0.23 nM). Although Matthews et al. [97] and Stoica et al. [96] did not report K_d values for ERβ, Kuiper et al. [98] and one other study by Escande et al. [82] reported values of 0.12 nM and 0.11 nM, respectively. Notably, these K_d values are approximately 20-fold lower than the value determined in our study (2.08 nM). Since it is known that the binding affinity of estrogens for the ER is species-dependent [83,97,109], the discrepancy between the K_d value determined in our study for ERβ and that of Kuiper et al. [98] may be attributed to the fact that these authors used human ERα and rat ERβ, whereas we used human ERα and human ERβ. Interestingly, Escande et al. [82] reported a K_d of 0.04 nM for E_2 for ERα which is approximately 10-fold lower than that reported by us and others [96–98], while not specifying which ER species they used in their study. We showed that the estrogens all displayed similar binding affinities to E_2 for ERα and ERβ, with the exception of EE which had a higher affinity for ERα (Fig. 1B), and E_1 which had a lower affinity for ERβ (Fig. 1D). Moreover, consistent with the literature available for E_2, E_3, E_1 and EE [81–83], we showed that all the estrogens have higher affinities for ERα than ERβ (Fig. 1F).

Receptor binding, however, does not always correlate to the biological activity of steroid hormones [19,110]. For example, although progesterone and the potent androgen receptor (AR) agonist dihydrotestosterone bind to the AR with similar affinities, progesterone is not an AR agonist, but rather an antagonist [60]. Thus, we next compared the agonist efficacies and potencies of the estrogens for transactivation and transrepression on synthetic promoter-reporter constructs in HEK293 cells overexpressing ERα or ERβ, and on endogenous pS2 and IL-6 gene expression in the MCF-7 BUS cell line endogenously expressing both ER subtypes. While the pS2 gene is a well-known marker of breast cancer that is upregulated in ER positive tumors and associated with disease progression [111], the pro-inflammatory cytokine, IL-6, is a marker of poor prognosis when upregulated in ERα positive tumors [112]. Despite the fact that EE had a higher affinity for ERα than E_2 (Fig. 1B), and E_1 a lower affinity for ERβ (Fig. 1D), we show that all the estrogens are full ERα (Fig. 2B) and ERβ (Fig. 2E) agonists. Interestingly, E_1, E_3 and EE were less potent than E_2 via ERα (Fig. 2C), while only E_1 was less potent via ERβ (Fig. 2F). In MCF-7 BUS cells expressing both ER subtypes, however, E_1 was less potent and efficacious than the other estrogens, the latter indicating that E_1 is a partial agonist for pS2 mRNA expression (Fig. 2J). In contrast, E_3 was as efficacious and potent as E_2 on both an endogenous (Fig. 2J and K) and a synthetic ERE-containing promoter (via ERβ) (Fig. 2E and F), suggesting that E_3 is a full agonist and not a weak estrogen as previously reported [19–21,25]. In agreement with our findings, one other study has also shown that E_3 is not a weak estrogen in terms of transactivation of endogenous ERE-containing genes in both the MCF-7 and T47D breast cancer cell lines [94]. While two previous studies have directly compared EC_{50} values of E_2, E_3, E_1 and EE for both ERα and ERβ [82,99], the EC_{50} values between the studies differed by up to 750-fold for some estrogens. Although both studies found that E_2 and EE were the most potent estrogens, E_3 was the least potent estrogen in one study [99], while E_1 was the least potent in the other [82]. Discrepancies between these studies may be ascribed to the different systems in which the agonist activities were measured, such as yeast versus mammalian, the specific hormone response element used in the promoter-reporter construct, and/or differences in ER species. In contrast to these studies comparing only the potencies of E_2, E_3, E_1 and EE via the individual ER subtypes, we compared the efficacies and potencies of EE, bE_2 and bE_3 relative to the estrogen standards via the individual human ER subtypes in the same model system. Moreover, we are the first to report efficacies and potencies of bE_2, E_3, bE_3, E_1 and EE on the expression of the endogenous pS2 gene in MCF-7 BUS cells expressing both ER subtypes. Our result showing that E_2 upregulated pS2 mRNA expression by 7.5-fold in MCF-7 BUS cells with a potency of 53.7 pM (Fig. 2I and L), is similar to a previous study reporting a 10-fold increase in pS2 mRNA expression and a potency of approximately 100 pM in MCF-7 cells [113]. Taken together, our results indicate that while the potencies may sometimes differ, all the estrogens have similar efficacies for transactivation via ERα and ERβ on a minimal ERE-containing promoter, while E_1 is less efficacious and potent than the other estrogens on the endogenous ERE-containing pS2 promoter in MCF-7 BUS cells expressing both ER subtypes.

In addition to transactivation, the estrogen-bound ER can also repress gene expression via protein-protein interactions with transcription factors such as NFκB [56,57], c-Jun [53–55] and Sp1 [58,59]. Although at least two studies have investigated the efficacy and potency of E_2 and/or EE for transrepression of gene expression via ERα [114,115], not much is known for the other estrogens used in this study or for the transrepressive activities via ERβ. Interestingly, Cerillo and co-workers [115] suggested that the ability of overexpressed ERα to modulate NFκB activity is cell-specific, as these authors showed ERα-mediated repression of NFκB activity by E_2 in HeLa and COS-1 cells, but not in HEK293 cells. In contrast, we found that E_2 and all the other estrogens could repress NFκB activity via either ER subtype in HEK293 cells, but not in COS-1 cells (Supplementary Fig. 5). Consistent with our transactivation results, we showed that all the estrogens are full agonists for transrepression via overexpressed ERα (Fig. 3B) or ERβ (Fig. 3E). Furthermore, we showed that although all estrogens were full agonists for transrepression of the endogenous IL-6 gene, E_1 displayed partial agonist activity on the endogenous pS2 gene. Our study is the first to directly compare efficacy and potency values for transrepression of EE, bE_2 and bE_3 relative to the estrogen standards (Fig. 3L).

To measure the potential combined effects of transactivation and transrepression of endogenous gene expression on the final cellular phenotype, we next investigated the effects of the estrogens on two hallmarks of cancer, cellular proliferation and metastasis [88]. Our results indicating no difference between the efficacies of the estrogens for proliferation (Fig. 4B), but that E_3, bE_3, E_1 and EE were all less potent than E_2 (Fig. 4C), correlate to the effects observed for transactivation via overexpressed ERα (Fig. 2C), but not ERβ (Fig. 2F). Furthermore, our results showing that E_3 was as efficacious as E_2 for proliferation, are in line with previous reports that E_2 and E_3 have similar effects on the proliferation of MCF-7 and T47D breast cancer cells [94,116] as well as on the induction of T47D cells into the S-phase during synchronization [95]. Moreover, anchorage-independent growth assays showed that the E_2, E_3 and E_1 standards, custom-compounded bioidentical E_2 and E_3, as well as synthetic EE, all increased the anchorage-independent growth of MCF-7 BUS cells to a similar extent (Fig. 5), suggesting that these estrogens may cause breast cancer metastasis to the same extent.

One of the justifications for the use of biest and triest formulations by proponents of bHT is that E_3 can antagonize the activity of E_2 [21,23,24,117,118] and that E_1 and E_3 are weak estrogens [19–21,25]. However, our results showed that E_3 is not an antagonist, but rather a full agonist for transactivation and transrepression of gene expression. In addition, although E_1 was a partial agonist for transactivation, it was a full agonist for transrepression and proliferation. Furthermore, in terms of the lack of antagonism by E_3 and E_1, we excluded the possibility of promoter-specific effects by showing similar results on the expression of the CTSD (Fig. 6D) and RANTES (Fig. 6H) genes. CTSD is an ERE-containing gene linked to breast cancer metastasis, invasion, relapse and short disease survival [119], while RANTES contains an NFκB-binding site in its promoter and is a major chemoattractant that has been shown to regulate progression of breast cancer tumors [120,121]. Moreover, we show that E_3 not only increases breast cancer cell proliferation (Fig. 4B) and anchorage-independent growth (Fig. 5B) to the same extent as E_2, but also does not antagonize E_2-induced effects

in these assays (Fig. 6I and Supplementary Fig. 2B).

5. Conclusion

Taken together, we show that the custom-compounded bioidentical estrogens mimic their respective commercial estrogen standards in all assays performed in this study, while also mimicking synthetic EE in most assays. Whether our findings are physiologically relevant, would depend on serum concentrations in women using these estrogens in HT. These serum levels range between 0.1 nM and 8.6 nM for E_2 [122–125], 0.5 nM and 0.6 nM for E_3 [126], 0.1 nM and 10 nM for E_1 [124,125,127], and 0.06 nM and 0.13 nM for EE [122,128]. The fact that we show maximal responses between 0.1 pM and 1 nM for regulation of gene expression and breast cancer cell proliferation highlights the potential of the estrogens evaluated in this study to display similar effects in vivo. Moreover, our data suggests that custom-compounded bHT may not be a safer alternative to conventional HT products. Notably, our results showing that E_3 and E_1 may not be weak estrogens, and that neither can antagonize the activity of E_2, suggest that the rationale behind custom-compounded biest and triest formulations should be re-evaluated. Moreover, our data suggests that there is no difference between the custom-compounded estrogens and commercially available estrogens suggesting no clear advantage in choosing bHT instead of HT. Finally, large-scale clinical trials are required to investigate the efficacy and safety of therapies currently administered by compounding pharmacies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2017.07.022.

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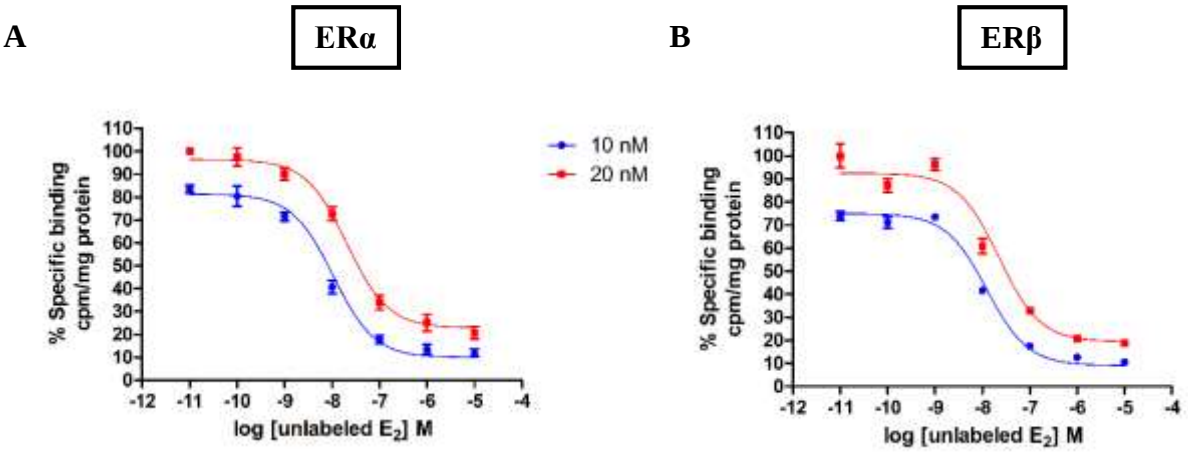
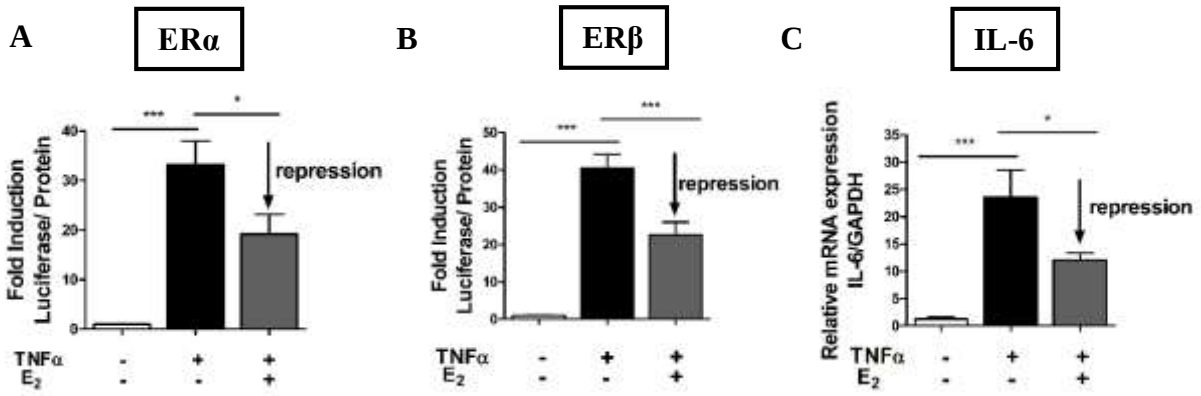
Supplementary Figures

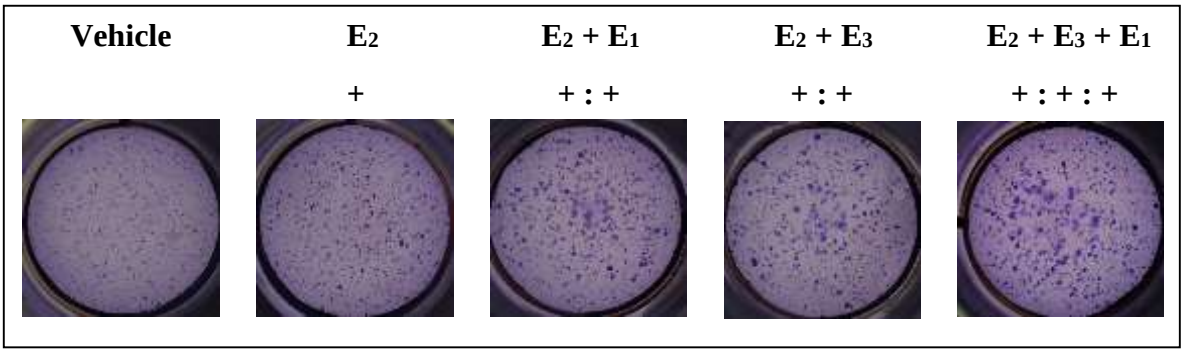


Supplementary Fig. 1. Determination of the equilibrium dissociation constant (K_d) of E_2 for ERα and ERβ from homologous displacement curves using a global fitting model. The COS-1 cell line transiently transfected with a human **(A)** ERα or **(B)** ERβ expression vector was incubated with 10 nM or 20 nM [^{3}H]-E_2 in the absence and presence of increasing concentrations of unlabeled E_2 for 4 hours. Counts per minute (cpm) were measured and normalized to protein concentration determined using the Bradford method [65]. Competition for binding is illustrated by the percentage of [^{3}H]-E_2 bound to the ER subtypes, where total binding ([^{3}H]-E_2 only) in the presence of 20 nM [^{3}H]-E_2 was set as 100% and all other results set relative to this. Results of at least three independent experiments with each condition performed in triplicate (± SEM) are shown.



Supplementary Fig. 2. E_2 significantly represses TNFα-induced upregulation of gene expression. The plots show the fold induction with 0.02 µg/ml TNFα and repression in the presence of 100 nM E_2. The HEK293 cell line transiently transfected with a human **(A)** ERα or **(B)** ERβ expression vector and the IL6-NFκB-luciferase promoter-reporter construct or the **(C)** the MCF-7 BUS cell line, was incubated with 0.1% EtOH (vehicle control) or 0.02 µg/ml TNFα in the absence and presence of 100 nM E_2. **(A, B)** Luciferase activity was measured in relative light units and normalized to protein concentration determined using the Bradford method [65]. **(C)** Real-time qPCR was performed to determine the mRNA expression levels of the IL-6 gene, using GAPDH as the internal standard. Relative gene expression of treated samples was calculated relative to the vehicle control (EtOH) which was set as 1, with all other responses set relative to this. One-way ANOVA analysis of variance with Newman-Keuls post-test was used to determine statistical significance of results.

A



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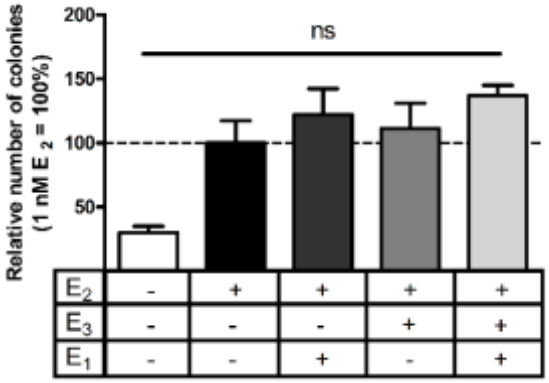
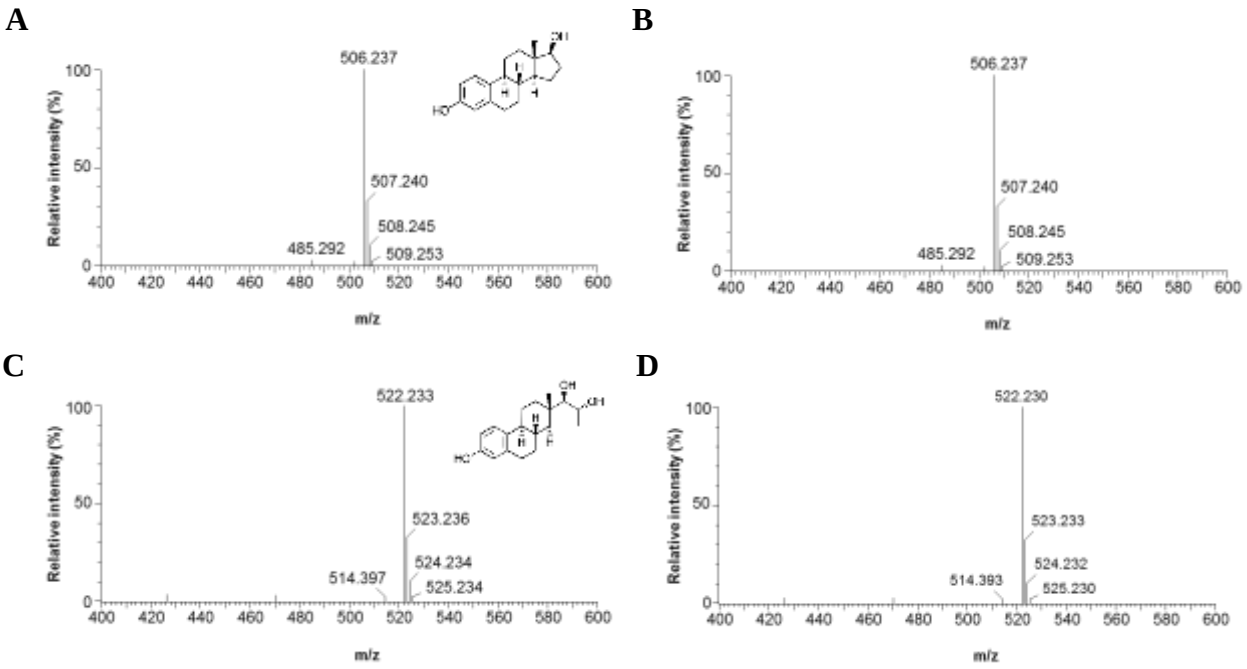


Supplementary Fig. 3. (A) The MCF-7 BUS cell line was incubated with 1 nM E_2 (+) in the absence and presence of 1 nM E_1 (+) and/or 1 nM E_3 (+) for 21 days, followed by staining of colonies with 0.005% crystal violet and **(B)** quantification using ImageJ software. The pictures in **(A)** are representatives of at least three independent experiments (± SEM). Anchorage-independent growth in the presence of 1 nM E_2 was set as 100% and all other responses, including the vehicle control, set relative to this. One-way ANOVA analysis of variance with Newman-Keuls post-test was used to determine statistical significance of results.

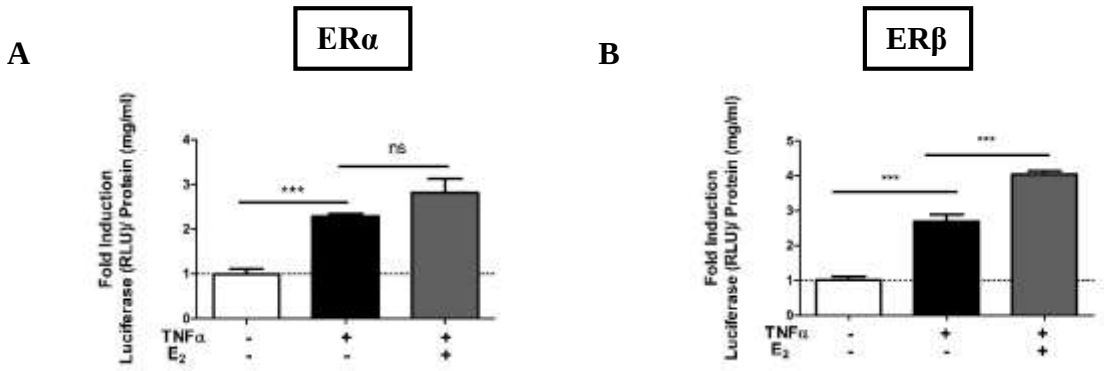


E

	Retention Time (min)	Observed Accurate Mass $(M+H)^+$	Derivatized Calculated Mass[a]	Calculated Mass[b]	Derivatized Formula[a]	Formula[b]
E_2	4.68	506.237	506.237	272.388	$C_{30}H_{36}NO_4S$	$C_{18}H_{24}O_2$
bE_2	4.70	506.237	506.237[c]	272.388[d]	$C_{30}H_{36}NO_4S$	$C_{18}H_{24}O_2$[d]
E_3	4.01	522.233	522.231	288.387	$C_{30}H_{36}NO_5S$	$C_{18}H_{24}O_3$
bE_3	4.01	522.230	522.231[c]	288.387[d]	$C_{30}H_{36}NO_5S$	$C_{18}H_{24}O_3$[d]

[a] after derivatization with dansyl chloride
[b] accepted for E_2 and E_3 standards prior to derivatization
[c] calculated from information given by supplier
[d] information given by supplier

Supplementary Fig. 4. MS spectra of **(A)** E_2 standard, **(B)** bE_2, **(C)** E_3 standard and **(D)** bE_3 after derivatization with dansyl chloride, as previously described [2]. Thereafter UPLC-MS was conducted using a Waters UPLC Ethylene Bridged Hybrid (BEH) C18 (2.1 mm × 50 mm, 1.7 µm) column (ACQUITY UPLC, Waters, Milford, USA). The UPLC mobile phases comprised of 1% formic acid (A) and acetonitrile (B). The estrogens were eluted at a flow rate of 0.350 ml/min using a linear gradient from 50% A to 100% B in 3.9 minutes, followed by a linear gradient from 100% B to 50% A in 0.1 minutes. An injection volume of 5 µl was used and accurate mass determinations were conducted using a Synapt G2 quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Milford, USA) in the positive ionisation mode (ESI+). The mass spectrometer was calibrated with sodium formate and leucine enkephalkin was used as the reference standard for lock mass. The remaining settings were used as previously described [123] and MassLynx V4.1 software (Waters, Milford, USA) was used to determine the molecular weights and elemental composition of the estrogens.



Supplementary Fig. 5. E_2 does not repress TNFα-induced gene expression in the COS-1 cell line. The COS-1 cell line was transiently transfected with either a human **(A)** ERα or **(B)** ERβ expression vector and the synthetic IL6-NFκB-luciferase promoter-reporter construct and incubated with 0.1% EtOH (vehicle control) or 0.02 µg/ml TNFα in the absence and presence of 10 µM E_2 for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration determined using the Bradford method [65].

Supplementary Table 1. Fractional occupancy of the ER subtypes at 100 nM, 1 µM and 10 µM ligand.[a]

Ligand	ERα			ERβ		
	100 nM	1 µM	10 µM	100 nM	1 µM	10 µM
E_2	99.64	99.96	99.99	97.96	99.79	99.98
bE_2	99.91	99.99	99.99	99.06	99.91	99.99
E_3	99.55	99.96	99.99	97.55	99.75	99.97
bE_3	99.69	99.97	99.99	99.15	99.91	99.99
E_1	99.25	99.92	99.99	91.54	99.08	99.96
EE	99.95	99.99	99.99	99.20	99.92	99.99

[a] The fractional occupancy refers to the fraction of receptors that are occupied by the specific ligand at equilibrium and was calculated using the equation: Fractional occupancy = [ligand] / ([ligand] + $K_d/_i$). The K_d values of E_2 and K_i values of the other ligands were obtained from Fig. 1E.

Chapter 3

Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy

The article in this chapter was published in *Biochemical and Biophysical Research Communications*, Volume 491, September 2017, pages 140-146, and is presented as it was published in combination with the supplementary data to form part of this thesis. Data that is referred to as 'data not shown' in the publication can be found in Appendix B.

The candidate and Dr. Renate Louw-du Toit share first authorship. The candidate planned and conducted all experimental work and data analysis pertaining to the estrogen receptor experiments, Dr. Renate Louw-du Toit performed the experimental work and data analysis pertaining to the androgen receptor. The candidate and Dr. Renate Louw-du Toit co-wrote the publication. Professor Janet Hapgood was a study collaborator and was involved with the critical evaluation of the study and editing of the publication. Prof. Donita Africander is the primary supervisor of the PhD study, the corresponding author, and was involved with the critical evaluation of the study, the writing and editing of the publication.

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Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy

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ABSTRACT

Progestins used in endocrine therapies bind to multiple steroid receptors and are associated with several side-effects. It is thus important to understand the relationship between steroid receptor cross-reactivity and the side-effect profile of progestins. In cell lines that express negligible levels of steroid receptors, we report for the first time the binding affinities, potencies and efficacies of selected progestins from different generations determined in parallel. We show that the progestins bind to the androgen receptor (AR) with similar affinities to each other and progesterone, while none bind estrogen receptor (ER)-β, and only norethisterone acetate, levonorgestrel and gestodene bind ERα. Comparative dose-response analysis revealed that progestins from the first three generations display similar androgenic activity to the natural androgen dihydrotestosterone for transactivation, while norethisterone acetate, levonorgestrel and gestodene are ERα agonists. We show for the first time that the anti-androgenic properties of progesterone and drospirenone are similar to the well-known AR antagonist hydroxyflutamide, while nomegestrol acetate is more potent and nestorone less potent than both hydroxyflutamide and progesterone. Moreover, we are the first to report that the older progestins, unlike progesterone and the fourth generation progestins, are efficacious ERα agonists for transrepression, while the selected progestins from the second and third generation are efficacious AR agonists for transrepression. Considering the progestin potencies and their reported free serum concentrations relative to dihydrotestosterone and estradiol, our results suggest that the progestins are likely to exert AR-, but not ERα- or ERβ-mediated effects *in vivo*.

1. Introduction

A variety of progestins, most of which are structurally related to the natural progestogen, progesterone (P_4), or testosterone [1,2], are used by women in contraception and menopausal hormone therapy (HT) [2,3]. Progestins are classified into four consecutive generations, with the fourth-generation reputed to be designed to have a greater affinity for the P_4 receptor (PR) and elicit biological effects more similar to P_4 than progestins from the earlier generations [3–5]. Despite their therapeutic benefits, clinical trials and epidemiological studies suggest that some progestins may result in side-effects including increased risk of developing breast cancer, cardiovascular disease, venous thromboembolism and increased susceptibility to genital tract infections [6–9]. Some of these adverse effects may be attributed to off-target actions via steroid receptors other than the PR [1,3,10].

Previously, the Hapgood laboratory directly compared the off-target mechanisms of the first-generation progestins medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A), and showed that they do not always mimic the effects of P_4 [11–13], and can differ from one another [11,13]. For example, P_4 displays anti-androgenic properties, while both MPA and NET-A are androgenic [12]. Furthermore, MPA, but not NET-A, is a partial glucocorticoid receptor agonist for transactivation [11]. However,

Abbreviations: AR, androgen receptor; ARE, androgen response element; DHT, dihydrotestosterone; DRSP, drospirenone; ER, estrogen receptor; ERE, estrogen response element; E_2, estradiol; GES, gestodene; HT, hormone therapy; LNG, levonorgestrel; MIB, mibolerone; MPA, medroxyprogesterone acetate; NES, nestorone; NET-A, norethisterone acetate; NoMAC, nomegestrol acetate; OHF, hydroxyflutamide; P_4, progesterone; PMA, phorbol 12-myristate 13-acetate; PR, progesterone receptor; TNFα, tumor necrosis factor alpha.

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numerous other progestins are available for clinical use, and it is surprising that studies comparing the activities of different progestins via individual steroid receptors are lacking.

Steroid receptors are ligand-activated transcription factors that activate or repress transcription of target genes [12,14]. Generally, transactivation refers to the increase in gene transcription caused by the ligand-activated steroid receptor such as the androgen receptor (AR) [12] or estrogen receptor (ER) [14] binding to androgen or estrogen response elements (AREs or EREs, respectively) in the promoters of target genes. Transrepression, on the other hand, can occur when the ligand-bound receptor inhibits gene transcription through protein-protein interactions with other transcription factors such as nuclear factor kappa-B (NFκB) or activator protein (AP)-1. This study is the first to directly compare the binding affinities and transcriptional activities of selected progestins from different generations relative to each other and P_4, in steroid receptor-deficient cell lines exogenously expressing human AR, ERα or ERβ.

2. Materials and methods

2.1. Inducing compounds

P_4, MPA, NET-A, levonorgestrel (LNG), gestodene (GES), nestorone (NES), nomegestrol acetate (NoMAC), drospirenone (DRSP), dihydrotestosterone (DHT), hydroxyflutamide (OHF), estradiol (E_2), fulvestrant (ICI 182,780) and phorbol 12-myristate13-acetate (PMA) were obtained from Sigma-Aldrich, RSA. Human tumor necrosis factor-alpha (TNFα) was obtained from Celtic Diagnostics, RSA. Unlabeled mibolerone (MIB) and [^{3}H]-MIB (84.3 Ci/mmol) were purchased from PerkinElmer Life and Analytical Science, RSA, while [^{3}H]-E_2 (100 Ci/mmol) was obtained from AEC-Amersham, RSA.

2.2. Cell culture

The COS-1 monkey kidney and HEK293 human embryonic kidney cell lines were obtained from the ATCC and cultured as previously described [12,15]. Only mycoplasma-negative cell lines were used in experiments.

2.3. Plasmids

The human AR expression vector (pSV-ARo) [16] was obtained from F. Claessens (University of Leuven, Belgium), while the human ERα and ERβ (pSG5-hERα and pSG5-hERβ) expression vectors [17] were received from F. Gannon (European Molecular Biology Laboratory, Germany). The pTAT-2xPRE-E1b-luciferase [18] and pGL3-2xERE-pS2-luciferase [19] constructs were gifts from G. Jenster (Erasmus University of Rotterdam, Netherlands) and B. Belandia (Institute for Biomedical Research, Spain), respectively. The 5xNFκB-luciferase plasmid was purchased from Stratagene (Houston, USA), while the p(IL6κB)350hu.IL6P-luciferase construct [20] was received from G. Haegeman (Ghent University, Belgium).

2.4. Whole cell binding assay

Competitive whole cell binding assays were performed in COS-1 cells as previously described ([12], Perkins et al., unpublished). Total binding ([^{3}H]-MIB or [^{3}H]-E_2 in the absence of unlabeled competitor) was set as 100% and binding of the unlabeled competitors plotted relative to this. K_d values were determined from homologous displacement curves using a global fitting model [21], whilst K_i values for the competing ligands were determined from heterologous displacement curves using the equation by Cheng and Prusoff [22].

2.5. Luciferase reporter assays

Promoter-reporter assays were performed essentially as previously described for the AR [12] and ER (Perkins et al., unpublished), with a few modifications. Briefly, COS-1 or HEK293 cells were seeded into 10 cm dishes at a density of 2×10^6 cells. After 24 h, the cells were transiently transfected using XtremeGene HP (Roche Molecular Biochemicals) as per the manufacturer's instructions. For transactivation assays, COS-1 cells were transfected with 2 μg AR and 20 μg pTAT-2xPRE-E1b-luciferase, while HEK293 cells were transfected with 0.15 μg ERα or ERβ and 6 μg (ERα) or 3 μg (ERβ) pGL3-2xERE-pS2-luciferase. For transrepression assays, COS-1 cells were transfected with 1.35 μg AR and 2.7 μg 5xNFκB-luciferase, while HEK293 cells were transfected with 0.15 μg ERα or ERβ and 1.5 μg p(IL6κB)$_3$50hu.IL6P-luciferase as transrepression after TNFα induction was not observed in COS-1 cells (data not shown). After 24 h, transfected cells were replated into 96-well plates at a density of 1×10^4 cells per well and treated with varying concentrations of test compounds in the absence (agonist dose-response) or presence of 0.1 nM MIB (AR) or 1 nM E_2 (ER) (antagonist dose-response) for 24 h (transactivation). For transrepression assays, the cells were treated for 24 h with either 10 ng/ml PMA (AR) or 20 ng/ml TNFα (ER) and increasing concentrations of the test compounds in the absence (agonist dose-response) or presence of 0.1 nM MIB (AR) or 1 nM E_2 (ER) (antagonist dose-response). The efficacies (maximal response) and potencies (EC_{50} values) were determined. Transcriptional activity of the ligands in the absence of transfected receptors was negligible in both cell lines (data not shown).

2.6. Data manipulation and statistical analysis

Graph Pad Prism® software version 5 was used for data analysis. Non-linear regression and one site competition were used for binding assays, while non-linear regression and sigmoidal dose-response were used for luciferase reporter assays. For both binding and dose-response analysis, fixed Hill slopes of 1 (transactivation) or -1 (competitive binding; transrepression) were chosen, which fitted the data with R^2 values of ≥0.9. One-way ANOVA analysis of variance and Newman-Keuls (compares all pairs of columns) post-test were used for statistical analysis. Statistically significant differences are indicated by different letters (a, b, c). Figures show pooled results and standard error of the mean (SEM) from at least two independent experiments performed in triplicate.

3. Results

3.1. Whilst all the selected progestogens bind to the AR, only NET-A, LNG and GES bind to ERα

Competitive whole cell binding assays in COS-1 cells expressing exogenous human AR, ERα or ERβ showed that all progestogens investigated bind to the AR (Fig. 1A), while only NET-A, LNG and GES bind to ERα (Fig. 1C and Supplementary Fig. 1A), and none bind to ERβ (Supplementary Fig. 1B). We confirmed the K_d/K_i values previously determined for MIB, DHT, P_4, MPA and NET-A [12], and showed that LNG, NoMAC and DRSP have similar binding affinities to each other and DHT for the AR, while GES exhibits a significantly higher affinity and NES a significantly lower affinity than the other progestogens and DHT (Fig. 1B; Supplementary Table 1). Surprisingly, NET-A displays a similar affinity to E_2 for ERα, while LNG and GES display similar binding affinities to each other, but significantly lower than that of E_2 and NET-A (Fig. 1C and D).

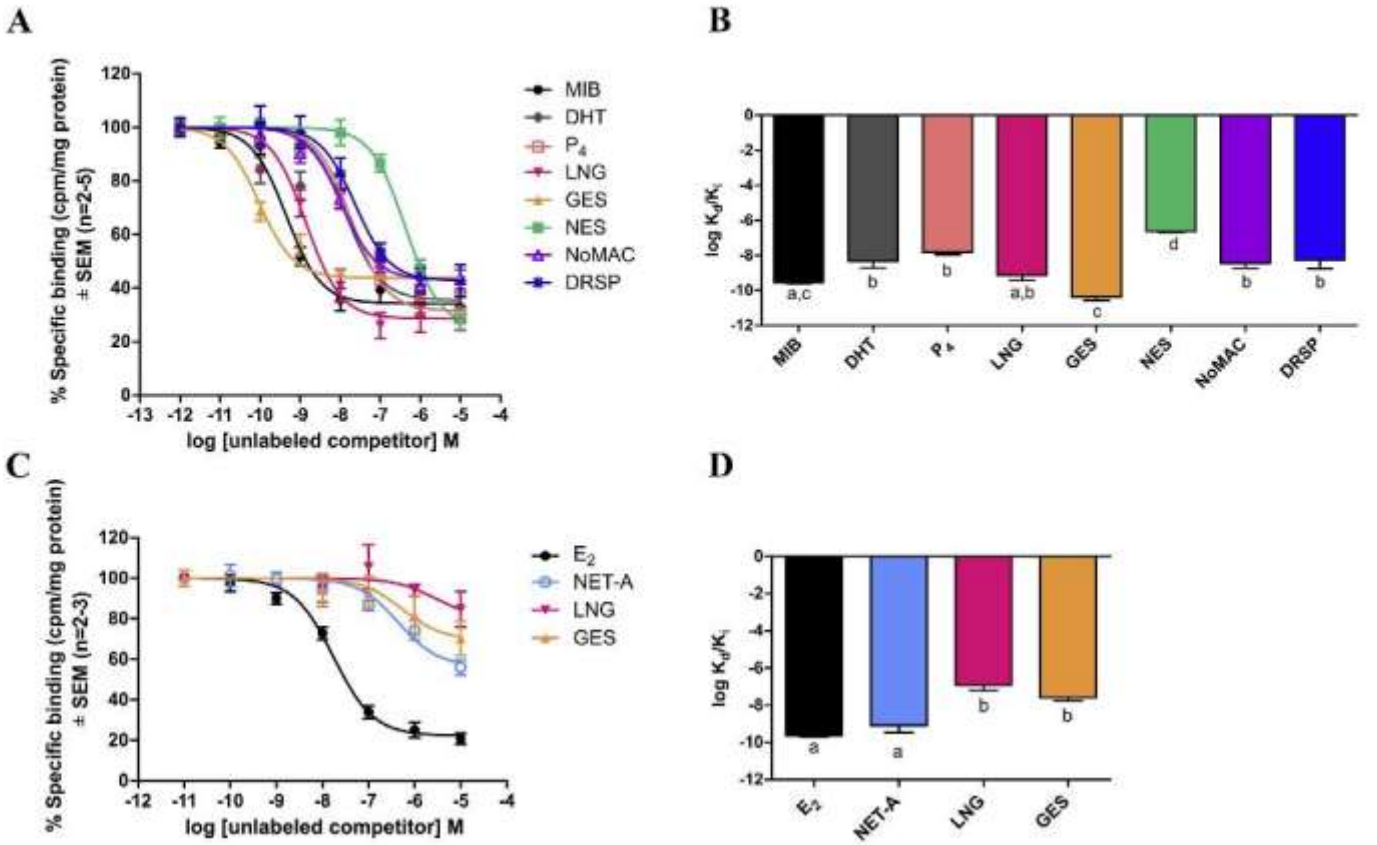

Fig. 1. (A) The selected progestogens all compete with [^{3}H]-MIB for binding to the human AR, (C) while only NET-A, LNG and GES bind to human ERα. COS-1 cells expressing the human (A) AR or (C) ERα expression vector, were incubated for (A) 16 h with 0.2 nM [^{3}H]-MIB in the absence or presence of increasing concentrations of either unlabeled MIB (●), DHT (◆), LNG (▼), GES (▲), NES (■), NoMAC (Δ), or DRSP (*) or (C) 4 h with 10 nM [^{3}H]-E_2 in the absence or presence of increasing concentrations of either unlabeled E_2 (●), NET-A (○), LNG (▼) and GES (▲). Counts per minute (cpm) were measured and normalized to protein concentration determined by the Bradford method [40]. Total specific binding of (A) [^{3}H]-MIB or (C) [^{3}H]-E_2 only was set as 100% and the binding of unlabeled competitors plotted relative to this. Log K_d/K_i values of the ligands for the (B) AR and (D) ERα were plotted.

3.2. NET-A, LNG and GES are agonists for both the AR and ERα, while NES, NoMAC and DRSP are AR antagonists

We next directly compared the relative agonist and antagonist efficacies and potencies (Supplementary Tables 2 and 3) of the progestogens for transactivation on an ARE-driven reporter construct via expressed AR in COS-1 cells or an ERE-driven reporter construct via expressed ERα in HEK293 cells. Results showed that LNG and GES display similar AR agonist efficacies and potencies to each other and DHT (Fig. 2A–C). In contrast, NES, NoMAC and DRSP, like P_4, display similar AR antagonist efficacies (Fig. 2D and E), but differential potencies (Fig. 2D and F). While P_4 and DRSP have similar potencies to the well-known AR antagonist OHF, NES is less potent and NoMAC more potent. We showed that although LNG and GES are full ERα agonists, while NET-A is a partial agonist (Fig. 2G and H), all three progestins display lower potencies than E_2 (Fig. 2G and I).

3.3. Fourth-generation progestins, unlike progestins from the first three generations, are partial AR agonists for transrepression, while NET-A, LNG and GES are ERα agonists for transrepression

We also compared the relative agonist and antagonist efficacies and potencies of the progestogens for transrepression (Supplementary Table 4) on an NFκB-containing promoter-reporter construct, in COS-1 cells expressing the AR and HEK293 cells expressing ERα. We showed that although all progestogens display similar agonist potencies to each other and to DHT (Fig. 3A and C), MPA, NET-A, LNG and GES, like DHT, are full AR agonists for transrepression, while P_4, NES, NoMAC and DRSP are partial agonists (Fig. 3A and B). Although P_4, NES, NoMAC and DRSP display similar antagonist efficacies, the progestins are less potent than P_4 (Supplementary Figs. 3A–3D). In terms of ERα, although NET-A and GES are full agonists for transrepression, while LNG is a partial agonist (Fig. 3D and E), NET-A and LNG are less potent than GES (Fig. 3D and F).

4. Discussion

Most studies comparing the binding of progestins to the AR report EC_{50} values (relative binding affinities) (reviewed in Refs. [2,12,23]) rather than precise equilibrium dissociation constants (K_i values), and mostly investigate binding to the rat AR or human AR in cell lines or tissue endogenously expressing other steroid receptors to which these ligands can bind (reviewed in Refs. [2,23]). We have recently reported accurate K_i values for P_4, MPA and NET-A for the human AR in COS-1 cells expressing negligible levels of endogenous steroid receptors [12]. Here we report for the first time accurate K_i values for LNG, GES, NES, NoMAC and DRSP (Supplementary Table 1). We performed detailed dose-response analysis and report both maximal responses and EC_{50} values for the progestins, relative to each other, natural P_4 and known androgens. We show for the first time that P_4 and DRSP are as potent as the well-known AR antagonist OHF, while NoMAC is

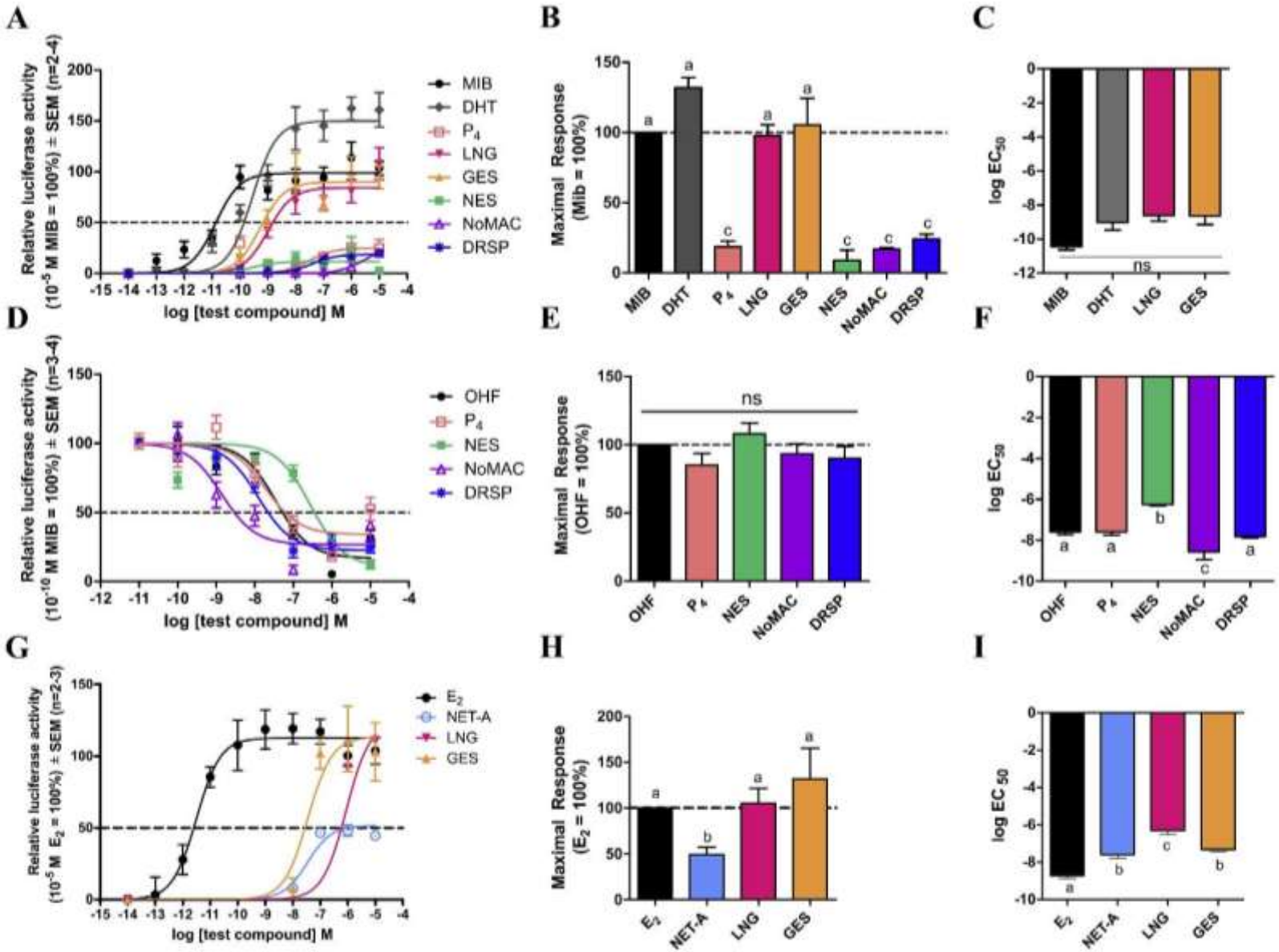

Fig. 2. (A) Second- and third-generation progestins display similar androgenic properties to DHT, while, like P_4, the fourth-generation progestins are AR antagonists. COS-1 cells, expressing the (A and D) human AR and the pTAT-2xPRE-E1b-luciferase reporter plasmid, were treated with varying concentrations of MIB (●), DHT (◆), P_4 (□), LNG (▼), GES (▲), NES (■), NoMAC (Δ) or DRSP (*) in the (A) absence or (D) presence of 0.1 nM MIB (set as 100%) for 24 h (G) LNG and GES are full agonists for transactivation via ERα, while NET-A is a partial agonist. The HEK293 cell line, expressing the (G) human ERα and the pGL3-2xERE-pS2-luciferase promoter-reporter plasmid, were treated with increasing concentrations of E_2 (●), NET-A (○), LNG (▼) and GES (▲) for 24 h. Luciferase activity was measured in relative light units and normalized to protein concentration determined by the Bradford method [40]. (B, E and H) Maximal responses and (C, F and I) log EC_{50} values were plotted. As P_4, NES, NoMAC and DRSP displayed very weak partial AR agonist activity, these log EC_{50} values were not depicted in (C) and should be interpreted with caution.

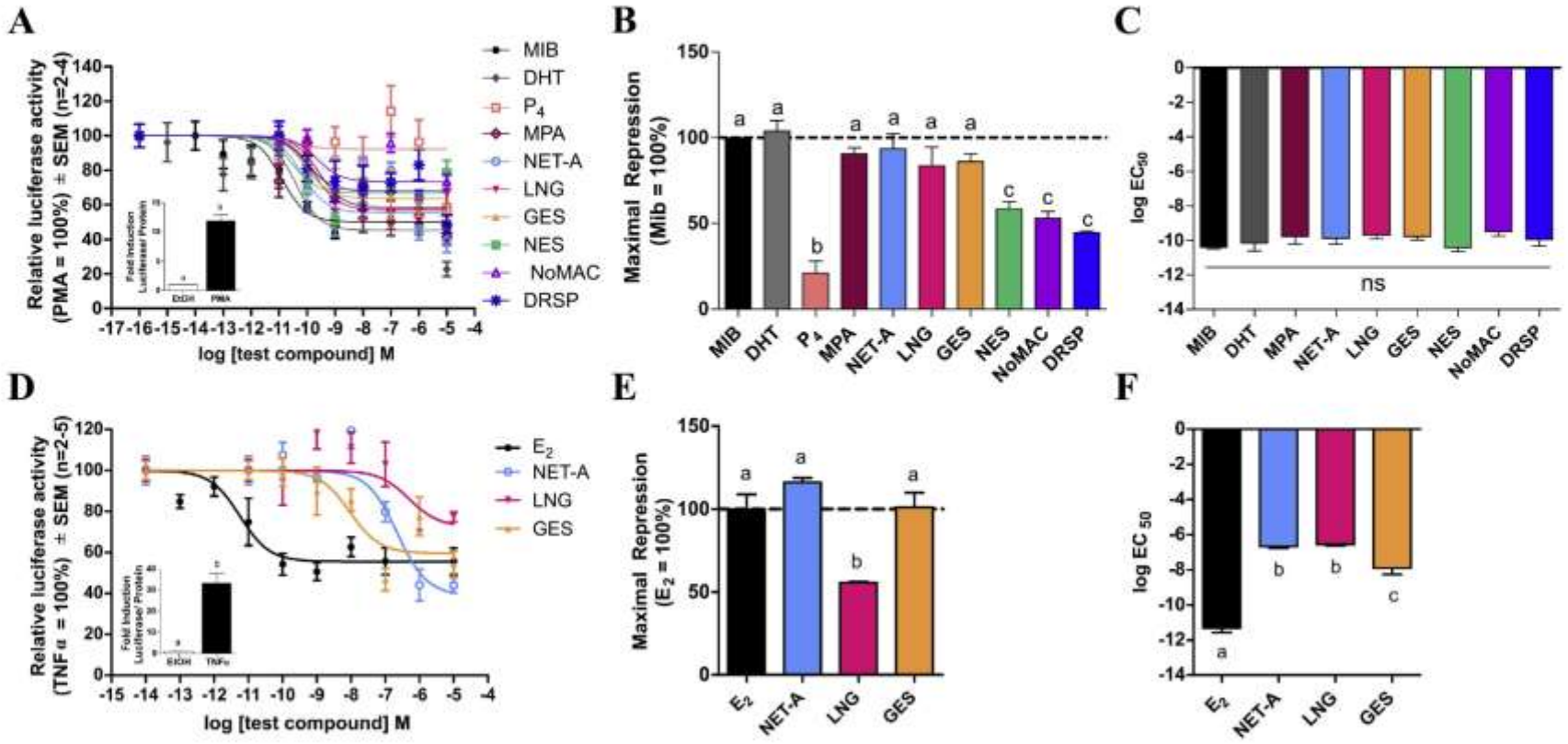

Fig. 3. (A) Progestins from the first three generations are full agonist for transrepression via the AR, while the fourth-generation progestins are partial agonists. COS-1 cells expressing human AR and the 5xNFκB-luciferase reporter plasmid, were treated with vehicle (EtOH) and 10 ng/ml PMA in the absence (set as 100%) or presence of increasing concentrations of MIB (●), DHT (◆), P_4 (□), MPA (◊), NET-A (○), LNG (▼), GES (▲), NES (■), NoMAC (Δ) or DRSP (*) for 24 h (D) NET-A and GES are full agonists for transrepression via ERα, while LNG is a partial agonist. HEK293 cells expressing human ERα and the p(IL6κB)$_3$50hu.IL6P-luciferase reporter plasmid, were treated with vehicle (EtOH) and 20 ng/ml TNFα in the absence (set as 100%) or presence of increasing concentrations of E_2 (●), NET-A (○), LNG (▼) and GES (▲) for 24 h. Luciferase activity was measured and normalized as in Fig. 2. Treatment with PMA or TNFα resulted in a ~12-fold and ~33-fold induction, respectively (Fig. 3A and D inserts). (B and E) Maximal repression and (C and F) log EC_{50} values were plotted.

more potent and NES less potent than P_4 and OHF (Fig. 2D–F). Although others have investigated the androgenic and anti-androgenic properties of some progestins for transactivation via the human AR [2,12,24–27], these studies often did not investigate progestins from different generations in parallel, did not include the relevant AR controls, used cell lines or tissues expressing multiple steroid receptors and do not investigate transrepression. This study is the first to show that progestins from the first three generations display similar androgenic properties for transrepression to each other and DHT, while P_4 and the fourth-generation progestins displayed anti-androgenic properties (Supplementary Fig. 3B) and partial agonist activity (Fig. 3A and B).

We showed that NET-A, LNG and GES, all structurally related to the estrogen precursor testosterone [28], bind to human ERα (Fig. 1C), but not human ERβ (Supplementary Fig. 1B), and are ERα agonists for both transactivation (Fig. 2G, Supplementary Fig. 2A) and transrepression (Fig. 3D, Supplementary Fig. 3E). Interestingly, previous studies investigated binding of progestin metabolites to ER subtypes rather than the parent compounds [29,30]. Results investigating binding of parent progestins such as MPA and NET-A are contradictory, possibly due to differences in model systems used (reviewed in Ref. [3]). Although the COS-1 and HEK293 cell lines express negligible levels of steroidogenic enzymes [31,32], we cannot exclude the possibility that the observed binding and estrogenic activity of these progestins may be due to progestin metabolites, as NET-A, LNG and GES have been shown to undergo metabolism [30], and it is known that some metabolites bind to and activate ERα [29,30]. However, as we showed no binding of NET-A, LNG and GES to ERβ, but others have reported binding of NET, LNG and GES metabolites [29,30], our results suggest that these progestins are probably not metabolized in our systems.

The physiological implications of our progestin results should be considered in the light of their affinities for the AR and ER, their serum concentrations in women using endocrine therapies and whether the progestins can bind to serum binding proteins, such as sex hormone binding globulin (SHBG). Steroids bound to SHBG are not available to enter target tissues, while unbound (free) steroids are, and can thus elicit a biological response [33]. Both DHT and E_2 bind to SHBG resulting in less than 1% of DHT and approximately 50% of E_2 being available to bind to the AR and ER, respectively, in target tissues [33,34]. While MPA, NES, NoMAC and DRSP do not bind to SHBG and are 100% available, NET-A, LNG and GES can bind and the availability of these progestins is approximately 65%, 50% and 25%, respectively [2,34,35]. Considering the above, and the affinities of DHT and the progestins for the AR, plus the fact that the progestin EC_{50} values for the AR in our study are within the range of serum concentrations reported for MPA (0.2–65 nM) [13], NET-A (17.6–36 nM) (Jinteli package insert, Teva Pharmaceuticals USA Inc.), LNG (4.4–16 nM) [36], GES (6.4–31 nM) [36], NES (0.1–27.3 nM) [37], NoMAC (3–33 nM) [38] and DRSP (26.7–253 nM) [39], it is likely that the progestins will compete with DHT for binding to the AR *in vivo*. However, considering the affinities of the progestins and E_2 for ERα, and that the EC_{50} values determined for NET-A, LNG and GES are 10–100-fold lower than the serum concentrations mentioned above, it is unlikely that these progestins will compete with E_2 for binding to ERα in target tissues. Taken together, our results showing that NES, NoMAC and DRSP elicit anti-androgenic and little to no androgenic activity, while lacking estrogenic effects, reassure the claims that the fourth-generation progestins are more similar to P_4 than progestins from the first three generations.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.07.063.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.07.063.

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SUPPLEMENTARY DATA

Supplementary Table 1. Binding affinities of the ligands for the AR, ERα and ERβ.[a]

Ligand	K_d or K_i (M) ± SEM		
	AR	ERα	ERβ
MIB	$3.19 \pm 0.58 \times 10^{-10}$	-	-
DHT	$1.63 \pm 1.39 \times 10^{-8}$	-	-
E_2	-	$2.35 \pm 0.34 \times 10^{-10}$ [b]	$2.08 \pm 0.68 \times 10^{-9}$ [b]
P_4	$1.59 \pm 0.46 \times 10^{-8}$	N.B.	N.B.
MPA	$2.21 \pm 0.65 \times 10^{-9}$	N.B.	N.B.
NET-A	$3.47 \pm 2.22 \times 10^{-9}$	$3.87 \pm 1.59 \times 10^{-9}$	N.B.
LNG	$1.32 \pm 0.86 \times 10^{-9}$	$2.40 \pm 1.22 \times 10^{-7}$	N.B.
GES	$4.66 \pm 1.90 \times 10^{-11}$	$2.80 \pm 1.01 \times 10^{-8}$	N.B.
NES	$2.41 \pm 0.39 \times 10^{-7}$	N.B.	N.B.
NoMAC	$5.76 \pm 3.62 \times 10^{-9}$	N.B.	N.B.
DRSP	$1.60 \pm 1.39 \times 10^{-8}$	N.B.	N.B.

[a]Data shown in Fig. 1A and 1E were analyzed using GraphPad Prism software, with non-linear regression analysis (one site competition). K_d ± SEM values for MIB and E_2 for the AR and ER subtypes, respectively, were determined from homologous displacement curves using a global fitting model [4], while K_i ± SEM values for the ligands were determined from heterologous displacement curves using the EC_{50} values, K_d ± SEM values for MIB or E_2 and the concentration of [^{3}H]-MIB or [^{3}H]-E_2, according to the equation by Cheng and Prusoff [22].

[b]Previously reported (Perkins et al., unpublished)

N.B. denotes no binding.

Supplementary Table 2. Relative agonist efficacies and potencies of the ligands for transactivation via the AR and ERα on a synthetic ARE- or ERE-containing promoter-reporter construct, respectively. [c]

Ligand	AR		ERα	
	MAX (%) ± SEM	EC_{50} (M) ± SEM	MAX (%) ± SEM	EC_{50} (M) ± SEM
MIB	100 ± 0.0	$4.42 \pm 1.33 \times 10^{-11}$	-	-
DHT	131.9 ± 7.27	$3.39 \pm 1.82 \times 10^{-9}$	-	-
E_2	-	-	100 ± 0.0 [e]	$3.09 \pm 1.07 \times 10^{-12}$ [e]
P_4	18.69 ± 4.16	$6.36 \pm 3.02 \times 10^{-6}$ [d]	N.D.	N.D.
MPA	134.2 ± 23.03	$1.32 \pm 0.84 \times 10^{-8}$	N.D.	N.D.
NET-A	67.15 ± 6.13	$5.01 \pm 1.47 \times 10^{-9}$	49.0 ± 8.34	$2.92 \pm 1.27 \times 10^{-8}$
LNG	97.65 ± 7.86	$2.33 \pm 0.15 \times 10^{-9}$	105.0 ± 16.18	$5.70 \pm 2.63 \times 10^{-7}$
GES	105.5 ± 19.01	$1.07 \pm 0.03 \times 10^{-9}$	103 ± 30.28	$5.12 \pm 1.10 \times 10^{-8}$
NES	9.08 ± 7.21	$1.33 \pm 1.23 \times 10^{-9}$ [d]	N.D.	N.D.
NoMAC	17.06 ± 1.04	$9.28 \pm 6.55 \times 10^{-6}$ [d]	N.D.	N.D.
DRSP	24.13 ± 3.54	$7.59 \pm 4.95 \times 10^{-7}$ [d]	N.D.	N.D.

[c]Data shown in Fig. 2A and 2G was analyzed to obtain the relative efficacies (maximal response (MAX) ± SEM) and potencies (EC_{50} ± SEM values) for each ligand for the AR or ERα, respectively.

[d]The EC_{50} values for NES, NoMAC and DRSP should be interpreted with caution as very weak agonist activity was observed.

[e]Previously reported (Perkins et al., unpublished)

N.D. denotes that no activity could be detected.

Since the progestogens do not bind to ERβ, no transcriptional activity was determined.

Supplementary Table 3. Relative antagonist efficacies and potencies of the ligands for transactivation via the AR on a synthetic ARE-containing promoter-reporter construct.[f]

Ligand	AR	
	MAX (%) ± SEM	**EC_{50} (M) ± SEM**
OHF	100 ± 0.0	$2.87 \pm 0.62 \times 10^{-8}$
P_4	85.25 ± 8.39	$3.14 \pm 1.16 \times 10^{-8}$
MPA	N.D.	N.D.
NET-A	N.D.	N.D.
LNG	N.D.	N.D.
GES	N.D.	N.D.
NES	108.1 ± 8.06	$5.80 \pm 0.89 \times 10^{-7}$
NoMAC	93.41 ± 7.12	$6.83 \pm 0.57 \times 10^{-9}$
DRSP	90.01 ± 8.96	$1.52 \pm 0.23 \times 10^{-8}$

[f]Data shown in Fig. 2D was analyzed to obtain the MAX ± SEM and EC_{50} ± SEM values for each ligand for the AR.

N.D. denotes that no activity could be detected.

Supplementary Table 4. Relative agonist efficacies and potencies of the ligands for transrepression via the AR and ERα on synthetic NFκB-containing promoter-reporter constructs.[g]

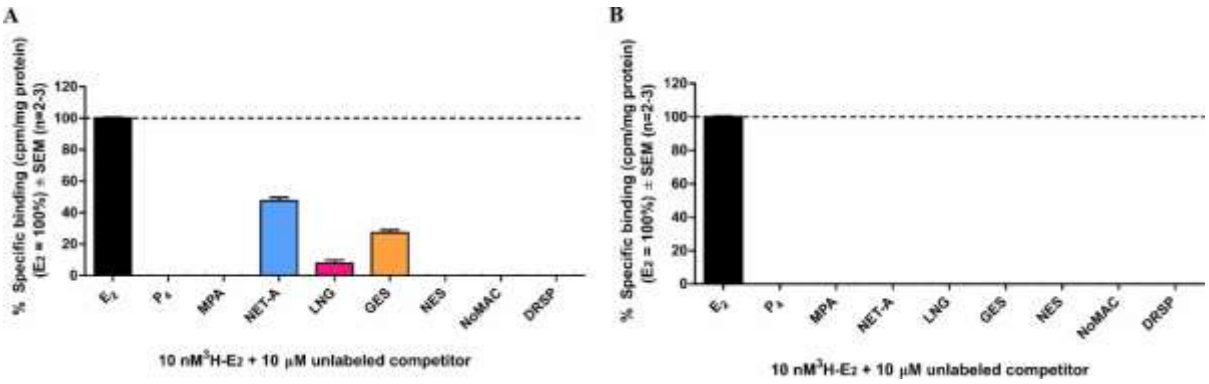
Ligand	AR		ERα	
	MAX (%) ± SEM	EC_{50} (M) ± SEM	MAX (%) ± SEM	EC_{50} (M) ± SEM
MIB	100 ± 0.0	$4.24 \pm 0.97 \times 10^{-11}$	-	-
DHT	104 ± 5.98	$2.26 \pm 2.02 \times 10^{-10}$	-	-
E_2	-	-	100 ± 0.0 [i]	$2.35 \pm 0.34 \times 10^{-10}$ [i]
P_4	20.92 ± 7.19	$1.15 \pm 1.03 \times 10^{-9}$ [h]	N.D.	N.D.
MPA	90.54 ± 3.63	$3.67 \pm 2.44 \times 10^{-10}$	N.D.	N.D.
NET-A	93.51 ± 8.70	$1.62 \pm 1.06 \times 10^{-10}$	116.1 ± 2.65	$2.18 \pm 0.49 \times 10^{-7}$
LNG	83.53 ± 11.05	$2.77 \pm 0.82 \times 10^{-10}$	55.7 ± 0.68	$2.82 \pm 0.43 \times 10^{-7}$
GES	85.96 ± 4.66	$1.88 \pm 0.80 \times 10^{-10}$	101.2 ± 8.69	$2.47 \pm 1.91 \times 10^{-8}$
NES	58.48 ± 4.14	$1.48 \pm 1.19 \times 10^{-10}$	N.D.	N.D.
NoMAC	53.12 ± 3.96	$4.88 \pm 2.70 \times 10^{-10}$	N.D.	N.D.
DRSP	44.54 ± 1.15	$2.48 \pm 1.97 \times 10^{-10}$	N.D.	N.D.

[g]Data shown in Fig. 3A and 3D was analyzed to obtain the MAX ± SEM and EC_{50} ± SEM values for each ligand for the AR or ERα, respectively.

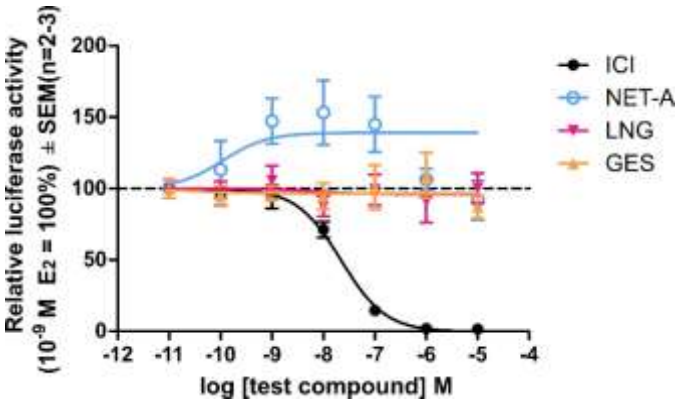
[h]As previously indicated in [12], the EC_{50} value for P_4 should be interpreted with caution as very weak agonist activity was observed.

[i]Previously reported (Perkins et al., unpublished)

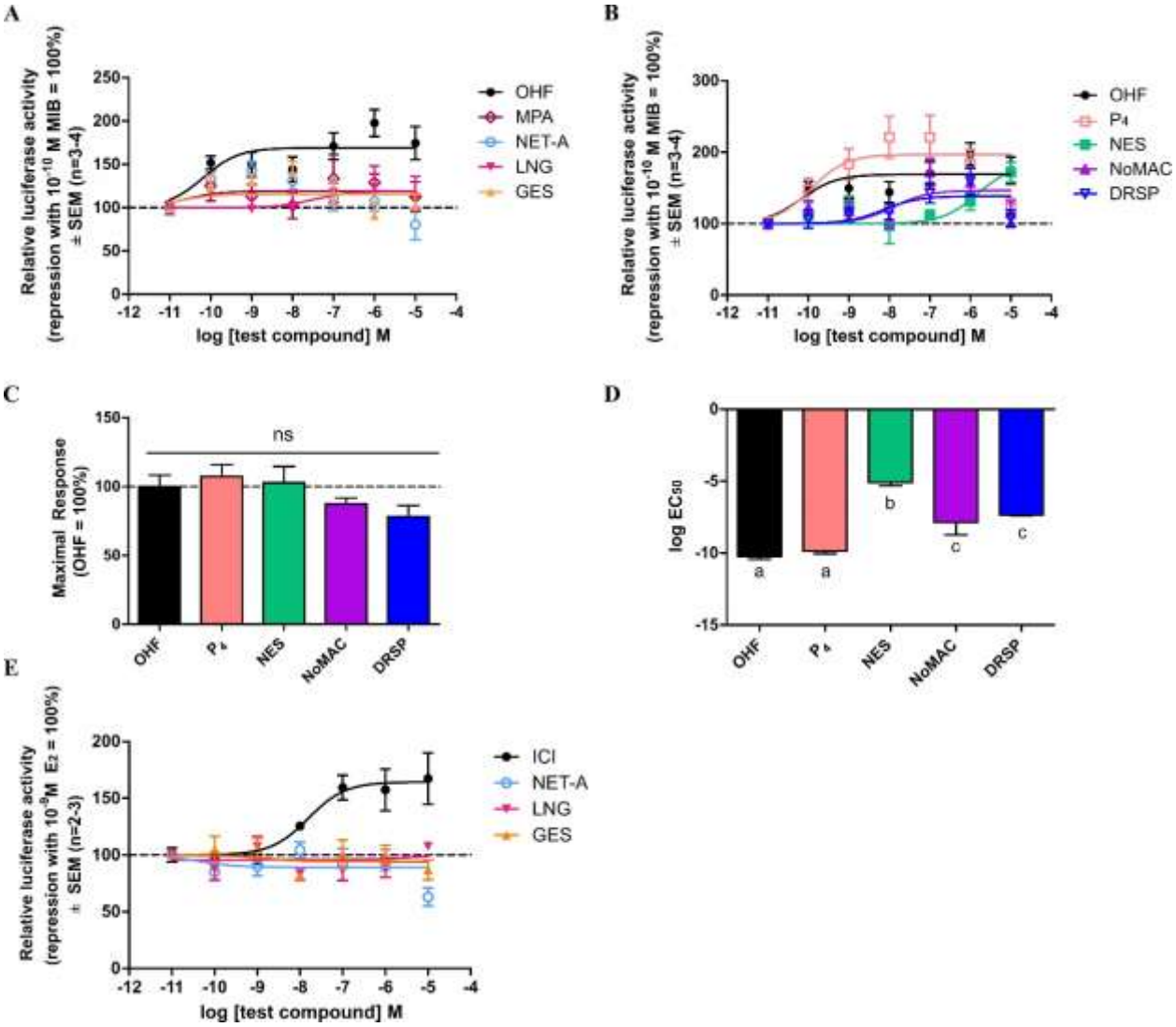
N.D. denotes that no activity could be detected.



Supplementary Figure 1. (A and B) While only NET-A, LNG and GES bind to human ERα, none of the progestogens bind to ERβ. COS-1 cells expressing either human (A) ERα or (B) ERβ were incubated for 4 hours with 10 nM [^{3}H]-E_2 in the absence or presence of 10 µM E_2, P_4, MPA, NET-A, LNG, GES, NES, NoMAC or DRSP. Counts per minute (cpm) were measured and normalized to protein concentration determined using the Bradford method [40]. Specific binding with unlabeled E_2 was set as 100% and the binding of the unlabeled competitors plotted relative to this.



Supplementary Figure 2. NET-A, LNG and GES do not display ER antagonist activity. HEK293 cells expressing human ERα and the pGL3-2xERE-pS2-luciferase promoter-reporter plasmid, were treated with 1 nM E_2 in the absence and presence of increasing concentrations of the well-known ER antagonist ICI 182,870 (ICI) (●), NET-A (○), LNG (▼) or GES (▲) for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration using the Bradford method [40]. Results are shown as relative luciferase activity with 1 nM E_2 set as 100% and all other responses set relative to this.



Supplementary Figure 3. (A and B) NES, NoMAC and DRSP, like P_4, antagonize MIB-induced transrepression. COS-1 cells expressing human AR and the 5xNFκB-luciferase reporter plasmid, were treated with 10 ng/ml PMA and 0.1 nM MIB in the absence and in the presence of increasing concentrations of P_4 (□), MPA (◊), NET-A (○), LNG (▼), GES (▲), NES (■), NoMAC (Δ), DRSP (*) and the AR antagonist OHF (●) for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration determined by the Bradford method [40]. Repression with 0.1 nM MIB was set as 100% and all other responses set relative to this. (C) Maximal response and (D) log EC_{50} values were plotted. (E) NET-A, LNG and GES do not display antagonist activity for transrepression via ERα. HEK293 cells expressing human ERα and the $p(IL6\kappa B)_3$50hu.IL6P-luciferase reporter plasmid, were treated with 20 ng/ml TNFα and 1 nM E_2 in the absence and presence of increasing concentrations of NET-A (○), LNG (▼), GES (▲) and the ER antagonist, ICI (●) for 24

hours. Luciferase activity was measured as above. Repression with 1 nM E_2 was set as 100% and all other responses set relative to this.

Chapter 4

Upregulation of estrogen receptor-regulated genes by first generation progestins requires both the progesterone receptor and estrogen receptor alpha

Upregulation of estrogen receptor-regulated genes by first generation progestins requires both the progesterone receptor and estrogen receptor alpha

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Abbreviations: AR, androgen receptor; CCND1, cyclin D1; CTSD, cathepsin D; DRSP, drospirenone; E_2, estradiol; ER, estrogen receptor; ERE, estrogen response element; GR, glucocorticoid receptor; HT, hormone therapy; ICI, fulvestrant; MPA, medroxyprogesterone acetate; NET, norethisterone; NET-A, norethisterone acetate; P_4, progesterone; PR, progesterone receptor; pS2, trefoil factor 1; RU486, mifepristone

Abstract

Various progestins, designed to mimic the activity of natural progesterone (P_4), are used globally in menopausal hormone therapy (HT). Although older progestins such as medroxyprogesterone acetate (MPA) and norethisterone (NET) have been implicated in increased breast cancer risk, it is controversial whether P_4 is associated with increased breast cancer risk and little is known regarding newer progestins. Considering that breast cancer is the leading cancer-related cause of mortality in women, establishing which progestins increase breast cancer risk and elucidating the mechanism behind the increased breast cancer risk is a global priority. In this study, we showed for the first time that the newer-generation progestin drospirenone (DRSP) is the least potent progestin in terms of proliferation of the estrogen-responsive MCF-7 BUS cell line, while natural P_4 and NET have similar potencies to estradiol (E_2), which is known to drive breast cancer cell proliferation. Notably, MPA, the progestin most frequently associated with increased breast cancer risk, was even more potent than E_2. In contrast to the differences in potencies for proliferation, we show for the first time that P_4, MPA, NET and DRSP all induce anchorage-independent growth of the MCF-7 BUS cell line to a similar extent as each other and E_2. Interestingly, the progestogen-induced proliferation and anchorage-independent growth occurs via a mechanism requiring both the progesterone receptor (PR) and estrogen receptor (ER). We also show that all the progestogens increased the formation of PR/ERα complexes and caused the recruitment of the complex to the promoters of the *cyclin D1* and *MYC* PR target genes under both non-estrogenic and estrogenic conditions in MCF-7 BUS cells. In contrast, only the older progestins, MPA and NET, caused the recruitment of the PR/ERα complex to the promoter region of the *trefoil factor 1* (*pS2*) and/or *cathepsin D* (*CTSD*) ER target genes, resulting in increased *pS2* and *CTSD* expression under both non-estrogenic and estrogenic conditions. These results suggest that progestins differentially regulate the manner in which the PR and ER cooperate to modulate the expression

of PR and ER regulated genes. Further studies are therefore required to underpin the clinical relevance of PR/ERα crosstalk in response to different progestins in both normal and malignant breast tissue. Our novel findings highlight differences between natural P_4 and progestins and emphasizes the importance of comparatively investigating the effects of individual progestins, rather than grouping them as a class and assuming that all progestins are the same.

1. Introduction

A variety of progestins are used globally in both contraception and postmenopausal hormone therapy (HT) [1–4]. For the latter, progestins are prescribed in combination with an estrogen to women with a uterus to prevent the proliferative effects of estrogens on the endometrium [2]. Progestins are synthetic progestogens (progesterone receptor (PR) ligands), that are classified into four consecutive generations, with the fourth-generation reported to have a greater affinity for the PR and elicit effects more similar to natural progesterone (P_4) than progestins from earlier generations [2,3,5]. For example, we have shown that fourth-generation progestins, like P_4, display anti-androgenic activity, while the earlier generation progestins display androgenic activity [6,7].

Both progestins and estrogens have previously been implicated in increased breast cancer risk [8]. However, HT containing progestins such as first-generation medroxyprogesterone acetate (MPA) [8–12] or norethisterone (NET) [8,10,11], have been associated with a higher risk than estrogen-only HT (reviewed in [12]). The role of progestins in breast cancer risk is, however, not straightforward as some clinical studies have suggested that progestins are not linked to increased breast cancer risk [13–21], while some progestins have also been used for breast cancer treatment [22–24]. An added complexity is the fact that a diverse range of progestins, known to elicit effects different to each other and P_4, are available for therapeutic use [6,25–27]. It is thus evident that large-scale clinical trials and more molecular studies are required to directly compare the effects of progestins on breast cancer risk.

Emerging evidence suggests that the estrogen receptor (ER) is not the only steroid receptor implicated in breast cancer pathogenesis, but that other steroid receptors such as the PR, previously considered to only be an indicator of a functional ER in breast cancer tumors [23,24,28], also plays an important role [29–39]. The role of the PR in breast cancer is however complex, as the PR isoforms, PR-A and PR-B, are generally expressed at equimolar ratios in

the normal mammary gland, while this ratio is usually dysregulated in breast cancer tissue [40–42]. Furthermore, the unliganded PR constitutively regulates a gene profile that is distinct from the profile regulated by the progestogen-activated PR (reviewed in [43]). Evidence also suggests that the formation of ERα and PR complexes can regulate the signaling of these receptors [34–36,39,44–50]. For example, a recent study revealed that unliganded PR-B enhances ERα-regulated gene expression and breast cancer cell proliferation [36], while another study showed that when the PR and ERα are activated, they associate and direct ERα to new chromatin binding sites, leading to a gene expression profile that is associated with a good prognosis in breast cancer [39]. Moreover, it has been shown that both ERα and the PR are required for MPA-induced increased gene expression and breast cancer cell proliferation [34].

Considering that there are many different types of progestins, this study aimed to directly compare the effects of selected progestins on breast cancer cell proliferation, anchorage-independent cell growth and the expression of ER target genes, while also elucidating the role of ER and PR signaling in mediating these processes. Since progestins are often co-administered with estrogens in hormone therapies [51] and breast cancer tumors often have high intratumoral estrogen levels [52], we also investigated the effects of estrogen-progestin combinations on the above-mentioned responses. Underpinning these mechanisms would further our understanding of the differential effects elicited by progestins and whether these effects are influenced by the presence of estrogen, all of which may assist in the design of hormone therapies with fewer side-effects.

2. Materials and Methods

2.1. Inducing compounds

Estradiol (E_2), P_4, MPA, NET, NET-acetate (NET-A), drospirenone (DRSP), mifepristone (RU486) and fulvestrant (ICI-182,780; ICI) were obtained from Sigma-Aldrich, RSA.

2.2. Cell culture

The human MCF-7 BUS breast cancer cell line, received from Prof. Ana Soto (Tufts University, Boston), was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/ml glucose (Sigma-Aldrich, RSA), 5% (v/v) heat-inactivated (HI)-fetal calf serum (FCS) (Biochrom GmbH, Germany) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, RSA) as previously described [53]. The HEK293 human embryonic kidney cell line was obtained from the American Type Culture Collection (ATCC) and maintained in DMEM containing 4.5 g/ml glucose, 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin as previously described [54]. All experiments were conducted in charcoal stripped (CS)-FCS and within the first 35 passages since the cell line was thawed from storage. Mycoplasma testing was routinely conducted using Hoechst staining [55], and only mycoplasma-negative cell lines were used.

2.3. Plasmids

Human ERα and ERβ encoding cDNA expression vectors (pSG5-hERα and pSG5-hERβ) [56] were received from Prof. Frank Gannon (European Molecular Biology Laboratory, Germany), while cDNA expression vectors encoding the human PR isoforms (pSG5-hPRA and pSG5-hPRB) [57] were a gift from Dr. Eric Kalkhoven (University Medical Centre Utrecht, The Netherlands). The pGL2basic cDNA expression vector containing no eukaryotic promoter or enhancer sequences was obtained from Promega, USA.

2.4. Cell viability assays

MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell viability assays were conducted as previously described [58] in order to evaluate effects on the proliferation of the MCF-7 BUS cell line. Briefly, the cells were treated with 0.1% EtOH (vehicle control), increasing concentrations of E_2, P_4, MPA, NET or DRSP or 100 nM of the progestogens in the absence and presence of 1 nM E_2, 10 µM ICI or 10 µM RU486. After 48 hours, the cells were incubated with pre-warmed MTT solution at a final concentration of 1.25 mg/ml for 4 hours. The medium was removed and 200 µl dimethyl sulfoxide (DMSO) added to each well. The absorbance at 550 nm was subsequently measured.

2.5. Anchorage-independent growth

Soft agar assays were conducted as previously described [58]. Briefly, MCF-7 BUS cells were incubated with 0.1% EtOH (vehicle control), 100 nM E_2, P_4, MPA, NET or DRSP or 100 nM of the progestogens in the absence and presence of 1 nM E_2, 10 µM ICI or 10 µM RU486 for 21 days. Thereafter, the colonies were fixed with 37% formaldehyde and stained with 0.005% crystal violet. Colonies were quantified using ImageJ software (Version 1.49) [59].

2.6. Small interfering RNA (siRNA) transfections

MCF-7 BUS cells were seeded into 10 cm^2 dishes at a density of 2 x 10^6 cells in phenol red-free DMEM supplemented with 5% HI-CS-FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin. The next day the cells were transfected with either 10 nM non-silencing scrambled sequence control (NSC) siRNA (Qiagen, USA) or siRNA directed against the human PR isoforms (GS5241; a combination of 4 target-specific siRNAs, Qiagen, USA), or 25 nM NSC siRNA or siRNA directed against human ERα (SC-29305; a combination of 4 target-specific siRNAs, Santa Cruz, Germany), using Dharmafect transfection reagent

(Dharmacon, USA) as per the manufacturer's instructions. After 24 hours, the cells were replated into 12-well plates at a density of 2 x 10^5 cells per well. The next day, cells were treated with 0.1% EtOH (vehicle control), or 100 nM MPA or NET in the absence and presence of 1 nM E_2, or 100 nM E_2 only for 24 hours. For the quantification of mRNA expression by real-time quantitative PCR (qPCR), total RNA was harvested and cDNA synthesized. Reduction in protein levels was confirmed by immunoblotting.

2.7. Isolation of total RNA, cDNA synthesis and real-time qPCR

MCF-7 BUS cells were plated and treated as described in Section 2.6. Total RNA was isolated using Tri-reagent (Sigma-Aldrich, RSA) and reverse transcribed using the ImProm-II™ Reverse Transcription System (Promega, USA) as per the manufacturer's instructions. Real-time qPCR was performed using the KAPA SYBR® FAST ABI Prism qPCR Kit (Roche Applied Science, RSA) according to the manufacturer's instructions. The mRNA expression of *pS2* (*trefoil factor 1*), *CTSD* (*cathepsin D*) and the reference gene *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenase*) was measured using the primer sets described in Table 1. Agarose gel electrophoresis and melt curve analyses were performed to confirm the presence of the amplicon of the correct size (data not shown). The primer efficiency of each primer set was determined using a cDNA dilution series to generate standard curves (data not shown). The efficiencies were 1.99, 1.93 and 1.86 for *pS2*, *CTSD* and *GAPDH*, respectively. Relative transcript levels were determined as previously described [60].

2.8. Co-immunoprecipitation (Co-IP) assays

Co-IP assays were conducted as previously described [61], with a few modifications. MCF-7 BUS cells were seeded into 10 cm^2 dishes at a density of 2 x 10^6 cells in phenol red-free DMEM supplemented with 5% HI-CS-FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin.

Table 1. Primers used for real-time qPCR.

Gene	Primer sequence	Amplicon Length	Ref.
pS2	5'-ATACCATCGACGTCCCTCCA-3' (fwd) 5'-AAGCGTGTCTGAGGTGTCCG-3' (rev)	147 bp	[62]
CTSD	5'-GCGAGTACATGATCCCCTGT-3' (fwd) 5'-CTCTGGGGACAGCTTGTAGC-3' (rev)	89 bp	[63]
GAPDH	5'-TGAACGGGAAGCTCACTGG-3' (fwd) 5'-TCCACCACCCTGTTGCTGTA-3' (rev)	307 bp	[64]

The following day cells were treated with 0.1% EtOH (vehicle control) or 100 nM E_2, MPA or NET for one hour. Cells were subsequently washed with ice-cold PBS and harvested in 500 µl RIPA buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.1% nondidet P-40 substitute (Roche Applied Science, RSA)), containing protease inhibitors. An aliquot was removed for input controls and the remaining lysate incubated for one hour with protein A/G PLUS agarose beads pre-blocked with salmon sperm DNA before an overnight incubation on a rotating wheel with antibodies against ERα (sc-8002, Santa Cruz Biotechnology, Germany), PR (PGR-312-L-CE, Leica Biosystems, UK) or IgG (IgG control, sc-2027, Santa Cruz Biotechnology, Germany). The antibody-bound proteins were released from the beads by boiling in 2xSDS-sample buffer [65] and the supernatants subjected to immunoblotting.

2.9. Immunoblotting

HEK293 cells were transiently transfected with the pGL2basic empty vector (negative control) or cDNA expression vectors for human ERα, PR-A and PR-B (positive controls). After 48 hours, cells were lysed with 2xSDS-sample buffer [65] and boiled at 97°C for 10 minutes. HEK293 cell lysates and MCF-7 BUS cell lysates from siRNA transfections (Section 2.6.) and Co-IP assays (Section 2.8.) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel, and subsequently transferred to nitrocellulose membranes (AEC Amersham, RSA). The membranes were then probed with primary antibodies specific for ERα, PR or the loading

control, GAPDH (0411, Santa Cruz Biotechnology, Germany), followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse, sc-2005, Santa Cruz Biotechnology, Germany). Proteins were visualized using enhanced chemiluminescence (Biorad, RSA) and a MyECL imager (Thermo Scientific, USA) and quantified using ImageJ software (version 1.49) [59].

2.10. Chromatin immunoprecipitation (ChIP) and re-ChIP assays

ChIP and re-ChIP assays were conducted as previously described [61,66–68], with a few modifications. Briefly, MCF-7 BUS cells were seeded into 10 cm^2 dishes at a density of 2 x 10^6 cells in phenol red-free DMEM supplemented with 5% HI-CS-FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin. After 24 hours, the cells were treated with 0.1% EtOH (vehicle control) or 1 nM MPA or NET in the absence and presence of 1 nM E_2, or 1 nM E_2 only for 2 hours. Cells were harvested in PBS containing protease inhibitors once the chromatin and proteins were cross-linked with 1% formaldehyde. The lysate was subsequently sonicated and 30 µg used as input controls. Approximately 100 µg of chromatin was immunoprecipitated with antibodies specific for ERα, PR or IgG, followed by the collection of chromatin using pre-blocked protein A/G-PLUS agarose beads. After thorough washing steps, the DNA-protein complexes were eluted. For ChIP assays a 1% SDS, 100 mM $NaHCO_3$ elution buffer was used, and for re-ChIP assays a 1% SDS, 10 mM dithiothreitol elution buffer containing protease inhibitors was used. For re-ChIP assays, an aliquot of the supernatant was used as confirmation that the first immunoprecipitation was successful, and the remaining chromatin was re-immunoprecipitated with anti-ERα, anti-PR or anti-IgG antibodies. The cross-linking of all the DNA-protein eluents was then reversed by adding NaCl, followed by incubation overnight at 65°C. Proteinase K (Roche Applied Science, RSA) was added to the samples the following day and incubated at 45°C for 1 hour for protein digestion. The input and immunoprecipitated

samples were subsequently purified using the Machery Nagel NucleoSpin® Extract II kit (Separations, RSA) as per the manufacturer's instructions. Purified DNA samples were analysed by real-time qPCR using the primer sets described in Table 2.

Table 2. ChIP and re-ChIP primers used for real-time qPCR.

Gene	Primer Sequence	Amplicon Length	Ref.
pS2	5'-ATTAGCTTAGGCCTAGAC-3' (fwd) 5'-CTGAGGGATCTGAGATTCA-3' (rev)	257 bp	[62]
CTSD	5'-TCCAGACATCCTCTCTGGAA-3' (fwd) 5'-GGAGCGGAGGGTCCATTC-3' (rev)	240 bp	[69,70]
CCND1	5'-CCTGCTGGGGCAACCCATCG-3' (fwd) 5'-CCCTCCCCCGCCGGGAATTA-3' (rev)	99 bp	[34]
MYC	5'-TCTCTGCTGACTCCCCCGGC-3' (fwd) 5'-CCGCGGGACCGGACTTCCTA-3' (rev)	71 bp	[34]

2.11. Data manipulation and statistical analysis

Data manipulation, graphical presentations and statistical analysis were performed using GraphPad Prism® version 5 (GraphPad Software). Non-linear regression analysis was used to determine efficacies and potencies. One-way ANOVA analysis of variance with Newman-Keuls (compares all pairs of columns) post-test was used to determine statistical significance of results. Statistically significant differences are indicated by the letters 'a', 'b', 'c', etc., where significantly different values are assigned a different letter, or * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). Non-significant differences are indicated by ns ($p>0.05$).

3. Results

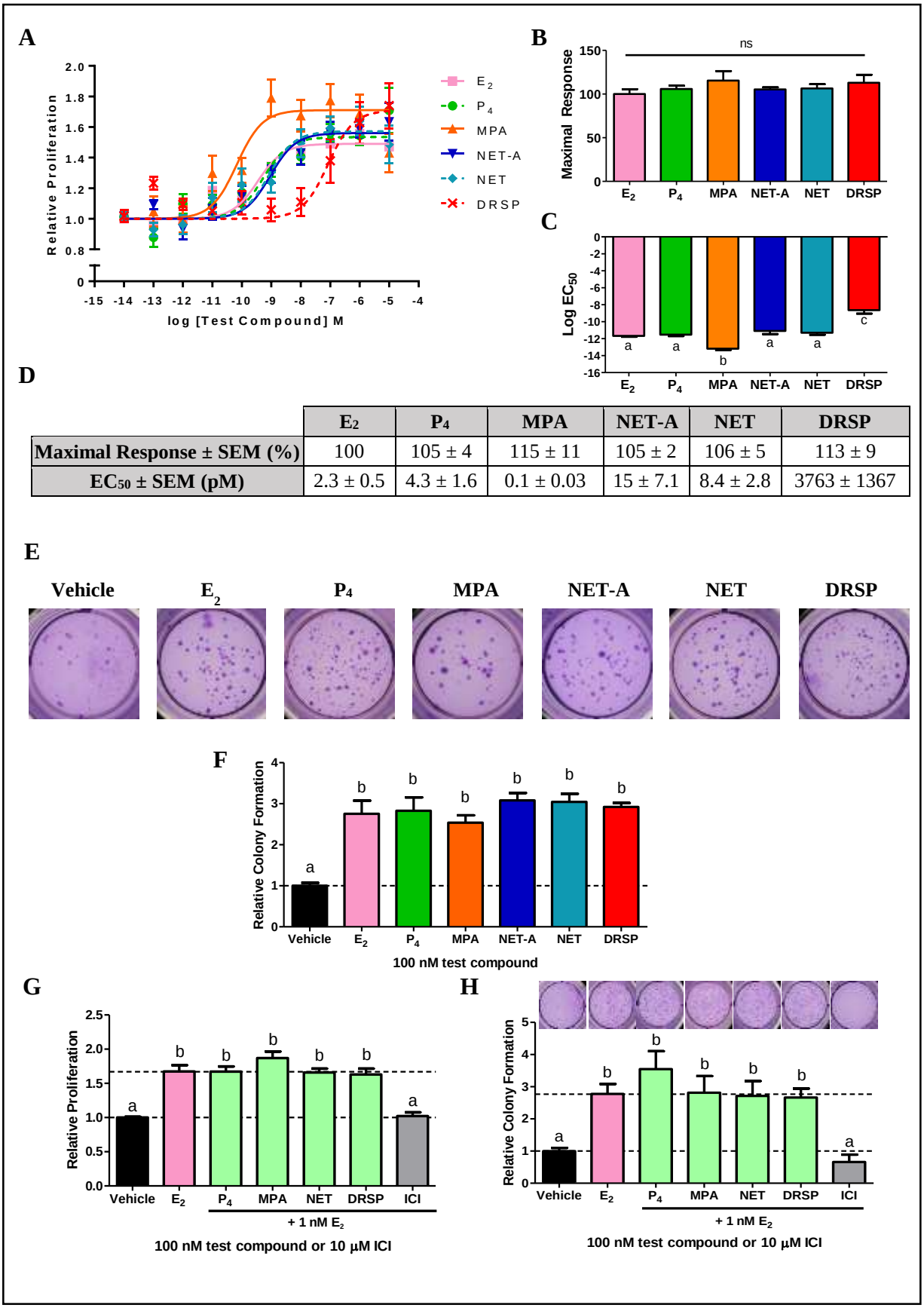
3.1. P_4, MPA, NET and DRSP increase proliferation and anchorage-independent growth of the estrogen-responsive MCF-7 BUS breast cancer cell line

Progestins often elicit biological effects that are distinct from each other and natural P_4 (reviewed in [2,26]). Indeed, studies have shown that some progestins are linked to increased breast cancer risk (reviewed in [12]), while most studies suggest P_4 is not [10,11,71]. We therefore directly compared the effects of the older first-generation progestins MPA and NET-A, as well as the newer fourth-generation progestin DRSP, relative to each other and natural P_4 on cell proliferation and anchorage-independent growth. In addition, we investigated whether the progestogens could antagonize the effects of E_2. MPA and NET-A were included in this study as they have both been linked to increased breast cancer risk [8], but are known to differentially activate steroid receptors such as the glucocorticoid receptor (GR) [25] and ERα [6]. DRSP was included as it is a progestin reported to elicit effects similar to P_4, but dissimilar to MPA and NET-A [6,72,73]. As NET-A administered in HT is rapidly metabolized to active NET [74], we included both NET-A and NET in this phase of the study to exclude the possibility that the acetate elicits different effects in the MCF-7 BUS cell line. This cell line was thus incubated with increasing concentrations of E_2, P_4, MPA, NET-A, NET and DRSP, or 1 nM E_2 in the absence and presence of 100 nM progestogens for 48 hours after which proliferation was quantified using the MTT cell viability assay. Since the MCF-7 BUS cell line is estrogen-sensitive [75] and highly proliferative in response to E_2 treatment, we included treatment with E_2 alone as a positive control. For anchorage-independent growth of the MCF-7 BUS cell line, the soft agar assay was used to quantify the number of colonies formed in the presence of 100 nM progestogens in the absence and presence of 1 nM E_2 for 21 days. Surprisingly, the selected progestins and P_4 had similar efficacies for proliferation of the estrogen-responsive MCF-7 BUS cell line to each other and E_2 (Fig. 1A and 1B). However,

while P_4, NET-A and NET were equipotent to E_2 and each other, MPA was approximately 20-fold more potent than E_2, and DRSP approximately 1600-fold less potent (Fig. 1C). The maximal responses (efficacies) and EC_{50} values (potencies) for proliferation are summarized in Fig. 1D. In terms of anchorage-independent growth, no differences were observed in the number of colonies obtained with any of the test compounds (Fig. 1F) and unlike the potent ER antagonist ICI, the progestogens did not modulate E_2-induced cell proliferation (Fig. 1G) or anchorage-independent growth (Fig. 1H). In agreement with the study by Govender *et al.* showing that NET-A and NET elicit similar effects to each other on gene expression in the HeLa and End1/E6E7 cell lines [25], we show that NET-A and NET induce similar increases in proliferation (Fig. 1A and 1D) and anchorage-independent growth (Fig. 1E and 1F). Further experiments were thus only performed with the active metabolite NET.

3.2. Progestogen-induced breast cancer cell proliferation and anchorage-independent growth is abrogated by ICI and RU486

Since the PR ligands displayed similar effects to E_2 on breast cancer cell proliferation and anchorage-independent growth in the estrogen-responsive MCF-7 BUS cell line, we next investigated the contributions of both the PR and ER towards mediating these effects. MCF-7 BUS cells were incubated with 100 nM P_4, MPA, NET or DRSP in the absence and presence of the ER antagonist, ICI, or the PR antagonist, RU486. Results show that both ICI and RU486 abrogated progestogen-induced proliferation (Fig. 2A) and anchorage-independent growth (Fig. 2B and 2C). Responses obtained in the presence of RU486 should, however, be interpreted with caution as it antagonizes not only the PR, but also the androgen receptor (AR) and GR [7,76,77]. Nevertheless, it is likely that the progestogen effects on proliferation and anchorage-independent growth are indeed mediated by the PR since we know that all these



	E2	P4	MPA	NET-A	NET	DRSP
Maximal Response ± SEM (%)	100	105 ± 4	115 ± 11	105 ± 2	106 ± 5	113 ± 9
EC50 ± SEM (pM)	2.3 ± 0.5	4.3 ± 1.6	0.1 ± 0.03	15 ± 7.1	8.4 ± 2.8	3763 ± 1367

Figure 1 legend on next page.

Figure 1. The progestogens display similar efficacies, but not potencies, for increased breast cancer cell proliferation and promote anchorage-independent growth of MCF-7 BUS cells to the same extent. The MCF-7 BUS cell line was incubated with EtOH (vehicle control) or **(A)** increasing concentrations of E_2 (■), P_4 (●), MPA (▲), NET-A (▼), NET (♦) or DRSP (×) or **(G)** 1 nM E_2 in the absence and presence of 100 nM progestogens or 10 µM ICI, for 48 hours. Cell proliferation was quantified using the MTT cell viability assay. **(B)** Plots of the maximal response and **(C)** log EC_{50} values of the test compounds for proliferation from Fig. 1A are shown and these values are reported in **(D)**. **(E, F and H)** MCF-7 BUS cells were incubated with EtOH (vehicle control) or 100 nM progestogens in the absence and presence of 1 nM E_2 for 21 days. After 21 days, colonies were stained with 0.005% crystal violet and **(F and H)** quantified using ImageJ software (Version 1.49). Results shown are representatives of at least three independent experiments, with the response obtained in the presence of the vehicle control set as one, and all other responses set relative to this. One-way ANOVA analysis of variance with Newman-Keuls post-test was performed to determine statistical differences**.**

progestogens are PR agonists, while NET and DRSP are not GR [73,78] agonists and P_4 and DRSP are AR antagonists [6].

3.3. P_4, MPA, NET and DRSP differentially regulate the mRNA expression of the ER-regulated pS2 and CTSD genes in an ER- and PR-dependent manner

Considering that we showed that progestogen-induced breast cancer cell proliferation and anchorage-independent growth requires the ER, we next investigated whether the selected progestogens could modulate the mRNA expression of the ER-regulated *pS2* and *CTSD* genes and whether these effects would be altered in the presence of E_2. The *pS2* gene is a well-known marker of breast cancer that is upregulated in ER positive tumors and associated with disease progression [79], while *CTSD* is linked to breast cancer metastasis, invasion, relapse and short disease survival [80]. MCF-7 BUS cells were thus incubated for 24 hours with 100 nM P_4, MPA, NET and DRSP in the absence and presence of 1 nM E_2, or with 100 nM E_2 only. Results showed that MPA was the only progestogen to increase *pS2* mRNA expression and to a similar extent as E_2 (Fig. 3A), while both MPA and NET significantly increased *CTSD* mRNA

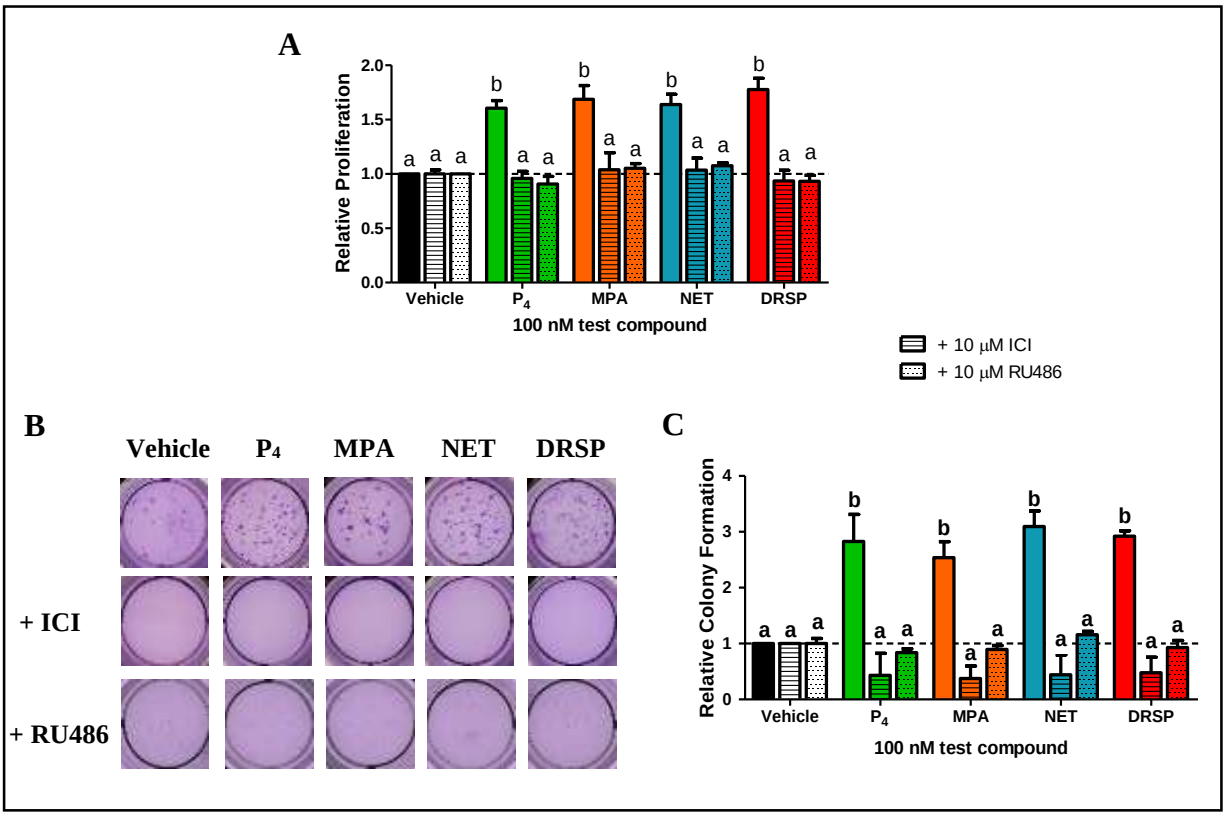


Figure 2. Progestogen-induced breast cancer cell proliferation and anchorage-independent growth is inhibited by both ICI and RU486. The MCF-7 BUS cell line was incubated with either EtOH (vehicle control) or 100 nM P_4, MPA, NET or DRSP in the absence and presence of 10 µM ICI or 10 µM RU486 for **(A)** 48 hours or **(B)** 21 days. **(A)** Cell proliferation was quantified using the MTT cell viability assay, while **(B)** anchorage-independent growth was quantified using the soft agar assay. **(C)** The colonies formed (in Fig. 2B) were quantified using ImageJ software (Version 1.49). Results are shown as **(A)** relative proliferation or **(C)** relative colony formation with the response obtained with the vehicle control set as one, and all other responses set relative to this. Results shown in (A) and (C) are the averages of at least three independent experiments, while (B) is a representative figure.

expression, albeit to a lesser extent than E_2 (Fig. 3B). Interestingly, treatment with progestogen in the presence of E_2 compared to E_2 alone, resulted in similar increases in *pS2* (Fig. 3C) and *CTSD* (Fig. 3D) mRNA expression. Knowing that both MPA and NET can bind to the PR, whilst NET-A, but not MPA, can bind to ERα [6], and that both the PR and ER are required for progestogen-induced breast cancer cell proliferation and anchorage-independent growth, we next investigated the role of the PR isoforms and ERα in mediating the effects of MPA and/or NET on these genes. MCF-7 BUS cells transfected with non-silencing control (NSC),

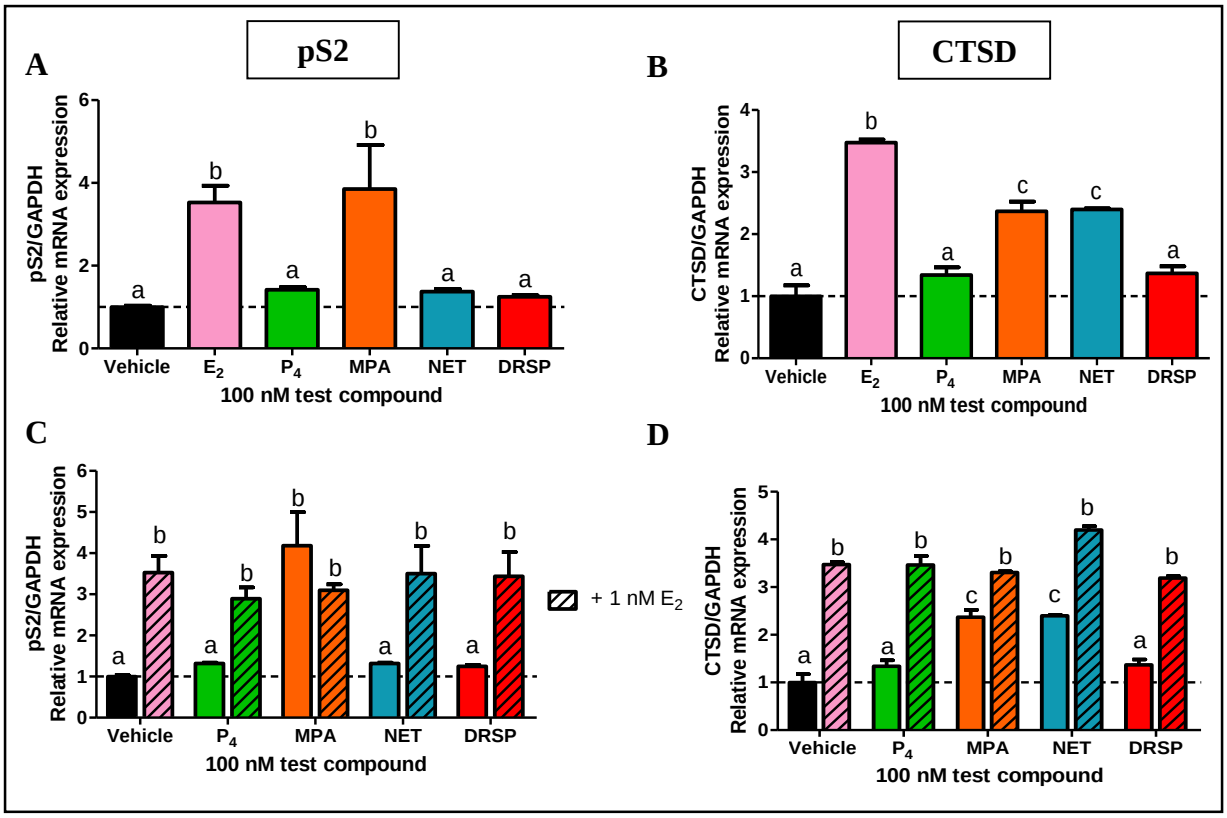


Figure 3. The progestogens differentially modulate *pS2* and *CTSD* mRNA expression and do not alter E_2-induced *pS2* and *CTSD* mRNA expression. MCF-7 BUS cells were treated with EtOH (vehicle control) or **(A and B)** 100 nM E_2, P_4, MPA, NET or DRSP, or **(C and D)** 100 nM progestogen in the absence and presence of 1 nM E_2 for 24 hours. Total RNA was isolated, reverse transcribed and real-time qPCR conducted to determine the relative expression of **(A and C)** *pS2* and **(B and D)** *CTSD* mRNA levels relative to that of *GAPDH* (reference gene). The vehicle control was set as one and the relative mRNA expression of *pS2* and *CTSD* in the treated samples set relative to this. Results shown are the averages of at least three independent experiments.

PR-A/B or ERα siRNA were incubated with 100 nM E_2, MPA or NET for 24 hours. Western blotting (Fig. 4A and 4B) confirmed that transfection of the MCF-7 BUS cell line with PR-A/B siRNA resulted in a 73% and 71% knockdown of PR-A and PR-B respectively, while ERα siRNA resulted in a 60% decrease in ERα expression, as well as 80% knockdown of PR-A and 86% knockdown of PR-B. The fact that knockdown of the ER causes a reduction in PR levels was not completely unexpected as it is well-known that the PR is an ER target gene [81], and this reduction has in fact been shown previously [82]. Both PR and ERα knockdown abrogated

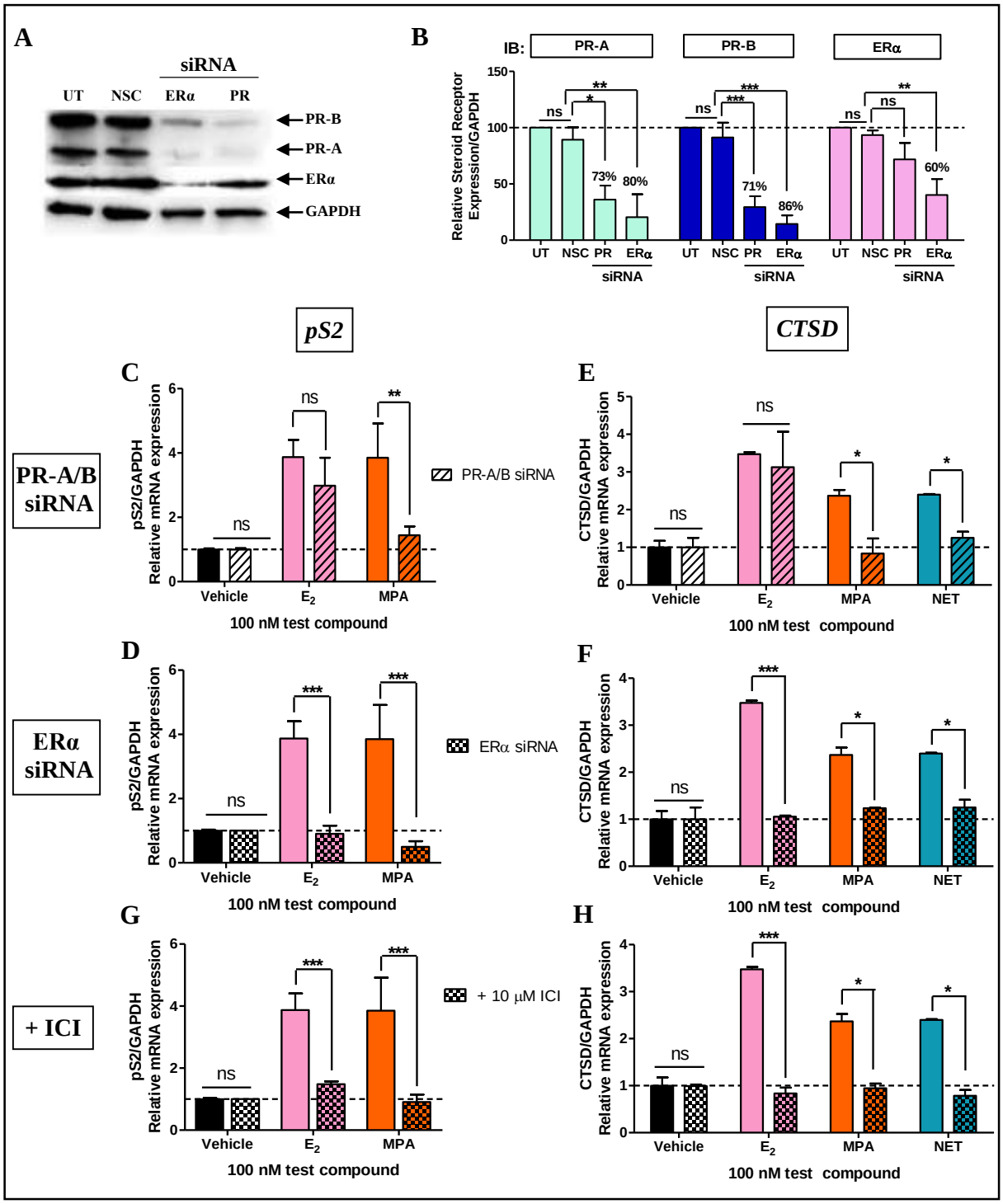


Figure 4. Both the PR and ERα are required the upregulation of ER-regulated genes by MPA and/or NET. MCF-7 BUS cells transfected with **(C, D)** 10 nM NSC or PR-A/B siRNA or **(E, F)** 25 nM NSC or ERα siRNA were treated with EtOH (vehicle control) or 100 nM E_2, MPA or NET for 24 hours. **(A)** For verification of PR-A/B or ERα knockdown, total protein from the MCF-7 BUS cells transfected as described above was harvested, and western blotting performed using antibodies specific for ERα, PR-A/B and GAPDH. A representative blot is shown and **(B)** PR-A, PR-B and ERα expression levels were quantified relative to the GAPDH loading control using ImageJ software (Version 1.49).

Western blots of three independent experiments were quantified to determine the percentage protein knocked down. **(G and H)** MCF-7 BUS cells were treated with EtOH (vehicle control) or 100 nM E_2, MPA or NET in the absence and presence of 10 µM ICI for 24 hours. **(C-H)** Total RNA was isolated, reverse transcribed and real-time qPCR was conducted to determine the relative expression of **(C, E, G)** *pS2* and **(D, F, H)** *CTSD* mRNA levels relative to *GAPDH*. The vehicle control of each condition was set as one and the relative mRNA expression in the treated samples set relative to this. Results shown are the averages of at least three independent experiments.

MPA-induced *pS2* (Fig. 4C and 4E) and *CSTD* (Fig. 4D and 4F) mRNA expression, as well as the NET-induced *CTSD* mRNA expression (Fig. 4D and 4F). As expected, ERα knockdown, but not PR knockdown, abrogated the E_2-induced increase in both *pS2* (Fig. 4C and 4E) and *CTSD* (Fig. 4D and 4F) mRNA expression. Considering that ERα knockdown also silenced PR expression, we confirmed that the ER is required for the upregulation of *pS2* (Fig. 4G) and *CTSD* (Fig. 4H) mRNA expression by MPA and/or NET using ICI to antagonize the ER, as ICI does not decrease PR levels [83].

3.4. MPA and/or NET treatment results in co-recruitment of the PR and ERα to the pS2 and CTSD promoters

Given that we show that both ERα and the PR are required for MPA- and/or NET-induced regulation of ER-target genes, we next investigated whether MPA or NET treatment results in the formation of PR/ERα complexes in the MCF-7 BUS cell line. The cells were incubated with 100 nM MPA, NET or E_2 for 1 hour followed by immunoprecipitation using a PR-A/B- or an ERα-specific antibody, and western blot analysis confirmed that PR-A, PR-B and ERα are present in all input samples (Fig. 5A). Results from Co-IP assays (Fig. 5B and 5C) confirmed previous findings that these steroid receptors occur in a complex both in the absence and presence of ligand [34,39] and revealed that both MPA and NET treatment resulted in increased PR-A and PR-B complexed with ERα (Fig. 5B - 5E). As expected, the E_2 control did

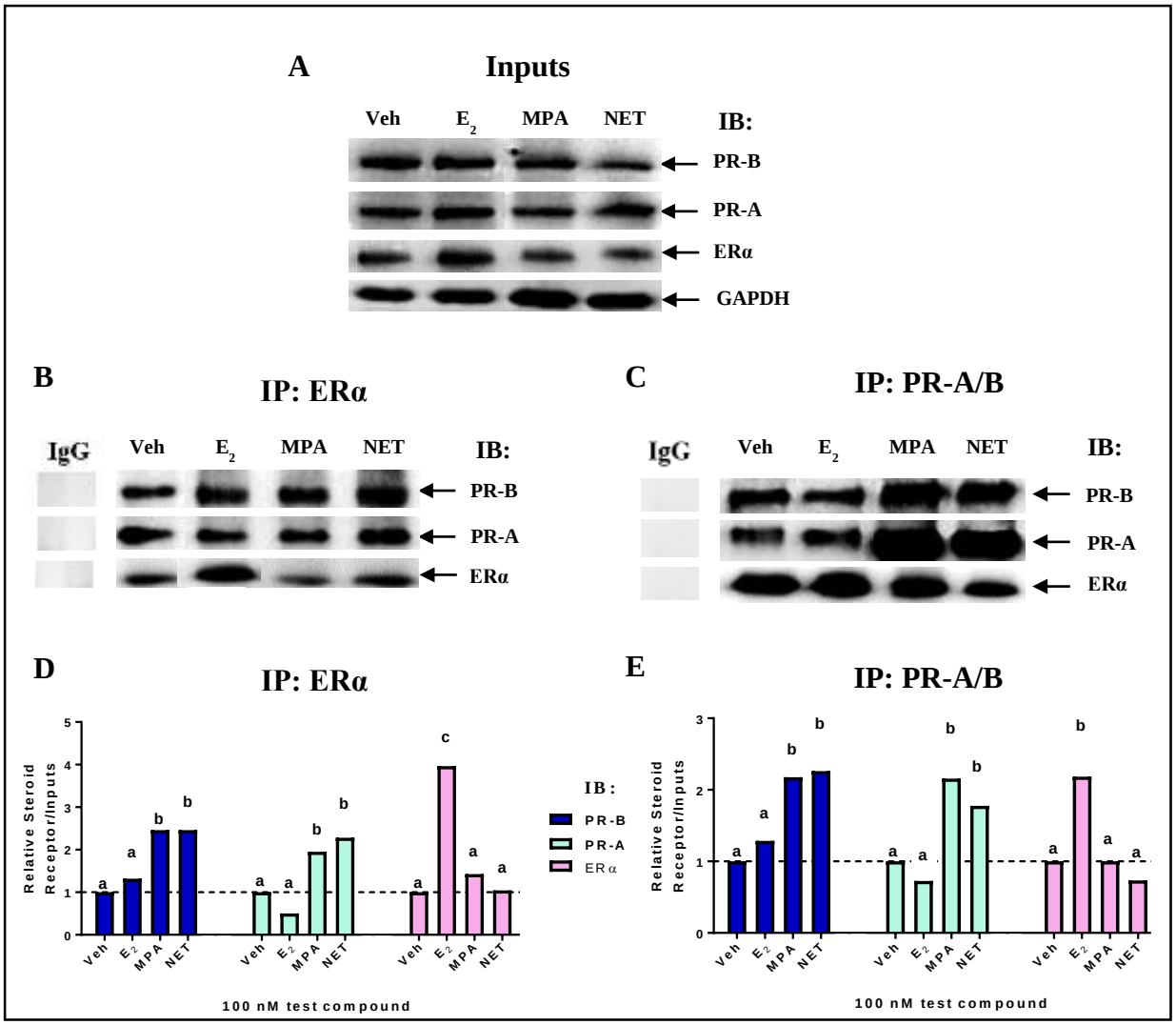


Figure 5. PR-A, PR-B and ERα occur in a molecular complex. MCF-7 BUS cells were incubated with 100 nM E_2, MPA or NET for 1 hour, after which cell extracts were immunoprecipitated (IP) with either an **(B)** ERα- or a **(C)** PR-A/B-specific antibody. **(A-C)** Immunoblotting (IB) was performed using antibodies specific for ERα, PR-A/B or GAPDH (internal control) and a representative blot is shown. **(D and E)** At least three independent experiments were quantified in terms of ERα, PR-A and PR-B expression levels relative to the respective input controls as well as GAPDH expression using ImageJ software (Version 1.49). The vehicle control of each condition was set as one and all other responses set relative to this.

not modulate the amount of PR-A and PR-B complexed with ERα (Fig. 5B - 5E). Considering that the PR isoforms and ERα occur in a complex in MCF-7 BUS cells, and that both receptors are required for MPA- and NET-induced *pS2* and/or *CTSD* expression, we next investigated whether MPA or NET treatment would cause the PR and ERα to be co-recruited to the

promoters of the endogenous *pS2* and *CTSD* genes. In Fig. 6A we show a schematic representation of the *pS2* and *CTSD* gene promoters, indicating the primers used in ChIP/re-ChIP/qPCR analysis and the relevant *cis*-elements. MCF-7 BUS cells were incubated for 2 hours with 1 nM MPA or NET in the absence and presence of 1 nM E_2, or with 1 nM E_2 only, after which ChIP and re-ChIP assays were performed. As expected, E_2-treatment resulted in the recruitment of ERα (Fig. 6B and 6D), but not the PR (Fig. 6C and 6E), to the *pS2* and *CTSD* promoters. In contrast, MPA treatment resulted in recruitment of both the PR and ERα to both promoters (Fig. 6B - 6E), while NET treatment also resulted in their recruitment to the *CTSD* promoter (Fig. 6D and 6E). We also showed that this recruitment was not modulated under estrogenic conditions (Fig. 6B - 6E). To determine whether the PR and ERα are in fact co-recruited to these promoters, we next performed re-ChIP assays. MCF-7 BUS cells were incubated with the test compounds and then subjected to immunoprecipitation with an anti-IgG antibody (negative control) or a PR-A/B-specific antibody followed by an ERα-specific antibody, and vice versa. Results show that the PR and ERα are co-localized on the endogenous *pS2* (Fig. 7A) and *CTSD* (Fig. 7D) promoters in the presence of MPA, and on the *CTSD* promoter in the presence of NET (Fig. 7E). E_2 did not modulated the progestin-induced co-localization (Fig. 7B and 7F) or cause any co-localization on its own (Fig. 7C and 7G).

3.5. ERα and the PR are co-recruited to PR binding sites in the CCND1 and MYC promoters

So far, this study has provided evidence that MPA and/or NET, but not P_4 and DRSP, can increase the expression of ER-target genes implicated in breast cancer via a mechanism involving the co-localization of the PR and ERα on the promoters of these genes. Interestingly, co-localization of these receptors in the presence of MPA has also been shown on known PR binding sites in the promoters of the progestogen-responsive proto-oncogenes *CCND1* (cyclin D1) and *MYC* in the T47D breast cancer cell line [34]. Considering that MPA has previously

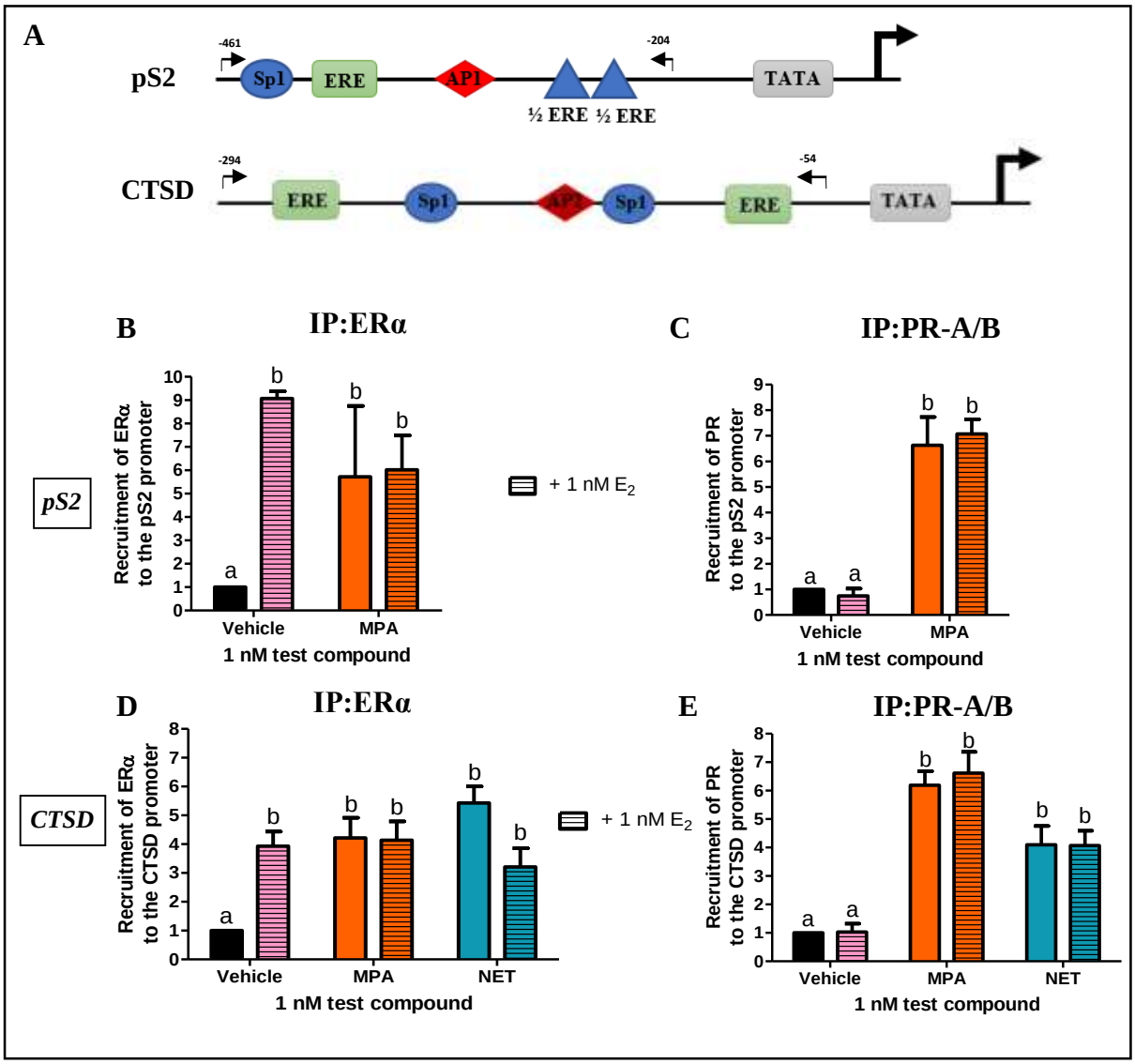


Figure 6. ERα and the PR are recruited to the *pS2* promoter in response to MPA treatment, and to the *CTSD* promoter in response to MPA or NET treatment. MCF-7 BUS cells were incubated with EtOH (vehicle control), 1 nM E_2, or 1 nM MPA or NET in the absence and presence of 1 nM E_2 for 2 hours, followed by the ChIP assay. **(A)** Schematic representation of *cis*-elements in the promoter regions of *pS2* (adapted from [84,85]) and *CTSD* (adapted from [80,86]) and the ChIP/qPCR primer positions. Cell lysates were immunoprecipitated (IP) with antibodies specific for IgG (negative control), **(B and D)** ERα, or **(C and E)** PR-A/B, followed by real-time qPCR analysis of the resulting immunoprecipitated DNA fragments and input controls. Data shown was normalized to input and IgG controls and expressed as the fold response relative to the vehicle control set as one. Results shown are the averages of three independent experiments.

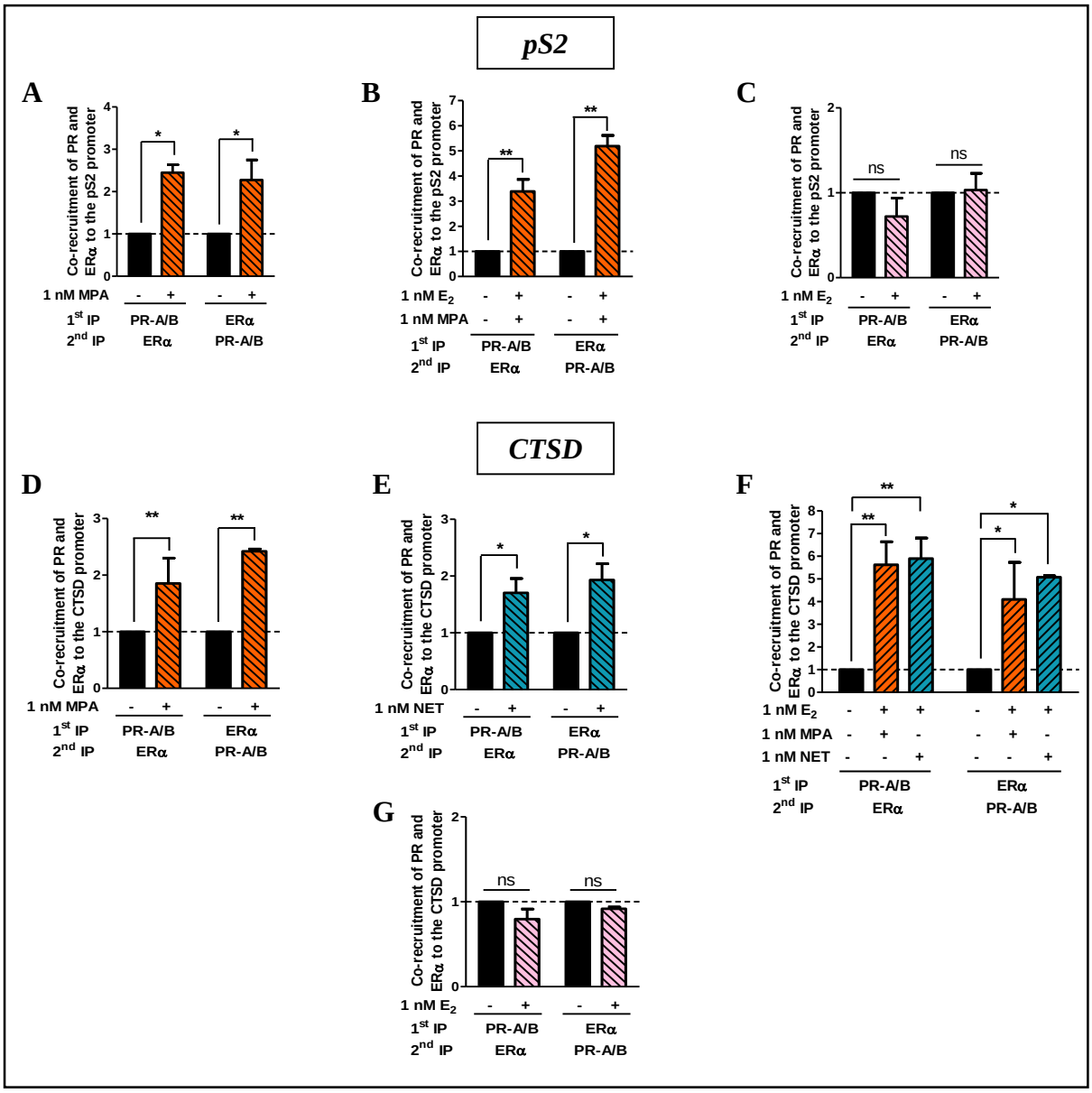


Figure 7. MPA causes the co-recruitment of ERα and the PR to the *pS2* promoter, while both MPA and NET result in co-recruitment to the *CTSD* promoter. MCF-7 BUS cells were incubated with EtOH (vehicle control), 1 nM MPA or NET in the absence and presence of 1 nM E_2, or with 1 nM E_2 only, for 2 hours followed by re-ChIP assays. Cell lysates were subjected to immunoprecipitation (IP) with an anti-IgG antibody (negative control) or an ERα-specific antibody followed by a PR-A/B-specific antibody, and vice versa, prior to real-time qPCR analysis of the resulting immunoprecipitated DNA fragments and input control. Data shown was normalized to input and IgG controls and expressed as the fold response relative to the vehicle control set as one. Results shown are the averages of three independent experiments.

been shown to regulate gene expression in a promoter- and cell-specific manner [68,87], we next determined whether MPA, as well as P_4, NET or DRSP, would induce PR and ERα co-localization on these gene promoters in MCF-7 BUS cell line. Moreover, we investigated these effects under estrogenic and non-estrogenic conditions. MCF-7 BUS cells were incubated with 1 nM P_4, MPA, NET or DRSP in the absence and presence of 1 nM E2, or with 1 nM E_2 only, for 2 hours after which ChIP and re-ChIP assays were performed. In agreement with the findings of Giulianelli and co-workers [34], our results indicated that MPA treatment induced PR and ERα co-localization on both the *CCND1* and *MYC* promoters in MCF-7 BUS cells (Fig. 8C and 8G). Furthermore, this co-localization was not unique to MPA, but was also observed in MCF-7 BUS cells treated with P_4 (Fig. 8B and 8F), NET (Fig. 8D and 8H) or DRSP (Fig. 8E and 8I). Moreover, progestin treatment under estrogenic conditions did not modulate the response observed under non-estrogenic conditions.

4. Discussion

In this study, we show that the first-generation progestins MPA and NET, the fourth-generation progestin DRSP, and natural P_4 increase breast cancer cell proliferation and anchorage-independent growth of the MCF-7 BUS breast cancer cell line (Fig. 1A-1F). Notably, the observed responses were similar both in the absence and presence of E_2 (Fig. 1G and 1H). We also show that DRSP is the least, and MPA the most potent progestin in terms of proliferation, suggesting that HT containing the newer generation progestin DRSP may pose less breast cancer risk than HT containing MPA or NET. Although inhibition with ICI abrogated the effects of the progestogens on both proliferation and anchorage-independent growth, suggesting a mechanism requiring the ER, co-treatment with RU486 also abrogated these responses (Fig. 2). Considering that RU486 can antagonize the PR, GR and AR, this result does not definitively reveal which of these receptors are required for progestogen-induced breast

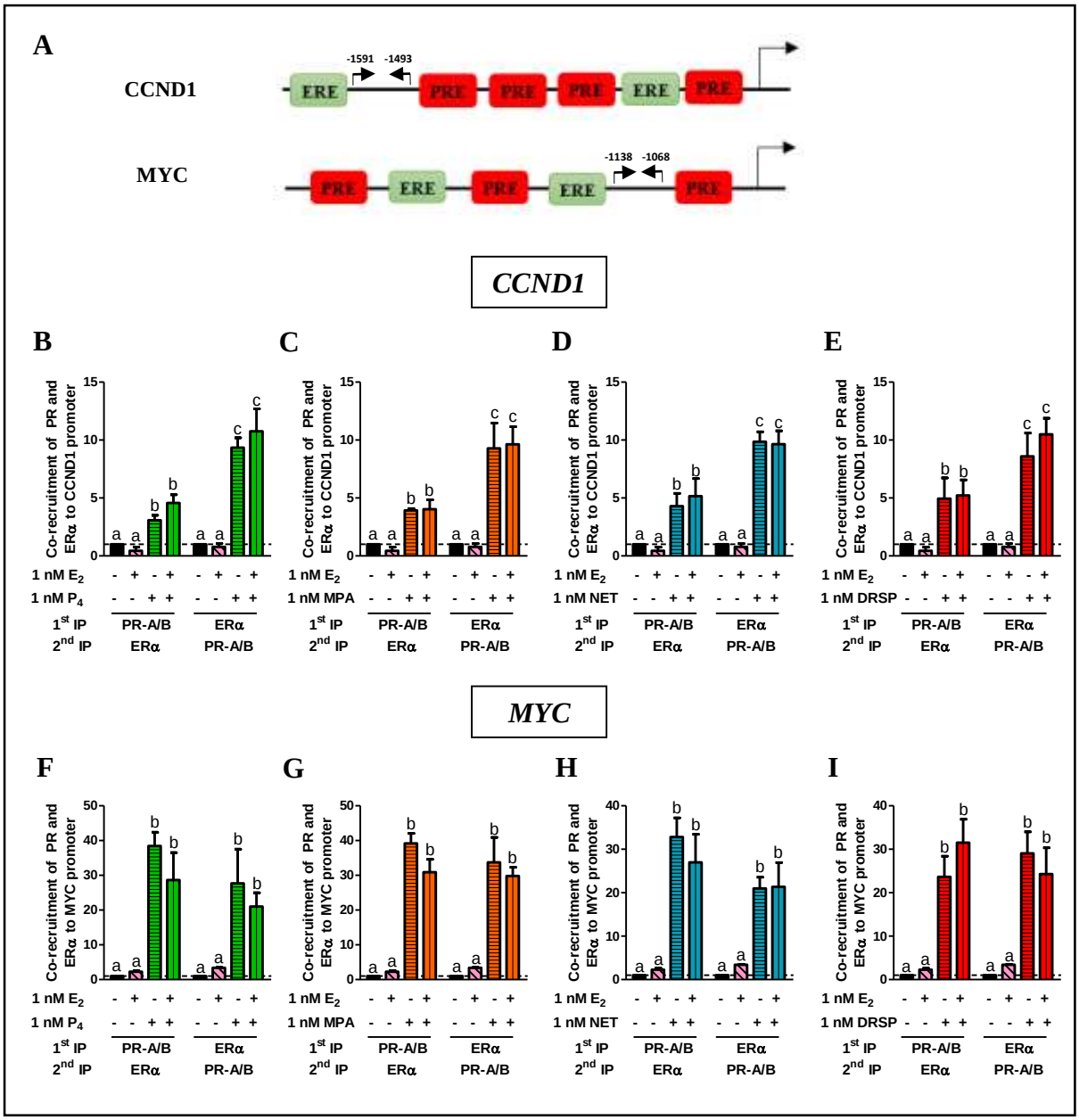


Figure 8. All the progestogens result in co-localization of the PR and ERα on both the *CCND1* and *MYC* promoters. MCF-7 BUS cells were incubated with EtOH (vehicle), 1 nM E_2, or 1 nM P_4, MPA, NET or DRSP in the absence and presence of 1 nM E_2 for 2 hours followed by re-ChIP assays. **(A)** Schematic representations of *cis*-elements in the promoter regions of *CCND1* and *MYC* (adapted from [34]), and the primer positions. Cell lysates were subjected to immunoprecipitation (IP) with an anti-IgG antibody (negative control) or a PR-A/PR-B-specific antibody followed by an ERα-specific antibody, and vice versa, prior to real-time qPCR analysis of the resulting immunoprecipitated DNA fragments and input controls. Data shown was normalized to input and IgG controls and expressed as the fold response relative to the vehicle control set as one. Results shown are the averages of at least two independent experiments.

cancer cell proliferation and anchorage-independent growth. However, we know that all of these progestogens are potent PR agonists [3,88], while P_4 and DRSP are not AR agonists [6], and DRSP and NET are not GR agonists [73,78]. P_4, although suggested to have partial GR agonist activity in some studies and not others [73,78], does not display any agonist activity at 100 nM [78]. Even though it is likely that the effects of the progestogens on proliferation and anchorage-independent growth of the MCF-7 BUS breast cancer cell line are indeed mediated by the PR, we cannot exclude a role for the AR or GR in mediating the effects of MPA, or for the AR in terms of NET. However, we do show that MPA and NET upregulate the expression of the ER-regulated *pS2* and/or *CTSD* genes in the same cell line (Fig. 3A and 3B), by inducing an interaction between the PR and ERα (Fig. 5). Moreover, we show the co-recruitment of the PR and ERα to the promoters of these genes (Fig. 7). As observed for the proliferation and anchorage-independent growth, the effects of MPA and NET on gene expression and the co-recruitment of the PR and ERα to the promoters of ER target genes was not modulated in the presence of E_2 (Fig. 3C and 3D). Although P_4 and DRSP also induced an interaction between the PR and ERα (Supplementary Fig. 1), these progestogens had no effect on *pS2* or *CTSD* expression (Fig. 3). Co-recruitment of the PR and ERα to known PR binding sites in the *CCND1* and *MYC* promoters in response to P_4, MPA, NET and DRSP treatment of the MCF-7 BUS cell line, under non-estrogenic and estrogenic conditions, was also shown (Fig. 8). The observed results in the absence of E_2 suggest that the previously reported PR and ERα co-recruitment to these genes in the T47D cell line in response to MPA treatment [34] is neither cell line- nor progestogen-specific.

Establishing the mechanism behind the increased breast cancer risk associated with estrogen-progestin HT is a global priority considering that breast cancer is the leading cause of cancer-related mortality in women in developed countries [89,90]. Furthermore, since estrogen-progestin HT is associated with higher breast cancer risk than estrogen only HT, and that

progestins such as MPA have been implicated in increasing breast cancer risk [8–10,12] while other progestins and P_4 have not [13–21], molecular studies are required to directly compare the effects of progestins relative to each other and P_4. Notably, the literature is contradictory as to whether progestogens elicit proliferative effects. For example, while some studies provide evidence indicating that P_4 [91,92] and progestins such as MPA [93,94], NET [93–95], gestodene [96–98] and levonorgestrel [95,96,98] increase proliferation in the ER- and PR-positive MCF-7 and T47D breast cancer cell lines, others show that P_4 [91,99–101] and progestins such as promegestone (R5020) [91,100,102], MPA [102], NET-A [100] and nomegestrol acetate [100] are anti-proliferative in these cell lines [91,99–104]. Some studies have even suggested that P_4 is proliferative for one cell cycle, after which it exerts anti-proliferative and pro-apoptotic effects in T47D cells [101,102]. These above-mentioned studies and others, suggest that progestogens elicit differential proliferative or anti-proliferative effects in different cell lines [94,95,105,106], highlighting the importance of characterizing the progestogens in parallel in the same model system. Interestingly, we report similar efficacies, but not potencies for proliferation for E_2, P_4, MPA, NET, and DRSP in the estrogen-responsive MCF-7 BUS cell line. For example, the newer-generation progestin DRSP, was 875-fold less potent than P_4, which most clinical studies have suggested does not increase breast cancer risk [10,71]. Remarkably, DRSP was less potent than MPA (37 630-fold) and NET (448-fold), both first generation progestins previously associated with increased breast cancer risk [8–10,12]. Not only was MPA the most potent progestogen in terms of proliferation of the estrogen-responsive MCF-7 BUS cell line, but it was also 20-fold more potent than E_2. This was surprising since this cell line is known to be highly proliferative in response to E_2 treatment [75]. In light of the above, and since E_2 is known to drive breast cancer cell proliferation, our results suggest that it is likely that MPA promotes breast cancer development and progression to a greater extent than E_2, as well as P_4, NET and DRSP. However, our results showing that

progestogens increased the anchorage-independent growth of the MCF-7 BUS cell line to the same extent as each other and E_2 (Fig. 1F), suggest that there is no difference in the metastatic potential of E_2 and the progestogens on breast cancer cells.

Our results showing that the progestogens increase breast cancer cell proliferation via a mechanism requiring both the PR and ER are consistent with previous studies showing a similar mechanism for MPA, norgestrel and gestodene [34,96,97]. Moreover, at least one study showed a PR and ERα-dependent mechanism for MPA-induced expression of PR regulated genes [34]. We next investigated whether these progestogens could regulate the expression of two ER-regulated genes, *pS2* and *CTSD*, and whether a similar PR and ERα-dependent mechanism is involved. Notably, only MPA significantly increased the mRNA expression of both *pS2* (Fig. 3A) and *CTSD* (Fig. 3B), while NET significantly increased the mRNA expression of only *CTSD* (Fig 3B). We then showed that the effects of MPA and NET were abrogated when PR and ERα expression was silenced, suggesting that both the PR and ERα are required for MPA- and/or NET-induced *pS2* and *CTSD* mRNA expression (Fig. 4). However, like others [82], we show that silencing of ERα, also resulted in decreased PR-A and PR-B expression (Fig. 4A and 4B), which raised the question as to whether both the PR and ER, or only the PR, are required. To exclude the latter, we confirmed that the ER is indeed required by showing that the effects on gene expression are abrogated in the presence of the ER antagonist, ICI, which reportedly does not affect PR levels [83]. Considering that we have previously shown that NET-A, but not MPA, can bind to ERα [6], these results suggest that at least the MPA-induced mRNA expression does not occur via a mechanism requiring binding to the ER, but rather suggests an indirect role for the ER.

The concept of crosstalk between the PR and ERα is not novel [35,107,108], however recent studies have emphasized that crosstalk between these steroid receptors may play a key role in breast cancer etiology [29,34,36,38,39]. These studies revealed that the PR can modulate both

the transcriptional activity and chromatin localization of ERα through the formation of PR/ERα complexes [29,34,36,39], resulting in a gene expression profile similar to that of PR alone, and one that is associated with decreased proliferation and an improved clinical outcome [39]. Although Giulianelli and co-workers [34] showed that the PR/ERα complex can be recruited to the promoters of PR target genes in response to MPA treatment, and we show similar recruitment with P_4, NET and DRSP, we are the first to show that the PR and ERα can also be co-recruited to the promoter regions of ER target genes in a ligand- and promoter-specific manner. This may be due to differences in the conformation of the receptor(s) in response to the different progestogens which may result in differential interactions of the ligand-bound receptor(s) with specific *cis*-elements in the *pS2* and *CTSD* promoters. It is known that the *pS2* and *CTSD* promoters contain different *cis*-elements [80,86] to which steroid receptors can bind [38,109–111]. In addition to the ERE or half-ERE sites to which ERα is known to bind and activate transcription [112,113], the promoters also contain activator protein 1 (AP1) sites, via which ERα has been shown to increase transcription [109,110]. Specificity protein 1 (Sp1) sites are also found in these promoters, and it has previously been shown that the PR increases the expression of the *PgR* [38] and *p21* [111] genes via these sites. Interestingly, we have also shown that the PR can increase gene expression on a synthetic ERE-containing promoter (Supplementary Fig. 2). Furthermore, the PR has previously been shown to interact with an ERE/Sp1 site in the *PR* promoter [38], suggesting that the PR/ERα complex may occupy the ERE/Sp1 site in the *pS2* and/or *CTSD* promoters. Further studies are required to delineate the precise mechanism whereby the PR/ERα complex mediates the regulation of ER target genes by MPA and/or NET.

5. Conclusion

Collectively, we show that both the PR and ER are required for the P_4, MPA, NET and DRSP

induced increase in breast cancer cell proliferation and anchorage-independent growth, as well as the MPA and/or NET induced upregulation of ER target genes. Moreover, we show that PR/ERα complexes are recruited to PR-regulated promoters in response to treatment with P_4, MPA, NET or DRSP, while recruitment to ER-regulated promoters is ligand- and promoter-specific. Interestingly, it has previously been suggested that treatment with P_4 or the progestin R5020, in the presence of E_2, induces a PR/ERα complex that causes ERα to be redirected away from ER binding sites to mostly PR binding sites that are associated with a good breast cancer prognosis [29,39]. Our results, independent of the presence of E_2, indicate that progestins differentially direct PR/ERα complexes, as P_4 and DRSP induce the formation of a PR/ERα complex that is recruited only to PR target genes, while the PR/ERα complex induced by MPA and NET is recruited to both PR and ER target genes. Although activation of the PR by P_4 or R5020, in the presence of an estrogen-activated ER complex, has been associated with a more favorable outcome [39], one cannot ignore the fact that the PR has previously been shown to increase breast cancer progression [114] and that we and others [34] show recruitment of the PR/ERα complex to the PR regulated *CCDN1* and *MYC* oncogenes. It is therefore critical that the manner in which the PR and ER cooperate to modulate the expression of PR and ER regulated genes in response to different progestins is understood. Further studies are thus warranted to investigate the clinical relevance of the interaction between the PR and ERα in response to progestins in both normal and malignant breast tissue.

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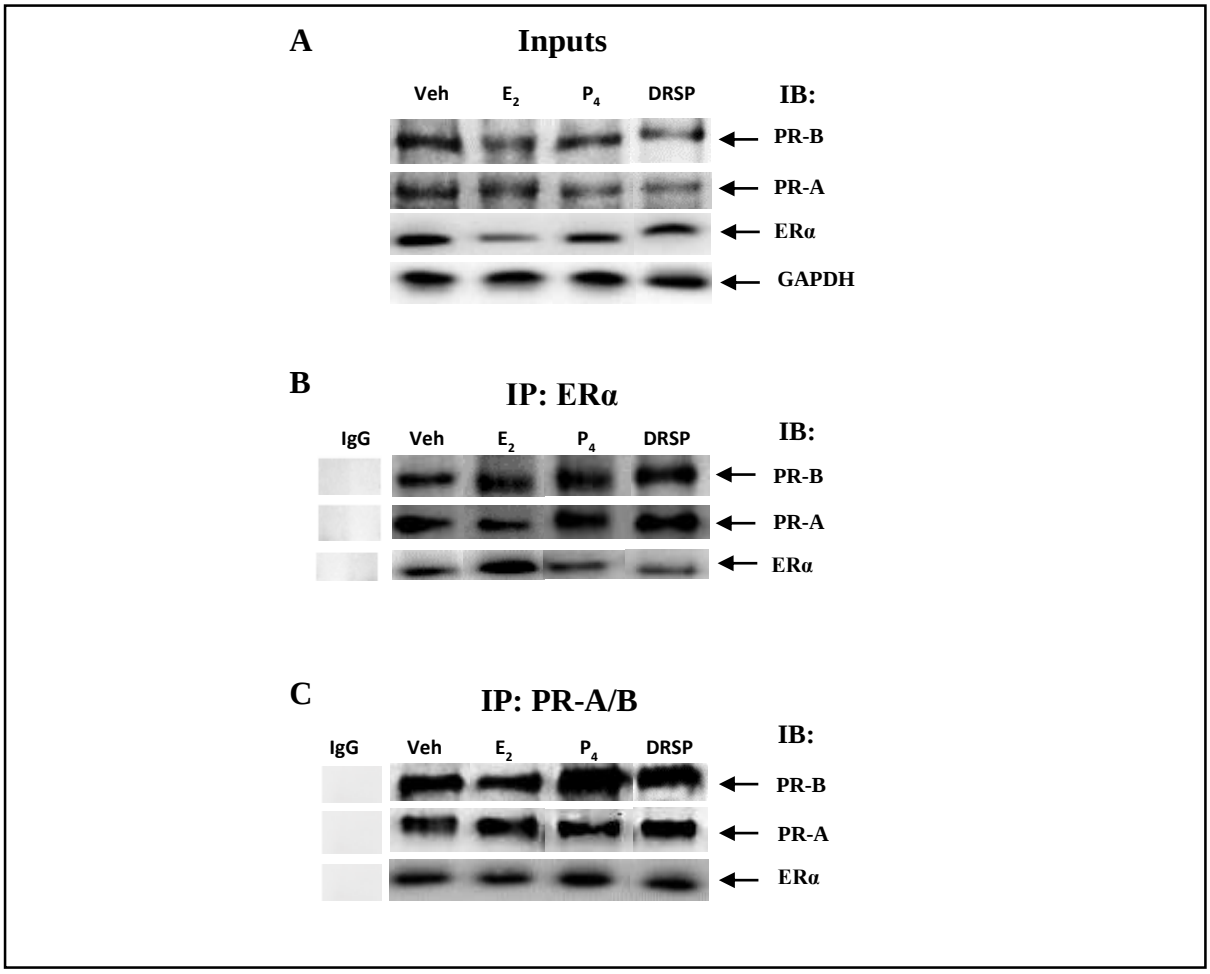
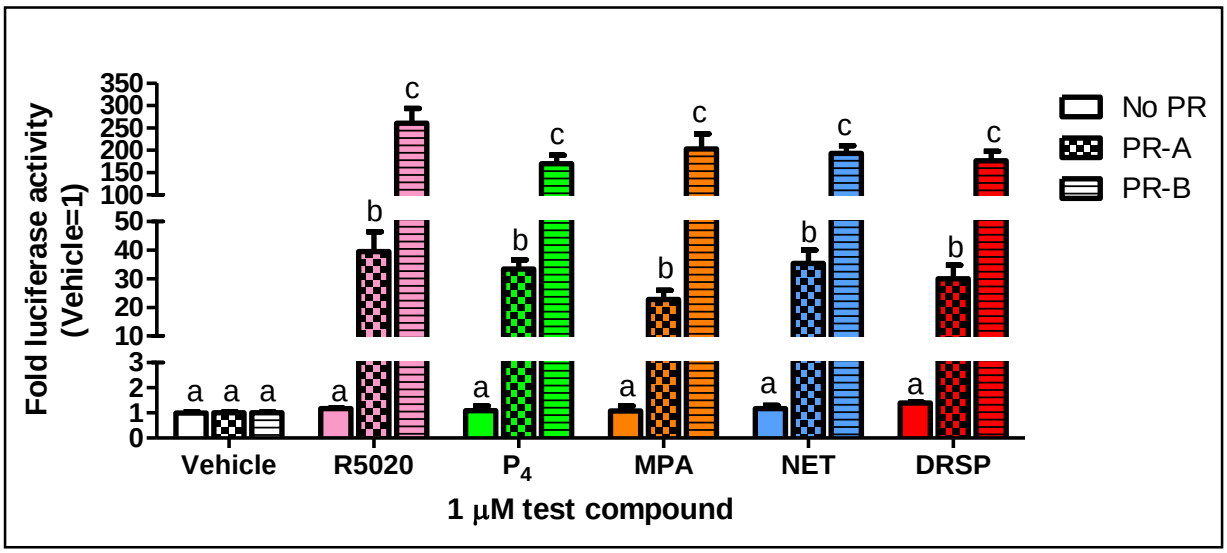
SUPPLEMENTARY FIGURES



Supplementary Fig. 1. P_4 and DRSP induce the formation of a PR/ERα complex. MCF-7 BUS cells were incubated with 100 nM E_2, P_4 or DRSP for 1 hour, after which cell extracts were immunoprecipitated (IP) with either an **(B)** ERα- or a **(C)** PR-A/B-specific antibody. **(A-C)** Immunoblotting (IB) was performed using antibodies specific for ERα, PR-A/B or GAPDH (internal control) and representative blots are shown.



Supplementary Fig. 2. Progestogens can activate a simple ERE-driven reporter construct via PR-A or PR-B. HEK293 cells transfected with 6000 ng pS2-ERE-luciferase promoter-reporter construct and 150 ng pGL2basic empty vector, PR-A or PR-B were incubated with EtOH (vehicle control), or 1 μM R5020, P_4, MPA, NET or DRSP for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration determined using the Bradford method [115]. Results are shown as fold luciferase activity where induction with the vehicle control is set as one and all other responses set relative to this. Results shown are the averages of two independent experiments.

Chapter 5

Conclusion and Future Studies

5. Concluding discussion

Estrogens and progestins used in menopausal HT have been associated with increased breast cancer risk, with the risk associated with estrogen-progestin combination HT reported to be higher than that of estrogen only HT (reviewed in [1]). However, the latter was shown for isolated progestins and considering the availability of a vast array of progestins with diverse structures and functions, risk of breast cancer should not be considered a class effect. Interestingly, although some clinical and observational studies indicate that natural P_4 may also be associated with increased breast cancer risk [1,2], most studies suggest no risk [3,4]. Moreover, experimental studies examining the effects of progestins and P_4 often report contradictory results, emphasizing the variability between different studies and the importance of directly comparing hormones used in HT in the same system. Considering the highly-publicized risks associated with conventional HT, and claims that bioidentical hormones are safer, natural alternatives that do not increase breast cancer risk, many women have turned to bHT. However, bHT can contain either a single hormone, or a mixture of several hormones, and little is known about the molecular mechanism of action of these hormones and whether they contribute to breast cancer risk. The primary goal of this study was thus to directly compare the transcriptional activities and breast cancer promoting effects of progestins relative to natural P_4, as well as natural, bioidentical and synthetic estrogens used in HT and bHT. The estrogens investigated in this study included the commercially available E_2, E_3 and E_1 standards (often used to represent the natural estrogens), custom-compounded bE_2 and bE_3, and synthetic EE, while the progestins included synthetic MPA, NET-A, LNG, GES, NES, NoMAC and DRSP. In the first part of this thesis (Chapter 2), the binding affinities and activities of the selected estrogens were evaluated in parallel via overexpressed ERα and ERβ in the COS-1 and HEK293 cell lines. Furthermore, we examined the effects of these estrogens on the proliferation and anchorage-independent growth of the estrogen sensitive MCF-7 BUS breast

cancer cell line. Since estrogen-progestin combination HT is associated with a greater breast cancer risk than estrogen only HT [1], and a conundrum exists on whether progestins can bind to the ER subtypes, we also compared the binding of selected progestins relative to each other and natural P_4 via overexpressed ERα and ERβ in the COS-1 cell line (Chapter 3). Precise equilibrium dissociation constants and the estrogenic properties were determined for those progestins that could bind. Considering that emerging evidence has highlighted an important role for crosstalk between ERα and the PR in breast cancer cell biology [5–8], the final part of this thesis (Chapter 4), investigated the role of interplay between the PR and ERα in mediating progestogen-induced gene expression, proliferation and anchorage-independent growth of the MCF-7 BUS cell line under both non-estrogenic and estrogenic conditions.

5.1. Comparing the estrogenic properties of natural, synthetic and custom-compounded bioidentical estrogens via ERα and ERβ

In the first part of Chapter 2, precise K_d/K_i values of the estrogens for ERα and ERβ were determined in the COS-1 cell line using competitive whole cell binding assays. Although a number of studies have previously examined the binding of natural and synthetic estrogens to the ER, these studies seldom used human ER, often failed to differentiate between the ER subtypes [9–13] and usually reported EC_{50} values or relative binding affinities (RBAs) [9–14] rather than accurate K_d/K_i values. This is important as K_d/K_i values remain constant between experimental systems, while EC_{50}/RBA values do not [15]. Collectively, our results showed that although all the estrogens investigated in this study mostly displayed similar binding affinities to E_2 for ERα and ERβ, they had higher affinities for ERα than ERβ. The bioidentical estrogens bind to both ER subtypes with similar affinities to their natural counterparts, while EE had a higher affinity than E_2 and the bioidentical estrogens for ERα, but a similar affinity for ERβ.

We also compared the effects of the estrogens on transactivation and transrepression of gene expression via overexpressed ERα or ERβ. For transactivation, we transfected a synthetic ERE-containing promoter-reporter construct into the HEK293 cell line, but also examined effects on an endogenous ERE-containing gene in the MCF-7 BUS cell line endogenously expressing both ER subtypes. The MCF-7 BUS cell line, also termed MCF-7 BOS, was cloned from the well-studied MCF-7 cell line and used as it is highly responsive to estrogens [16]. COS-1 cells were used for competitive whole cell binding assays, while the HEK293 cell line was used for all promoter-reporter assays as we were unable to optimize transrepression assays in COS-1 cells. We showed that although all the estrogens investigated were full ERα and ERβ agonists on the synthetic ERE-containing promoter, E_1, E_3 and EE were less potent than E_2 via ERα, and only E_1 was less potent via ERβ. Furthermore, although no differences were observed in the efficacies of the estrogens for ERα versus ERβ, most estrogens displayed higher potencies via ERα, except E_3 and bE_3. In terms of the regulation of an endogenous ERE-containing gene, the *pS2* gene was selected as it is a well-known marker of breast cancer that is upregulated in ER positive tumors [17], and frequently used to assess the regulation of ER-target genes [18–25]. Notably, bE_2, E_3, bE_3 and EE were as efficacious and potent as E_2 in upregulating *pS2* mRNA expression, while E_1 was the least efficacious and potent. The result showing that E_3 is a full ER agonist is contrary to previous claims that E_3 is a weak estrogen [26–29], but in line with at least one study showing that E_3 elicits similar transcriptional effects to E_2 on both synthetic and endogenous ERE-containing promoters [30].

Although transrepression of gene expression is a well-accepted mechanism of steroid receptor action, few studies have characterized the ERα- and ERβ-mediated transrepression induced by estrogens. Our study is the first to directly compare the efficacies and potencies of custom-compounded bioidentical estrogens relative to the commercial estrogen standards and synthetic EE for transrepression via overexpressed ERα and ERβ in HEK293 cells, and on the

endogenous NFκB containing *IL-6* gene in MCF-7 BUS cells. IL-6 is a pro-inflammatory cytokine that contributes towards breast cancer disease progression and is a marker of poor prognosis in ERα positive breast cancer tumors [31,32]. For promoter reporter assays, we transfected a p(IL6κB)350hu.IL6P-luciferase construct containing three copies of the NFκB binding site into HEK293 cells, as no E_2-induced repression was observed in COS-1 cells transfected with this construct, or in the COS-1 and HEK293 cell lines transfected with a 5xNFκB-luciferase construct containing five copies of the NFκB binding site (Addendum 1, Fig A6). These findings show that the ability of ERα to repress NFκB activity is both cell line- and promoter-specific, suggesting that the transrepression mechanism is complex. For the estrogens used in this study, we showed for the first time that they were all full ER agonists for transrepression, but displayed differential potencies. E_1 was more potent than E_2 via ERα on the synthetic promoter, while EE was more potent via ERβ. On the endogenous *IL-6* promoter in the MCF-7 BUS cell line expressing both ER subtypes, E_3, bE_3 and EE were less potent than E_2. Considering that all the estrogens used in this study repressed gene expression at concentrations reflecting serum concentrations in women using HT, it is likely that these estrogens may similarly repress the expression of pro-inflammatory genes *in vivo*. Understanding the physiological implications of repression of pro-inflammatory genes such as *IL-6* in breast cancer is not straightforward. Considering that *IL-6* contributes towards breast cancer disease progression, possibly due to its role in promoting inflammation [33,34] and resisting apoptosis [31,32], repression of *IL-6* expression is a potential mechanism whereby estrogens may in fact protect against breast cancer. Indeed, follow up studies to the WHI clinical trial investigating the risks associated with the use of conjugated equine estrogens (CEE) alone, reported a decrease in breast cancer risk [35]. However, our results suggesting protection against breast cancer by the inhibition of a pro-inflammatory cytokine gene but promotion of breast cancer by the upregulation of an ER target gene, emphasize that the role

of estrogens in breast cancer is complex and dependent on numerous factors, including a balance between transactivation and transrepression of gene expression.

Since proliferation and anchorage-independent growth are phenotypic responses that represent an integrated model to which both transactivation and transrepression contribute [36–38], we next compared the effects of the estrogens on these hallmarks of breast cancer in the MCF-7 BUS cell line. The results for proliferation indicated a similar trend to transactivation via ERα, suggesting that the proliferative effects of the estrogens may predominantly be ERα-mediated. This is not surprising since ERα is known to drive breast cancer cell proliferation [39–49]. Moreover, results from anchorage-independent growth assays suggested that all the estrogens investigated in this study may similarly increase breast cancer metastasis, as they all increased the anchorage-independent growth of MCF-7 BUS cells to a similar extent.

According to proponents of bHT, there are two main justifications for the use of E_3 and/or E_1 in biest or triest formulations in bHT; firstly that E_1 and E_3 are weak estrogens [26–29,50] and secondly that E_3 can antagonize the activity of E_2 [28,50–54]. For example, a study using a cell-free transcription assay has previously showed that E_3 is a weaker estrogen than E_2, and that 500 nM or 1 µM E_3 could antagonize the effects of 100 nM E_2 [50]. Our results at physiological concentrations of E_1 and E_3 showed that these were not weak estrogens in our model systems, and thus it was not surprising that we showed that neither estrogen could antagonize E_2-induced gene expression, cell proliferation or anchorage-independent growth. Consistent with our result for E_3, Diller and co-workers [30] have previously shown that E_3 elicits similar effects to E_2 on gene expression and proliferation of the MCF-7 and T47D breast cancer cell lines. Furthermore, we showed that the lack of antagonist effects of E_1 and E_3 on gene expression was not promoter-specific, as similar effects on the *pS2* and *IL-6* genes were shown on the *CTSD* and *RANTES* genes. *CTSD* is frequently used as a marker of ER-regulated gene expression [7,21,55,56] and its overexpression in breast tumors has been linked to

processes such as metastasis and invasion [57]. RANTES on the other hand, is a pro-inflammatory chemoattractant known to regulate breast cancer tumor progression [58].

Taken together, we show that the custom-compounded bioidentical estrogens mimic their respective commercial estrogen standards and often also mimic synthetic EE in terms of binding affinity, gene expression, breast cancer cell proliferation and anchorage-independent growth. Overall these findings suggest that the custom-compounded estrogens, commercially available estrogens and synthetic EE have similar effects on breast cancer risk, indicating no clear advantage in choosing bHT instead of conventional HT.

5.2. Comparing the estrogenic properties of progestins used in HT via ERα and ERβ

In the publication constituting Chapter 3 of this thesis, the androgenic and estrogenic properties of selected progestogens were evaluated. As only the characterization of the estrogenic properties of the progestogens were an aim of this thesis, the androgenic properties will not be discussed here. Although some studies have previously shown that progestins such as MPA and NET can bind to the ER and elicit estrogenic activity, others have indicated that they cannot bind to the ER (reviewed in [59]), or that progestin metabolites, rather than parent progestins, may bind [60,61]. These contradictory findings may be due to differences in model systems used, as some cell lines endogenously express a number of steroid receptors to which progestins may bind [62,63]. These discrepancies, in addition to the fact that studies frequently fail to distinguish between the ER subtypes [64,65], may also result in the reporting of inaccurate results. For this study, we thus used the COS-1 and HEK293 cell lines as in Chapter 2, to comparatively evaluate the binding and activity of selected progestins from different generations, relative to each other and P_4, via the individual ER subtypes. Both these cell lines are frequently used for characterization studies as they lack the expression of significant levels of endogenous steroid receptors [66,67]. Our results revealed that only NET-A, LNG and GES,

progestins structurally derived from the estrogen precursor testosterone, bind to human ERα. We are the first to report accurate K_i values of these progestins for ERα and showed that none of the progestins or natural P_4 could bind to human ERβ.

Similar to the characterization of the estrogens in Chapter 2, we next determined the efficacies and potencies of NET-A, LNG and GES for ERα-mediated transactivation and transrepression of gene expression. Our results showed that while LNG and GES displayed full ERα agonist activity for transactivation, and NET-A partial agonist activity, these progestins displayed lower potencies than E_2. We are the first to evaluate ERα-mediated repression of gene expression by progestins and show that while NET-A and GES displayed full agonist activity for transrepression, and LNG partial agonist activity, NET-A and LNG were less potent than GES. As previous studies have suggested that progestin metabolites rather than the parent progestins bind to the ER and elicit estrogenic effects, we cannot exclude that the results observed in our study may be due to progestin metabolites. However, it is likely that the progestins are not metabolized in our model cell lines considering that NET-A, LNG and GES metabolites have previously been shown to bind to and transactivate gene expression via ERβ [60,61], while we do not observe binding to ERβ in COS-1 cells, or ERβ-mediated transactivation or transrepression in HEK293 cells.

To understand the possible physiological implications of our results, one should consider that NET-A, LNG and GES have approximately a 16-, 1021- and 119-fold lower affinity for ERα than E_2, respectively, and that the EC_{50} values of the progestins for transactivation and transrepression are lower than the serum concentrations of these progestins in women using endocrine therapies. It is thus unlikely that NET-A, LNG or GES will compete with E_2 for binding to ERα in target tissues. Although it is unlikely that the progestins will bind to and elicit ERα-mediated effects *in vivo*, our study provides clarity regarding the activity of selected

progestins from different generations via individual ER subtypes. Lastly, these findings underline the fact that not all progestins elicit similar activity at the molecular level and emphasize that the current grouping of the effects of progestins as a class should be avoided.

5.3. Comparing the effects and underlying mechanisms of selected progestins on ER target gene expression and hallmarks of breast cancer

Considering that some progestins have been associated with increased breast cancer risk while natural P_4 and dydrogesterone (a P_4 isomer) have not [68], in Chapter 4 we compared the effects of selected progestins relative to P_4 on gene expression, breast cancer cell proliferation and anchorage-independent growth in the estrogen-sensitive MCF-7 BUS breast cancer cell line. Moreover, we performed a detailed investigation into the underlying mechanisms of these effects. We showed that P_4, MPA, NET and DRSP had similar efficacies, but not potencies for proliferation, supporting the concept that individual progestins do not have the same effects on breast cancer risk. More specifically, P_4 and NET displayed similar potencies to each other and E_2, while MPA was the most potent and DRSP the least potent. Considering that MPA has been linked to increased breast cancer risk in multiple clinical trials [1,3,69,70], it was not surprising that this first-generation progestin was the most potent. However, the fact that P_4 displayed a similar potency to E_2 was surprising since E_2 is known to drive breast cancer (reviewed in [71,72]), while clinical and observational studies have mostly suggested that P_4 does not increase breast cancer risk [3,4]. Furthermore, some studies have reported an association between NET-A and breast cancer, while others have not [73,74]. Our results showing that NET-A displayed a similar potency to E_2 suggest that NET-A may indeed increase breast cancer risk. The serum concentrations of DRSP (5.0 - 12.4 nM), MPA (1.8 - 12.4 nM) and NET-A (1.1 - 31.4 nM) are similar in women using HT [75–79], yet we showed that the potency of DRSP for proliferation was significantly less than MPA and NET-A, suggesting that DRSP

may promote proliferation to a lesser extent than progestins such as MPA and NET-A *in vivo*. Moreover, we showed that the progestins promoted proliferation both in the absence and presence of E_2, which is contrary to the proposed idea that progestins promote proliferation in the absence of E_2, while antagonizing proliferation in the presence of E_2 (reviewed in [80]). Similarly, we also showed that the presence of E_2 did not affect the progestogen-induced increase in the anchorage-independent growth of the MCF-7 BUS cell line.

Initial experiments blocking progestogen-induced proliferation and anchorage-independent growth with ICI or RU486 indicated that the ER, and possibly the PR, are involved in mediating these effects. The result showing the requirement of the ER for MPA-induced effects on proliferation is consistent with a previous study indicating that ICI inhibited MPA-induced proliferation [6,81]. Although RU486 is not a PR-specific antagonist, a role for the PR in mediating at least the effects of MPA and NET was confirmed at the level of gene expression using siRNA targeting the PR. Support for a role of both the ER and PR are gained from studies showing that MPA and R5020 inhibited proliferation in MDA-MB-231 cells expressing only the PR [82], while they promoted proliferation in cell lines expressing both the PR and ERα [81,83]. Interestingly, our Co-IP results showed an association between the unliganded PR and ERα in the MCF-7 BUS cell line, and an increased interaction in response to P_4, MPA, NET and DRSP treatment. Similar results have previously been shown for P_4 in MCF-7 cells [5] and for P_4 and MPA in T47D cells [5,6]. Considering that the observed progestogen-induced breast cancer cell proliferation and anchorage-independent growth required the ER and PR, we next investigated whether the selected progestins and P_4 could modulate the mRNA expression of the ERE-containing ER-regulated *pS2* and *CTSD* genes previously used in Chapter 2. Remarkably, only MPA increased *pS2* mRNA expression, while both MPA and NET-A increased the mRNA expression of *CTSD*, suggesting ligand- and promoter-specific regulation by these progestins. Here, we also showed that both the PR and ER were required for the effects

of MPA and NET on *pS2* and/or *CTSD* expression, as these effects were abrogated in the presence of the ER antagonist ICI, and upon PR and ERα knockdown. Although the activated PR traditionally binds to PREs, in this chapter we have shown that both PR-A and PR-B can activate gene expression via a synthetic ERE in the presence of P_4, R5020, MPA, NET and DRSP.

In Chapter 3, we showed that NET-A, but not MPA, binds to ERα, thus it is evident that effects induced by MPA occur via a mechanism that does not require direct binding of MPA to ERα, while effects of NET may be partly mediated by ERα. Interestingly, a similar requirement for both the PR and ERα had previously been shown for the effects of MPA on the regulation of PRE-containing PR target genes. Specifically, co-recruitment of the PR and ERα to the promoter regions of *CCND1* and *MYC* PR target genes were shown [6]. Although we did not compare the effects of the progestogens on the expression of the *CCND1* and *MYC* genes, we also showed recruitment of the PR/ERα complex to the promoters of these genes in response to P_4, MPA, NET and DRSP treatment in the MCF-7 BUS cell line. We showed for the first time that this PR/ERα complex is also recruited to the *pS2* promoter upon MPA treatment and to the *CTSD* promoter upon MPA or NET treatment. Contrary to the idea that progestins elicit differential effects in the absence and presence of E_2 [80], our study showed that the recruitment of the PR/ERα complex to ER and PR target genes in response to selected progestins was not modulated by the presence of E_2. These findings are in line with our proliferation, anchorage-independent growth and gene expression results in which the effects elicited by the progestins and P_4 in the MCF-7 BUS cell line were not influenced by the presence of E_2.

The recruitment of the PR/ERα complex to ER-target genes implicated in breast cancer in response to MPA and NET but not P_4 or DRSP, suggests that P_4 and DRSP may promote breast cancer pathogenesis to a lesser extent and thus may be safer options in terms of HT. Although it has recently been proposed that the redirection of activated ERα in the presence of a PR

ligand, whether an agonist or antagonist, results in the activation of genes associated with an improved breast cancer outcome (reviewed in [80]), our results showing progestin-specific effects in the presence of both unliganded and E_2-activated ERα suggests that this may not be the case for PR agonists. Moreover, as PR antagonists are known to lead to a different conformation of the PR compared to PR agonists [84], it is possible that the antagonist-bound PR will elicit differential effects on ER signaling. Further studies to determine the relevance of the interaction between the PR and ERα in the clinical setting in both normal and cancerous breast tissue are essential, with specific focus on whether PR/ERα crosstalk is linked to good or poor prognosis in the presence of different progestins.

5.4. Future studies

In Chapter 2 of this thesis, we compared the binding affinities and transcriptional activities of custom-compounded bE_2 and bE_3 to that of natural E_2, E_3, E_1 and synthetic EE via overexpressed ERα and ERβ in the COS-1 and HEK293 cell lines. In addition, we compared the activities of these estrogens on endogenous gene expression, proliferation and anchorage-independent growth of the MCF7 BUS breast cancer cell line expressing both ERα and ERβ. To provide insights into the role of each subtype in mediating the effects observed in the MCF-7 BUS cells, siRNA targeting ERα or ERβ could be employed. Studies performing pharmacological characterizations of the binding affinities and relative efficacies and potencies for gene regulation of progestins from different generations via individual steroid receptors are lacking. Chapter 3 of this thesis has subsequently addressed this paucity of knowledge for ERα and ERβ. However, a limitation of this study is that we did not investigate the influence of the ERα-mediated effects of NET-A, LNG and GES on any endogenous ERE- or NFκB-containing promoters. Considering that we demonstrated promoter-specific regulation of ER-target genes by NET in Chapter 4, the effects of NET-A, LNG and GES should be further pharmacologically

characterized on several endogenous genes such as *PgR*, *CTSD*, *IL-6* and *RANTES*. Although we, and a collaborator, have performed thorough pharmacological characterizations of several progestins for the AR [66,85], and of MPA and NET-A for the GR and MR [86–88], it would also be interesting to conduct similar experiments for other progestins used in HT via the GR, MR and PR isoforms. This is important given the link between progestins and increased breast cancer risk, and the prominent role of all steroid receptors in breast cancer biology. To provide insight into the role of specific steroid receptors in mediating the effects of specific progestins in breast cancer, it would be interesting to perform experiments investigating the effects of progestins on the various hallmarks of breast cancer in the absence and presence of steroid receptor-selective antagonists and steroid receptor-specific knockdown or overexpression. This is critical as understanding the molecular mechanism of action of progestins via specific steroid receptors may lead to the identification of novel breast cancer therapies.

Considering that many progestins are known to undergo metabolism [61], and it has been suggested that some metabolites rather than the parent compound bind to and activate the ER subtypes [60,61], discriminating between the binding of the parent compound versus its metabolites is not an easy task. However, as a first step, it would be interesting to investigate the metabolism of various progestins in different cell line and tissue models. Work in this regard using ultra-performance liquid chromatography/tandem mass spectrometry analysis has already begun in our laboratory and that of our collaborator.

An association between the PR and ERα was first reported more than ten years ago [89,90], but recent studies have highlighted a critical role for the interplay between these two receptors in breast cancer biology. The evidence indicates that ERα and the PR are co-recruited to the promoters of PR-regulated oncogenes in response to MPA [6], while a genome-wide study showed that treatment with P_4 and R5020 resulted in the PR modulating ER target gene activity by redirecting activated ERα to genes associated with good prognosis [5]. Indeed, in Chapter

4 we have shown that P_4, MPA, NET and DRSP also lead to the recruitment of a PR/ERα complex to the promoters of the *CCND1* and *MYC* PR target genes. However, we also showed that only MPA and/or NET could cause the upregulation of ER target genes via co-recruitment of the PR and ERα to the *pS2* and *CTSD* promoters. Our results showing ligand and promoter-specific effects for the progestins used in this study (Chapter 4) are consistent with evidence in the literature indicating ligand-, promoter- and cell line-specific effects of progestins [86,87,91,92]. To exclude the possibility of cell line-specific effects, gene expression, ChIP and re-ChIP assays should be repeated in at least one other breast cancer cell line such as the ER- and PR-positive T47D cells. The above-mentioned progestin-specific effects reiterate the importance of investigating effects of individual progestins, rather than drawing conclusions for all progestins based on studies using isolated progestins or natural P_4. Thus, genome-wide studies using ChIP-seq analysis should be performed to investigate the binding of the PR/ERα complex in the presence of different progestins, under both non-estrogenic and estrogenic conditions.

Further experiments are required to gain insight into the precise mechanism whereby PR and ERα may be regulating the expression of the *pS2* and *CTSD* genes. Although we proposed that PR and/or ERα may be interacting with the ERE in these gene promoters, our ChIP primers were not specific to the ERE region but included other *cis*-elements such as Sp1, AP1 and AP2 to which these receptors may bind. For example, it is known that the PR can increase the expression of the *PgR* [93] and *p21* [94] genes by interacting with the Sp1 sites in the promoters of these genes. Similarly, it has previously been shown that two Sp1 sites in the promoter of the *PgR* gene are required for E_2-activation of this gene via ERα [95,96]. To identify specific *cis*-elements involved, primers that are more specific to the promoter region containing only the ERE could be designed. However, considering the close proximity of the *cis*-elements in the promoters, this may not be possible. In this instance, ChIP and re-ChIP assays could be

conducted in the presence of siRNA targeting transcription factors such as AP1 or Sp1 to exclude the tethering of the PR/ERα complex to transcription factors bound to these *cis*-elements in the promoters of ER target genes. Interestingly, the DBDs of the ER [97] and AR [98] have previously been mutated so as to characterize the binding of these receptors to hormone response elements. It may thus be interesting to perform similar experiments for ERα and the PR to determine whether one or both receptors are directly interacting with a specific response element in the promoters of target genes.

Despite the above-mentioned studies all investigating the role of ERα in breast cancer biology, it is well-known that the ERβ subtype also plays a role in breast cancer. Evidence in the literature indicates that ERβ can inhibit ERα-driven proliferation in ER-positive breast cancer, while promoting proliferation in the absence of ERα [39–44,46–49,99]. To the best of our knowledge, no studies have investigated the possibility of an association between ERβ and the PR in breast cancer. Our attempts at investigating such as interaction have been unsuccessful due to lack of a suitable commercial ERβ antibody. Indeed, a recent comparison of commonly used commercial ERβ antibodies, including one of the antibodies we tested (Santa Cruz Biotechnology; sc8974), reported limitations in antibody specificity [100]. Similarly, since PR antibodies, including the one used in this study, tend to detect both PR-A and PR-B, further studies are required to determine whether PR-A and PR-B play distinct roles in PR/ER crosstalk. To determine whether the ER subtypes preferentially form heterodimers with a specific PR isoform, and whether this is influenced by receptor levels or the presence of different progestins, could be investigated using three-color spectral Förster resonance energy transfer (3sFRET) microscopy [101]. This technique would entail the labeling of PR-A and PR-B with distinguishable fluorophores that could both be excited by the emission spectrum of a third fluorophore tagged to ERα or ERβ, thus allowing the determination of whether ERα or ERβ preferentially binds a specific PR isoform.

Similar to our results showing that MPA was the most, and DRSP the least potent in terms of proliferation, a previous study has reported a similar trend for the promotion of breast cancer cell migration and invasion [102]. It would thus be interesting to directly compare the effects of different progestins on migration and invasion, as well as other hallmarks of cancer such as apoptosis. Finally, it would be beneficial to examine the oncogenic effects of different progestins relative to natural P_4, on breast tumor explants since these tissues represent a more physiological model system and have previously been used to validate findings from cell line experiments [5,8].

5.5. Conclusion

Taken together, the results presented in this thesis show that custom-compounded bE_2 and bE_3 mimic their respective commercially available natural estrogen standards and synthetic EE, suggesting that bHT is not necessarily a safer alternative to conventional HT. Furthermore, the fact that similar maximal responses were observed for these estrogens in terms of gene expression and breast cancer cell proliferation at concentrations reflecting serum estrogen levels, highlights the potential of these estrogens to display similar effects *in vivo*. Moreover, our results show that E_3 and E_1 are not weak estrogens, and that they do not antagonize the activity of E_2. This finding implies that the rationale behind using E_3 and E_1 in custom-compounded biest and triest bHT formulations should be re-evaluated.

The results investigating the binding and activity of selected progestins from different generations via overexpressed ERα or ERβ in COS-1 and HEK293 cells show that NET-A, LNG and GES bind only to ERα and display differential agonist activity. Considering the potencies of these progestins for gene regulation however, and their reported serum concentrations relative to that of E_2, our results suggest that the progestins would not elicit estrogenic effects *in vivo*. We do, however, show that MPA, NET-A, DRSP and P_4 may

contribute to breast cancer progression by stimulating breast cancer cell proliferation and anchorage-independent growth via a mechanism requiring the ER and PR, with DRSP being the least potent and MPA the most. This suggests that the fourth-generation progestin DRSP may be a safer conventional HT option than MPA and NET in terms of breast cancer risk. Moreover, the result showing that P_4, MPA, NET and DRSP all induced the co-recruitment of ERα and the PR to the promoters of known PR-regulated oncogenes, while only MPA and NET caused co-recruitment to the promoters of ER target genes, suggests that progestins such as MPA and NET may promote breast cancer pathogenesis by regulating both PR and ER target genes. Overall, the results of this thesis add to the understanding of estrogens and progestogens used in menopausal hormone therapies and highlight the concept that all progestins used in conventional HT are not equal. Finally, this study contributes to the knowledge and understanding of the physiological responses elicited by estrogens and progestins, while providing the potential underlying mechanisms.

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Appendix A

Additional Experimental Data

A1. Increasing the ratio of the PR isoforms relative to the ER subtypes increases the efficacy of both ERα and ERβ

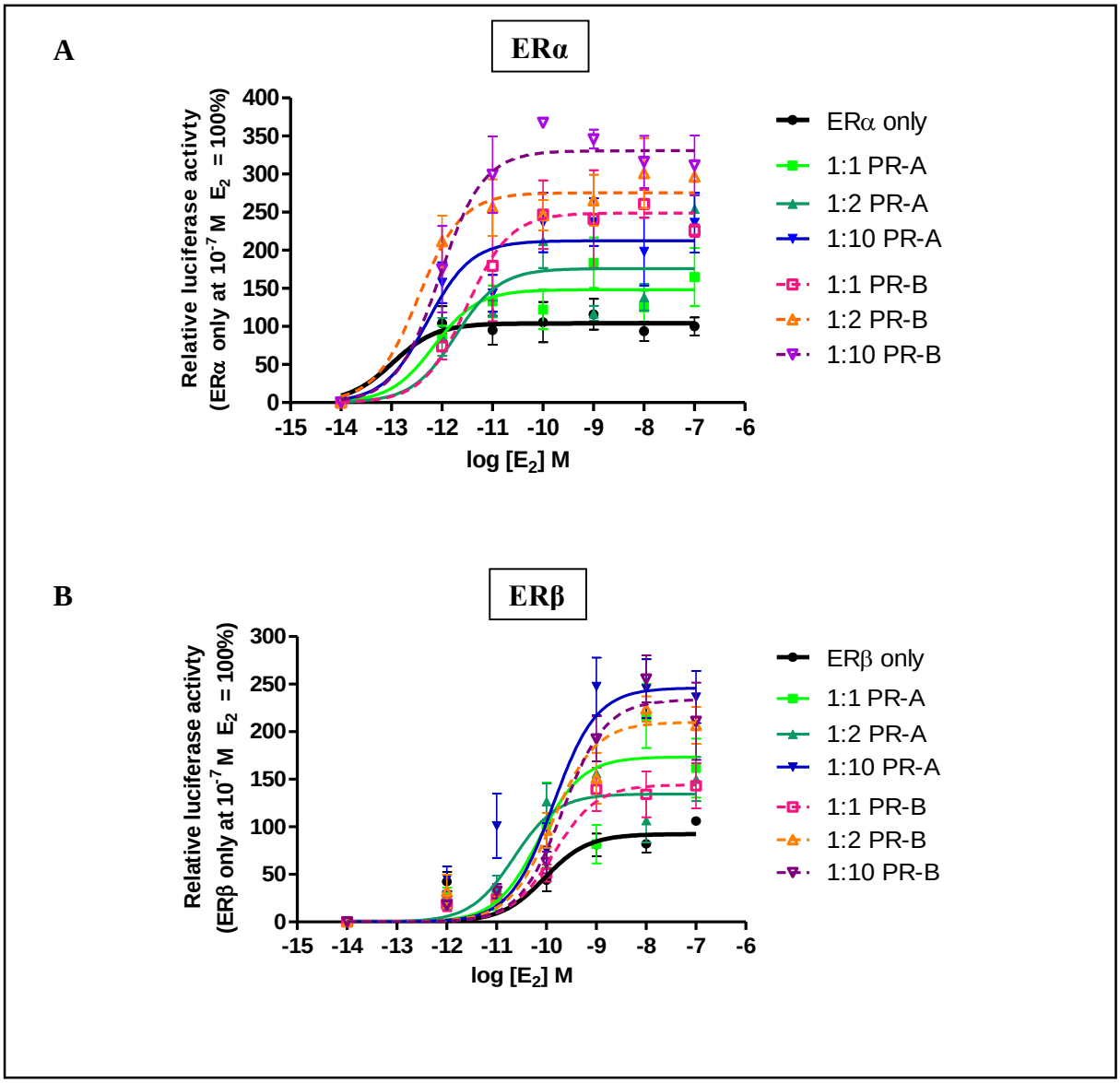


Figure A1. Unliganded PR-A and PR-B differentially modulate the transcriptional activity of ERα and ERβ. HEK293 cells expressing the pS2-ERE-luciferase promoter-reporter construct, **(A)** ERα or **(B)** ERβ, and increasing concentrations of either PR-A or PR-B was incubated with increasing concentrations of E_2 for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration. Results are shown as relative luciferase activity where induction via ERα or ERβ only at 10^{-7} M E_2 was set as 100% and all other responses set relative to this. Maximal response and EC_{50} values are reported in Table A1. Results shown are representatives of at least three independent experiments.

A2. The PR isoforms increase the transcriptional activity of ERα in a progestin-specific manner

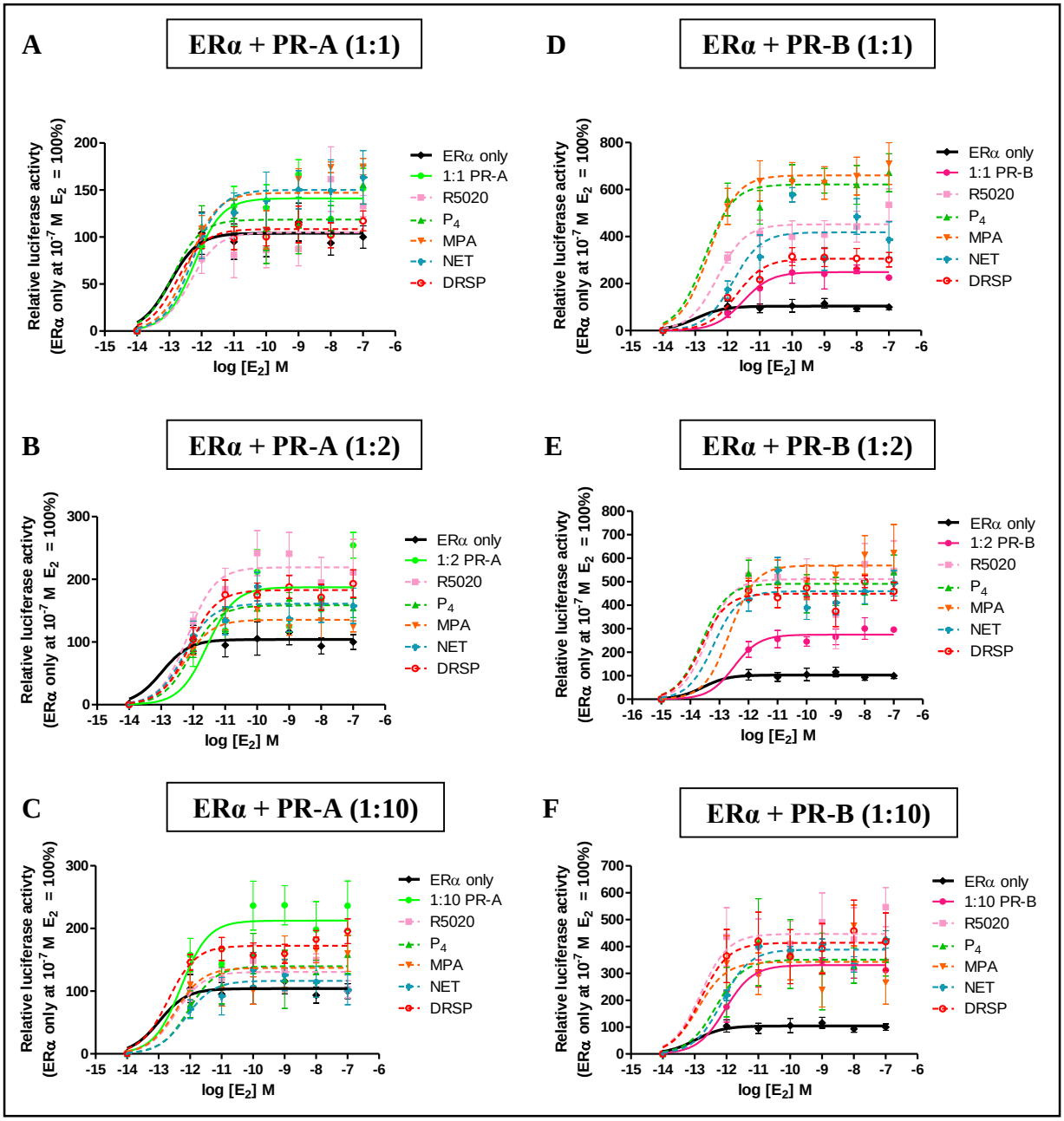


Figure A2. Ligand activated PR-A and PR-B increase the efficacy and potency of ERα. HEK293 cells expressing ERα and the pS2-ERE-luciferase promoter-reporter construct in the absence and presence of increasing concentrations of **(A-C)** PR-A or **(D-F)** PR-B, were treated with increasing concentrations of E_2 in the absence and presence of 1 µM R5020 (■), P_4 (▲), MPA (▼), NET (⁎) or DRSP (○) for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration. Results are shown as relative luciferase activity where induction via ERα only at 10^{-7} M E_2 was set as 100% and all other responses set relative to this. Maximal response and EC_{50} values are reported in Table A1. Results shown are representatives of at least three independent experiments.

A3. The progestin-bound PR isoforms differentially increase the transcriptional activity of ERβ

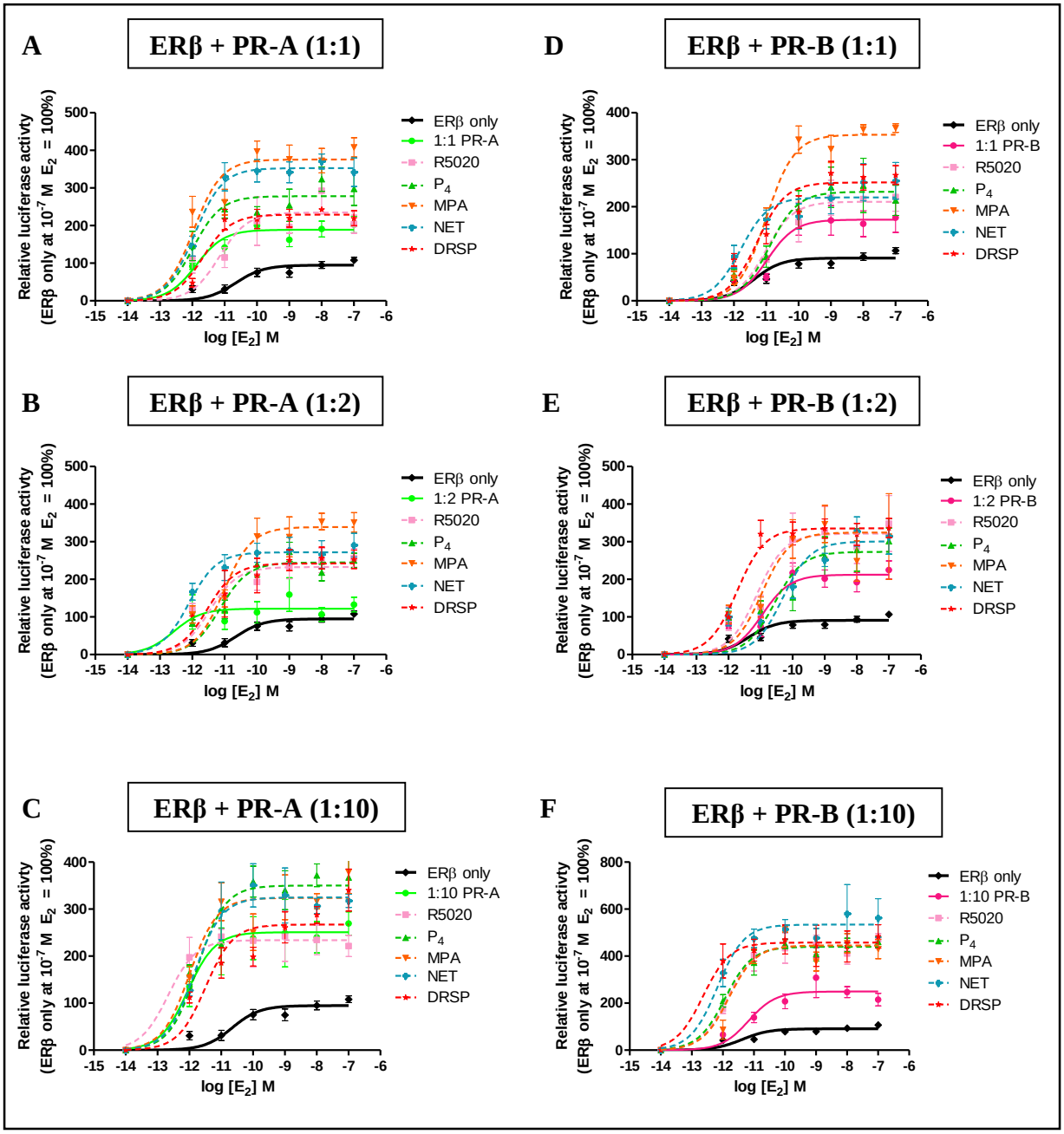


Figure A3. Ligand activated PR-A and PR-B increase the efficacy and potency of ERβ. HEK293 cells expressing ERβ and the pS2-ERE-luciferase promoter-reporter construct in the absence and presence of increasing concentrations of **(A-C)** PR-A or **(D-F)** PR-B were treated with increasing concentrations of E_2 in the absence and presence of 1 µM R5020 (■), P_4 (▲), MPA (▼), NET (⁎) or DRSP (○) for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration. Results are shown as relative luciferase activity where induction via ERβ only at 10^{-7} M E_2 was set as 100% and all other responses set relative to this. Maximal response and EC_{50} values are reported in Table A1. Results shown are representatives of at least three independent experiments.

A4. The unliganded and progestin-activated PR isoforms differentially increase the transcriptional efficacy and potency of ERα and ERβ

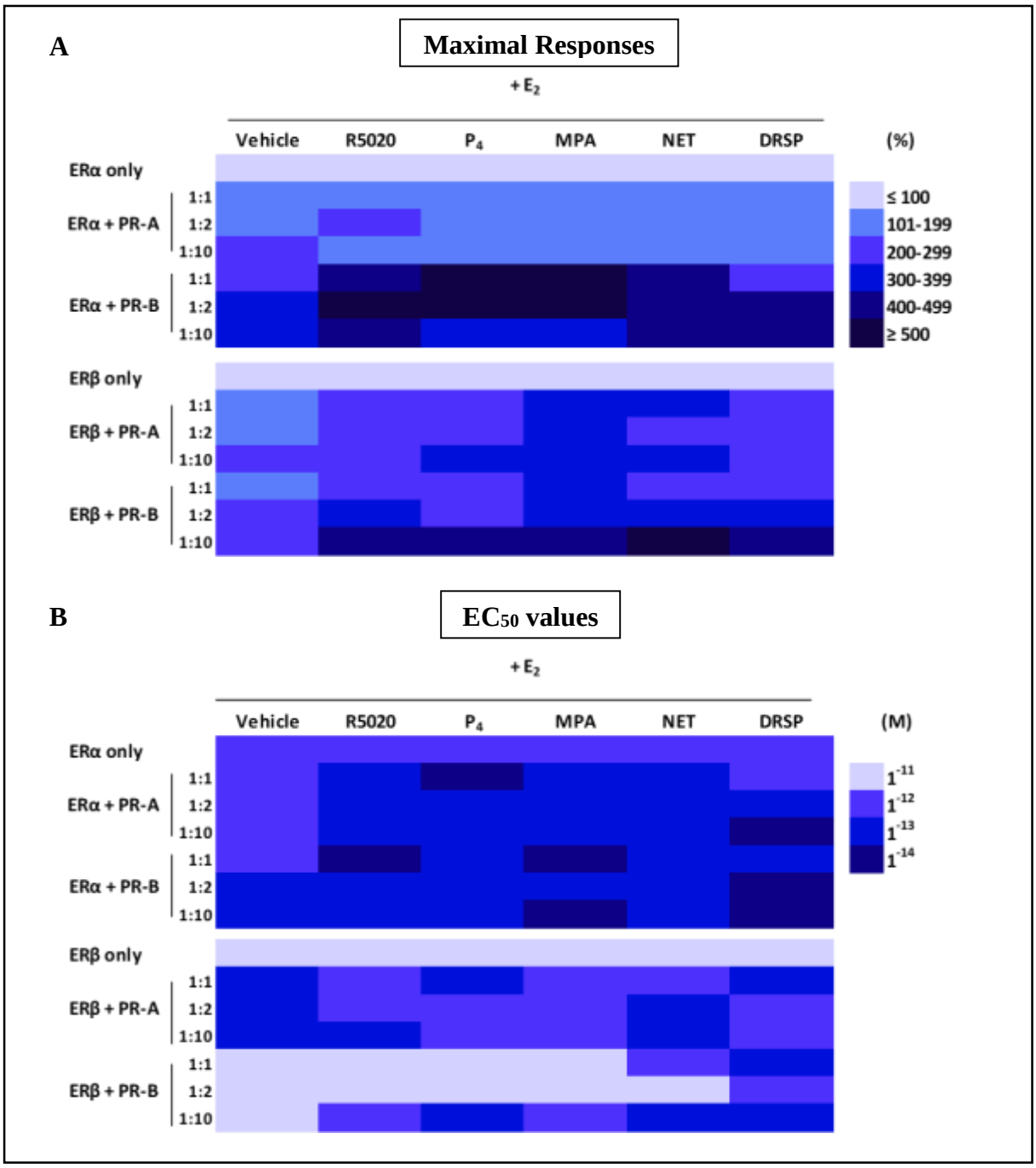


Figure A4. Progestogen-activated PR-A or PR-B differentially modulate the transcriptional activity of ERα and ERβ. Dose response curves (Figures A1-A3) and non-linear regression analysis were used to determine **(A)** maximal response and **(B)** EC_{50} values. The relative values are reported in Table A1 and are visually represented here using heat maps.

Table A1. Relative agonist efficacies and potencies of E_2 for transactivation via the ER on a synthetic ERE-containing promoter-reporter construct in the absence and presence of increasing concentrations of unliganded or progestogen-bound PR-A or PR-B.

Maximal Response ± SEM (ERα only = 100 ± 0 %)							
Ratio		**Vehicle**	**R5020**	**P_4**	**MPA**	**NET**	**DRSP**
ERα + PR-A	**1:1**	144.8 ± 35.0	105.6 ± 0.5	117.8 ± 72.1	148.4 ± 15.2	154.7 ± 11.0	106.3 ± 2.1
	1:2	182.9 ± 34.0	216.6 ± 19.5	164.8 ± 15.9	126.5 ± 19.9	168.4 ± 2.7	178.2 ± 45.6
	1:10	269.4 ± 38.9	129.7 ± 10.2	132 ± 20.8	130.6 ± 3.2	118.2 ± 0.1	185.4 ± 65.6
ERα + PR-B	**1:1**	241.5 ± 42.0	484.4 ± 91.4	680.4 ± 183.4	724.6 ± 167.3	466.1 ± 114.2	294.7 ± 32.14
	1:2	326.0 ± 70.7	548.6 ± 130.5	512.6 ± 120.1	592.3 ± 9.6	484.9 ± 69.5	478.5 ± 184.2
	1:10	328.4 ± 34.2	420.4 ± 105.6	334.9 ± 82.9	331.2 ± 66.8	399.5 ± 55.6	414.9 ± 121.9
EC_{50} ± SEM (ERα only = 3.9 ± 1.8 pM)							
Ratio		**Vehicle**	**R5020**	**P_4**	**MPA**	**NET**	**DRSP**
ERα + PR-A	**1:1**	4.3 ± 1.1	0.3 ± 0.06	0.06 ± 0.004	0.4 ± 0.1	0.4 ± 0.2	1.8 ± 0.07
	1:2	3.6 ± 0.6	0.3 ± 0.1	0.4 ± 0.3	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.04
	1:10	6.7 ± 1.4	0.4 ± 0.2	0.6 ± 0.1	0.7 ± 0.6	0.4 ± 0.2	0.06 ± 0.01
ERα + PR-B	**1:1**	2.4 ± 0.6	0.02 ± 0.001	0.2 ± 0.03	0.02 ± 0.008	0.8 ± 0.5	0.3 ± 0.08
	1:2	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	0.2 ± 0.08	0.06 ± 0.02
	1:10	0.5 ± 0.4	0.4 ± 0.2	0.2 ± 0.01	0.04 ± 0.02	0.3 ± 0.1	0.08 ± 0.007
Maximal Response ± SEM (ERβ only = 100 ± 0 %)							
Ratio		**Vehicle**	**R5020**	**P_4**	**MPA**	**NET**	**DRSP**
ERβ + PR-A	**1:1**	187 ± 20	230 ± 8.4	280 ± 55	380 ± 69	340 ± 70	230 ± 78
	1:2	130 ± 0.08	220 ± 36	250 ± 76	360 ± 58	280 ± 110	230 ± 11
	1:10	250 ± 100	240 ± 70	350 ± 150	310 ± 80	310 ± 100	270 ± 45
ERβ + PR-B	**1:1**	150 ± 27	210 ± 9.7	230 ± 43	360 ± 53	220 ± 37	250 ± 49
	1:2	210 ± 57	320 ± 72	280 ± 70	320 ± 60	300 ± 78	330 ± 71
	1:10	240 ± 15	450 ± 150	460 ± 140	440 ± 65	510 ± 120	450 ± 78
EC_{50} ± SEM (ERβ only = 51.1 ± 13.4 pM)							
Ratio		**Vehicle**	**R5020**	**P_4**	**MPA**	**NET**	**DRSP**
ERβ + PR-A	**1:1**	0.9 ± 0.8	5.7 ± 5.3	0.7 ± 0.6	1.4 ± 0.3	1.4 ± 1.4	0.3 ± 0.1
	1:2	0.7 ± 0.003	2.1 ± 1.0	9.0 ± 0.7	7.2 ± 2.0	0.7 ± 0.5	2.3 ± 1.6
	1:10	0.8 ± 0.04	0.3 ± 0.1	2.5 ± 2.0	1.2 ± 0.4	0.2 ± 0.07	6.7 ± 2.2
ERβ + PR-B	**1:1**	38 ± 22	35 ± 12	30 ± 20	18 ± 8.3	1.8 ± 0.5	0.5 ± 0.2
	1:2	62 ± 19	11 ± 4.1	51 ± 36	26 ± 6.5	31 ± 6.4	1.1 ± 0.6
	1:10	18 ± 14	1.4 ± 0.8	1.0 ± 0.7	4.9 ± 4.3	0.2 ± 0.1	0.4 ± 0.2

A5. Mycoplasma-negative COS-1, HEK293 and MCF-7 BUS cells

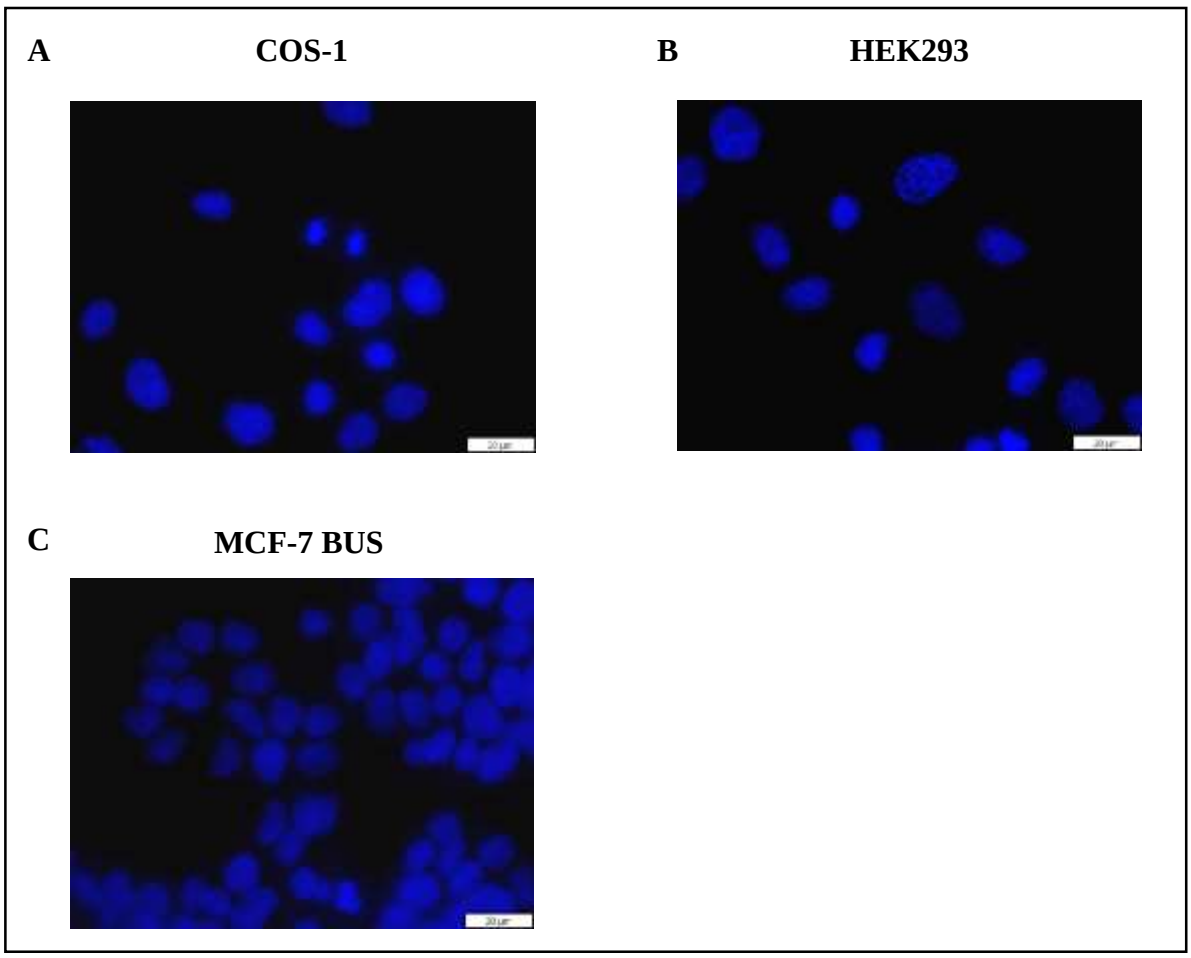


Figure A5. Mycoplasma-negative COS-1, HEK293 and MCF-7 BUS cells. All cell lines used in this study were routinely tested for mycoplasma infection and only mycoplasma-negative cells were used in experiments. **(A)** COS-1, **(B)** HEK293 and **(C)** MCF-7 BUS cells were stained with DNA Hoechst 33258 dye and visualized using the Olympus IX81 microscope.

A6. E_2 represses TNFα-induced gene expression via overexpressed ERα or ERβ in a promoter-specific manner

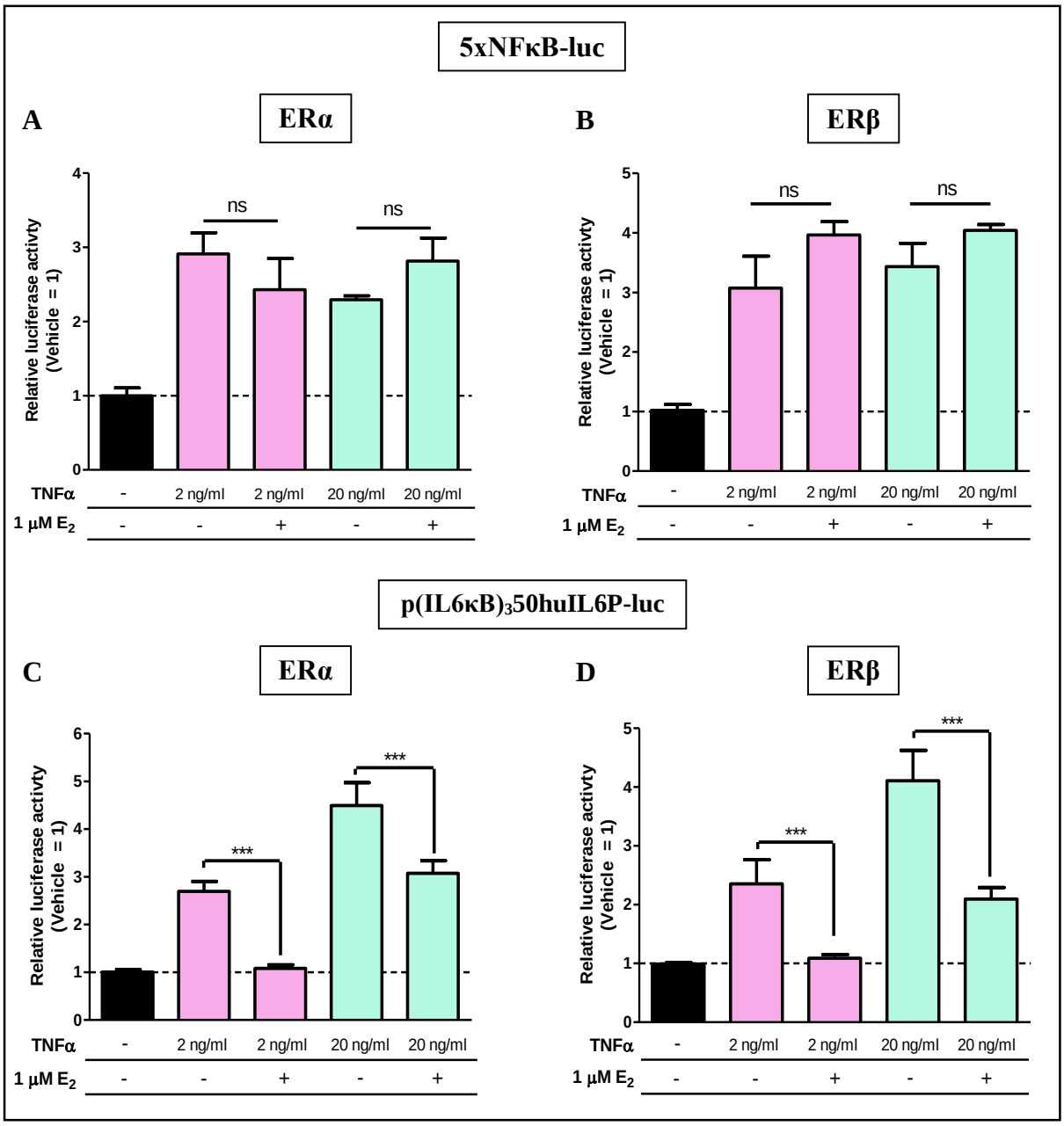


Figure A6. E_2 represses TNF-induced gene expression via ERα or ERβ in a promoter-specific manner. HEK293 cells expressing the **(A, B)** 5xNFκB- luciferase- or the **(C, D)** p(IL6κB)$_3$50huIL6P-luciferase promoter-reporter construct and the **(A, C)** pSG5-hERα or **(B, D)** pSG5-hERβ cDNA expression vector, were treated with 0.1% EtOH (vehicle control) or 2 ng/ml or 20 ng/ml TNFα in the absence or presence of 1 μM E_2 for 24 hours. Luciferase activity was measured in relative light units and normalized to protein concentration. Results are shown as relative luciferase activity where induction with the vehicle control is set as one and all other responses set relative to this.

A7. Representative RNA gel showing intact RNA

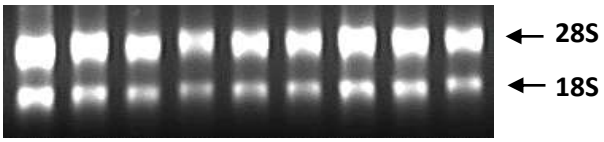
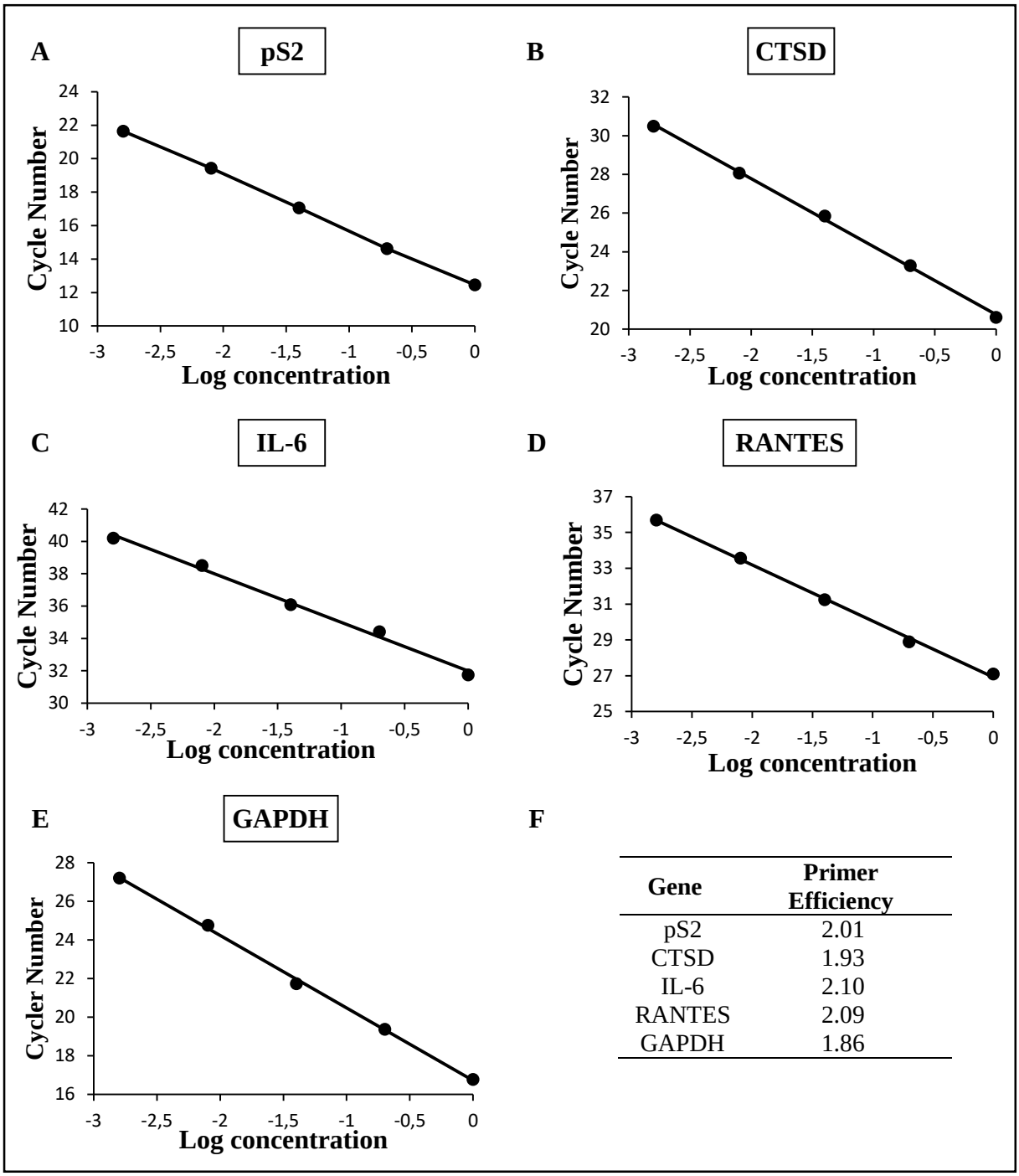


Figure A7. A representative 1% denaturing agarose gel. RNA was isolated from MCF-7 BUS cells using Tri-reagent (Sigma-Aldrich, South Africa) as per the manufacturer's instructions, and 1 µg of each RNA sample was subjected to electrophoresis on a 1% denaturing formaldehyde agarose gel to confirm the presence of 28S and 18S subunits in a ratio of approximately 2:1 as an indicator of intact RNA.

A8. Primer efficiencies of the primer pairs were determined from standard curves showing cycle number versus log cDNA concentration



Gene	Primer Efficiency
pS2	2.01
CTSD	1.93
IL-6	2.10
RANTES	2.09
GAPDH	1.86

Figure A8. Representative standard curves that were used to determine primer efficiencies. A cDNA dilution series was used to generate standard curves indicating the cycle number versus the log concentration of the amplified cDNA. Representative standard curves for **(A)** pS2, **(B)** CTSD, **(C)** IL-6, **(D)** RANTES and **(E)** GAPDH are shown and the reported **(F)** primer efficiencies (or exponential amplification values; E) are the average of two individual experiments performed in triplicate, calculated using the equation by Pfaffl ($E = 10^{[-1/slope]}$).

A9. Melt curve analysis for pS2, CTSD, IL-6, RANTES and GAPDH

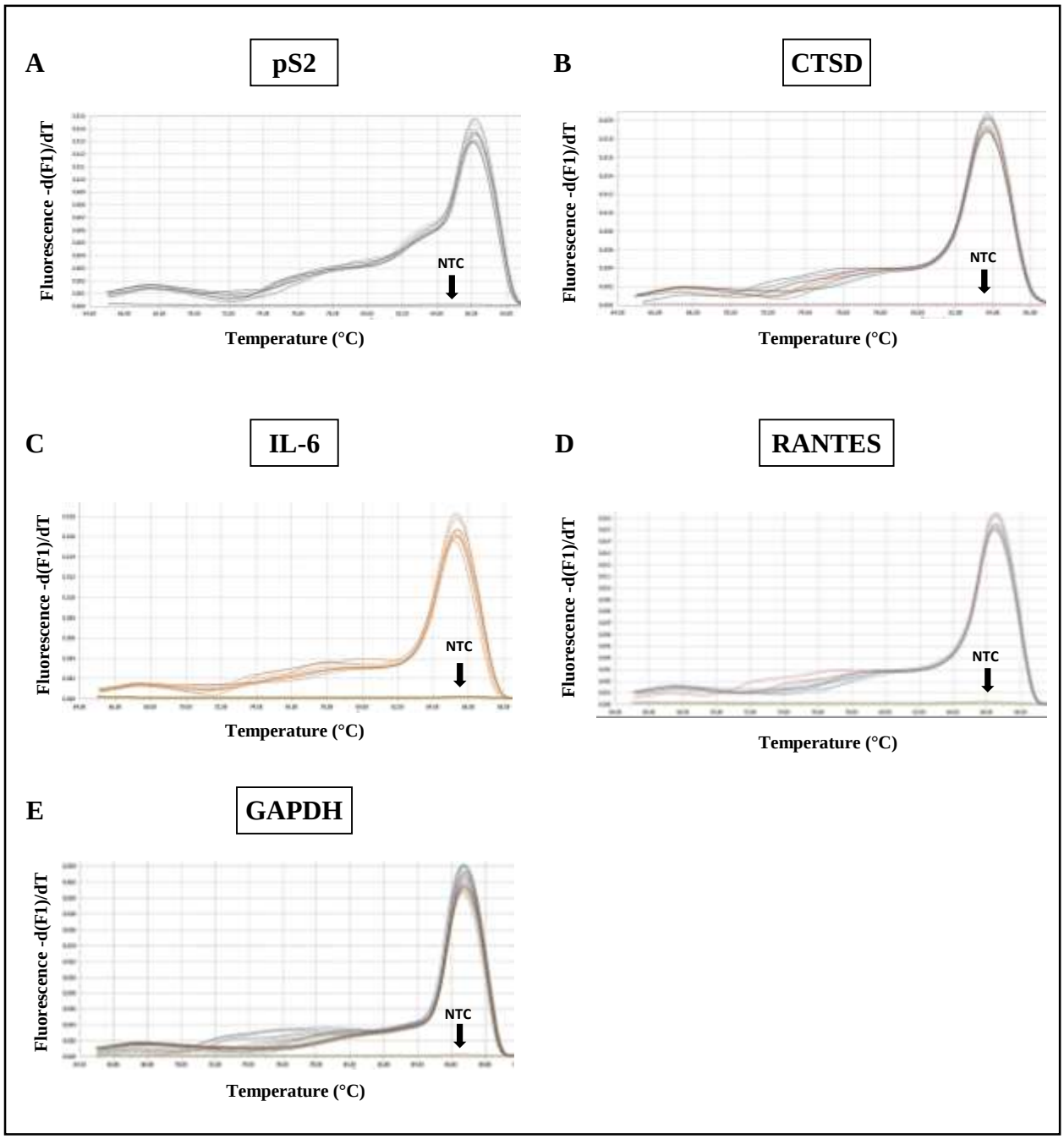


Figure A9. Representative melt curves are shown for pS2, CTSD, IL-6, RANTES and GAPDH. Melt curve analysis was conducted for every realtime qPCR experiment to ensure amplicon specificity and that the product is not present in the no template control (NTC). Melting curve analysis can be used to differentiate between different PCR products, non-specific amplicons as well as primer dimers.

A10. Representative agarose gel of sonicated chromatin

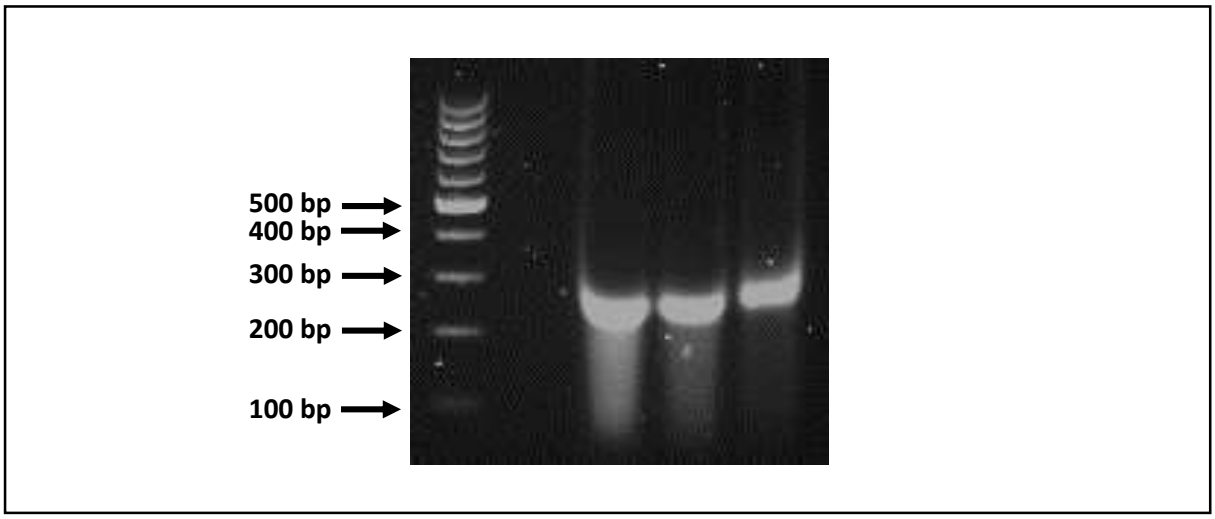


Figure A10. An example of an agarose gel indicating sonicated DNA fragments between 200 bp and 300 bp in size. A representative agarose gel indicating the size of DNA fragments after sonication on 100% power for 60 cycles of 60 seconds each, with 20 second intervals between cycles using the Misonix sonicator (Qsonica, RSA).

Appendix B

Oral and Poster Outputs of the PhD study and

Contributions to Publications Not Part of This Study

Oral and Poster Outputs of the PhD Study

1. **Perkins, M.**, Louw-du Toit, R**.**, Africander, D. Bioidentical hormones used in hormone replacement therapy: Implications for breast cancer. SASBMB congress, Goudini Spa Resort, South Africa. 6 - 9 July 2014. **(Poster Presentation)**

2. **Perkins, M.**, Louw- du Toit, R., Africander, D. Investigating the mechanism of action of hormones used in hormone replacement therapy via estrogen receptor subtypes and influence of the progesterone receptor. FEBS advanced lecture course, Nuclear receptor signalling in physiology and disease, Spetses Island, Greece. 23 - 28 August 2015. **(Oral and Poster Presentation)**

3. **Perkins, M.S.**, Louw- du Toit, R., Africander, D. Both the ER and PR are required for progestin-induced effects on breast cancer cell proliferation. FASEB Conference, Cell Signalling in Cancer: from Mechanisms to Therapy, Snowmass Village, Colorado, USA. 5 - 10 June 2016. **(Poster Presentation)**

4. **Perkins, M.S.**, Louw-du Toit, R., Africander, D. The Rationale Behind Compounded Bioidentical Hormone Therapy Should be Reassessed. 25th SASBMB congress, East London Convention Centre, East London, South Africa. 10 - 14 July 2016. **(Oral Presentation)**

5. **Perkins, M.S.**, Louw- du Toit, R., Africander, D. Progestin-induced breast cancer: A role for estrogen- and progesterone receptor crosstalk. Gordon Research Conference, Hormone-dependent Cancers, Sunday River, Newry, Maine, USA. 6 - 11 August 2017. **(Poster Presentation by Africander, D.)**

Contributions to publications not part of this study

1. R. Louw-du Toit, **M.S. Perkins**, J.L. Snoep, K.-H. Storbeck, D. Africander, Fourth-generation progestins inhibit 3β-hydroxysteroid dehydrogenase type 2 and modulate the biosynthesis of endogenous steroids, PLoS One. 11 (2016) e0164170. doi:10.1371/journal.pone.0164170.

For this publication, the candidate was involved with the investigation, formal analysis and visualization. The candidate was also involved with reviewing and editing the final publication draft.

2. E. Pretorius, D.J. Africander, M. Vlok, **M.S. Perkins**, J. Quanson, K.-H. Storbeck, 11-Ketotestosterone and 11-ketodihydrotestosterone in castration resistant prostate cancer: Potent androgens which can no longer be ignored, PLoS One. 11 (2016) e0159867. doi:10.1371/journal.pone.0159867.

For this publication, the candidate performed the whole cell binding experiments, analyzed the data, and wrote the methods section pertaining to these binding experiments. The candidate also involved with critical reviewing and editing of the final document.

PLOS | ONE

RESEARCH ARTICLE

Fourth-Generation Progestins Inhibit 3β-Hydroxysteroid Dehydrogenase Type 2 and Modulate the Biosynthesis of Endogenous Steroids

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Abstract

Progestins used in contraception and hormone replacement therapy are synthetic compounds designed to mimic the actions of the natural hormone progesterone and are classed into four consecutive generations. The biological actions of progestins are primarily determined by their interactions with steroid receptors, and factors such as metabolism, pharmacokinetics, bioavailability and the regulation of endogenous steroid hormone biosynthesis are often overlooked. Although some studies have investigated the effects of select progestins on a few steroidogenic enzymes, studies comparing the effects of progestins from different generations are lacking. This study therefore explored the putative modulatory effects of progestins on *de novo* steroid synthesis in the adrenal by comparing the effects of select progestins from the respective generations, on endogenous steroid hormone production by the H295R human adrenocortical carcinoma cell line. Ultra-performance liquid chromatography/tandem mass spectrometry analysis showed that the fourth-generation progestins, nestorone (NES), nomegestrol acetate (NoMAC) and drospirenone (DRSP), unlike the progestins selected from the first three generations, modulate the biosynthesis of several endogenous steroids. Subsequent assays performed in COS-1 cells expressing human 3βHSD2, suggest that these progestins modulate the biosynthesis of steroid hormones by inhibiting the activity of 3βHSD2. The K_i values determined for the inhibition of human 3βHSD2 by NES (9.5 ± 0.96 nM), NoMAC (29 ± 7.1 nM) and DRSP (232 ± 38 nM) were within the reported concentration ranges for the contraceptive use of these progestins *in vivo*. Taken together, our results suggest that newer, fourth-generation progestins may exert both positive and negative physiological effects via the modulation of endogenous steroid hormone biosynthesis.

conclusions or recommendations expressed in this material are those of the authors, and therefore the NRF does not accept any liability in regard thereto.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Synthetic progestogens (progestins), were developed to have similar progestogenic properties, but greater bio-availabilities, half-lives and potencies than the rapidly metabolized natural progestogen, progesterone (Prog) [1, 2]. Progestins are mostly derived from parent compounds such as Prog and testosterone (reviewed in [3]), with those structurally related to Prog referred to as 17α-hydroxyprogesterone (17OH-Prog) and 19-norprogesterone derivatives, and those related to testosterone known as 19-nortesterone derivatives. A variety of these structurally diverse compounds are available, and are classified into four consecutive generations. Like Prog, these progestins mediate their biological effects by binding to the progesterone receptor (PR), and are used in many applications in female reproductive medicine including contraception and hormone replacement therapy (HRT) [4, 5] (reviewed in [3, 6]). A number of side-effects have however been reported with their clinical use and include weight gain, acne, increased risk of invasive breast cancer, cardiovascular disease (CVD) and modulation of immunity in the female genital tract (reviewed in [6]).

To date it has been suggested that the mechanism underlying most of these adverse effects are most likely due to some progestins interacting with steroid receptors other than the PR [7–13]. Thus, the newer, fourth-generation progestins were developed to be "purer" progestogens by having stronger affinities for the PR. Although these progestins may also bind to other steroid receptors, their activities are similar to the natural PR ligand, Prog, in that they are devoid of estrogenic, androgenic, glucocorticoid and mineralocorticoid activity, with some, like Prog, eliciting anti-androgenic and/or anti-mineralocorticoid effects [5, 14] (reviewed in [3, 6]). However, some recent studies indicate that these newer generation progestins also display adverse effects. For example, the risk of developing venous thromboembolism (VTE) has been shown to increase with the use of combined oral contraceptives (COC) containing the fourth-generation progestin drospirenone (DRSP) [15–17].

This raises the possibility that a mechanism other than off-target steroid receptor-mediated effects may be involved. One possibility, and an area of research that has received little attention, is the influence of progestins on adrenal steroid biosynthesis. It is well documented that abnormal hormone levels due to the modulation of adrenal steroidogenesis are associated with numerous undesirable conditions [18–21] (reviewed in [22]). The limited number of studies that have in fact investigated the effects of progestins on adrenal steroid biosynthesis in humans have primarily focussed on the first-generation progestin, medroxyprogesterone acetate (MPA), and showed a reduction in the serum levels of the endogenous glucocorticoid cortisol [23–26], the endogenous androgen precursors androstenedione (A4) and dehydroepiandrosterone sulphate (DHEA-S) [25], and the endogenous androgen testosterone [27]. Recent studies examining the effects of progestins developed after the first generation, such as levonorgestrel (LNG), nomegestrol acetate (NoMAC) and DRSP, also showed decreased concentrations of androgens and their precursors [28–30]. The biosynthesis of steroid hormones are dependent on the function of steroidogenic enzymes, which consists of substrate-selective cytochrome P450 enzymes (CYP's) and hydroxysteroid dehydrogenases (HSD's) (Fig 1) (reviewed in [22, 31–33]). Interestingly, only a few studies have examined the influence of progestins on the activity of these enzymes, and most of the studies focus on the effects of MPA. For example, MPA has been shown to inhibit the activity of both human [34] and rat [35, 36] 3β-hydroxysteroid dehydrogenase (3βHSD), while suppressing the activity of rat, but not human, cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17A1) [34, 36, 37]. Although some studies have investigated the effects of other progestins, such as norethisterone (NET) and LNG, on the activity and/or mRNA expression of steroidogenic enzymes, these studies are limited to rat [38] and fish [39] models. Considering that different species express different enzyme isoforms,

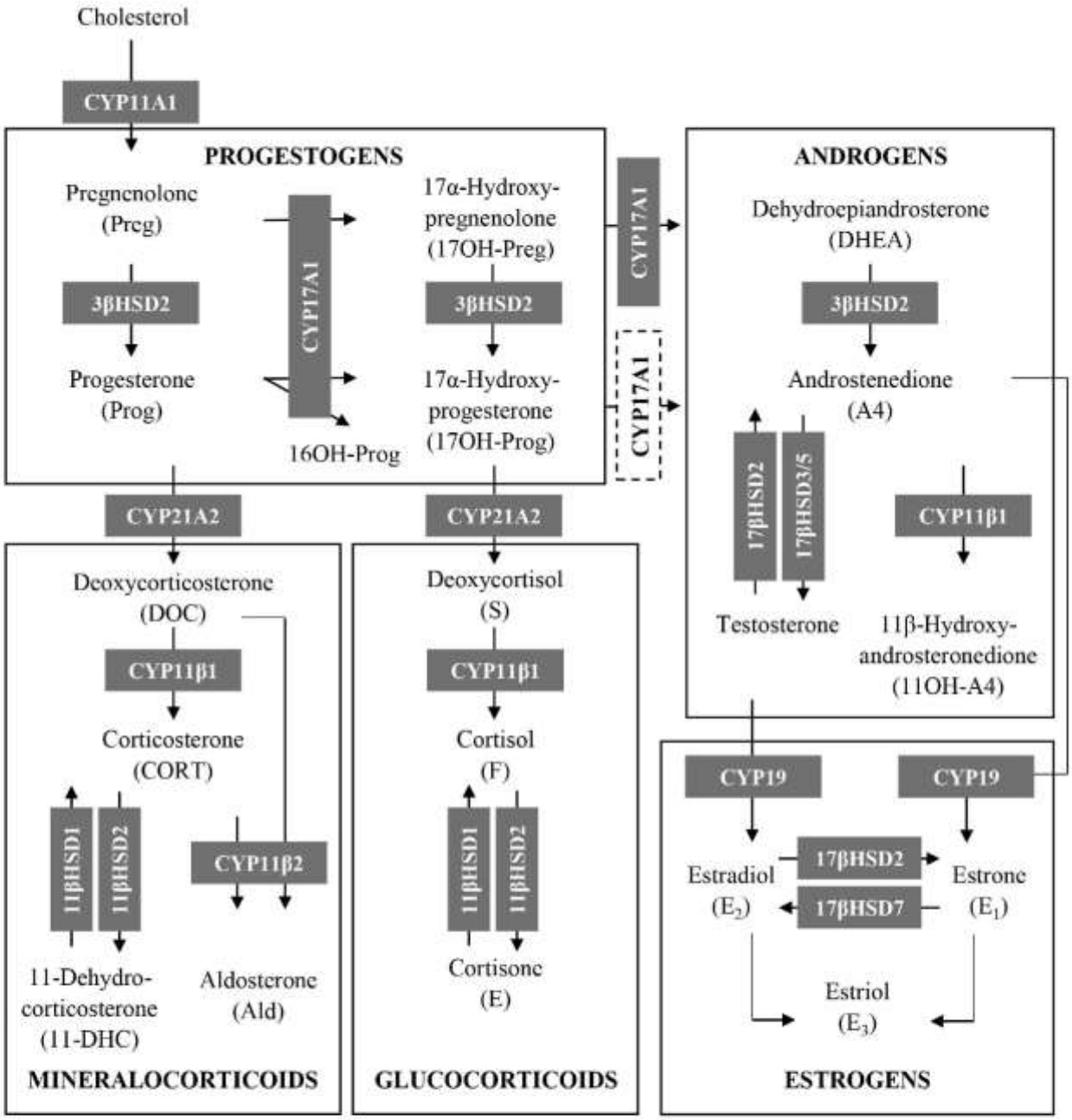

Fig 1. The biosynthesis of human steroid hormones consists of multiple reactions which are catalysed by specific steroidogenic enzymes (grey boxes). The conversion of 17α-hydroxyprogesterone (17OH-Prog) to androstenedione (A4) by CYP17A1 is shown as a dashed box as 17OH-Prog is a poor substrate for the 17,20-lyase activity of human CYP17A1 [22, 31].

doi:10.1371/journal.pone.0164170.g001

which have different functions and substrate specificities [40–42], it is probable that the effects of progestins in animal models will not reflect their actions on human enzymes. It is thus clear that studies investigating the effects of progestins on human steroidogenic enzymes are needed, and more so, a direct comparative study of the influence of progestins from the different generations.

The present study thus directly compared the effects of select progestins from different generations on the biosynthesis of steroids by the H295R human adrenocortical carcinoma cell line, which expresses all the steroidogenic enzymes required for the biosynthesis of progestogens, mineralocorticoids, glucocorticoids and adrenal androgen [43–46]. The comparison included the first-generation progestins MPA and NET acetate (NET-A), the second-generation progestin LNG, the third-generation progestin gestodene (GES) and the fourth-generation progestins nestorone (NES), NoMAC and DRSP (Fig 2). Specifically, we used ultra-performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) to measure not only the end products of the progestogenic, mineralocorticoid, glucocorticoid and androgenic pathways, but also to identify the steroid intermediates which are affected by the progestins. Furthermore, we also determined whether the progestins themselves are metabolized in the H295R cell line. Our results indicate that fourth-generation progestins modulate endogenous steroid biosynthesis due to the inhibition of human 3βHSD2 and/or CYP17A1 activity. Moreover, we determined inhibition constant (K_i) values for 3βHSD2 in the nanomolar range for NES, NoMAC and DRSP, with the mechanisms of inhibition best fitted to the experimental data indicating that NES and DRSP are non-competitive inhibitors of 3βHSD2, while NoMAC is a competitive inhibitor of this enzyme.

Materials and Methods

Test compounds and standards

MPA, NET-A, LNG, GES, NES, NoMAC, DRSP, pregnenolone (Preg), Prog, 17OH-Preg, 17OH-Prog, 16OH-Prog, deoxycorticosterone (DOC), corticosterone (CORT), 11-dehydrocorticosterone (11-DHC), aldosterone (Ald), deoxycortisol, cortisol, cortisone, dehydroepiandrosterone (DHEA), A4, testosterone, forskolin (FSK) and trilostane, were obtained from Sigma–Aldrich, South Africa, while 11β-hydroxyandrostenedione (11OH-A4) was purchased from Steraloids, USA. All test compounds, as well as FSK, were prepared in dimethylsulfoxide (DMSO), and added to the culturing medium at a final concentration of 0.2% DMSO. The deuterated internal standards, d2-testosterone, d9-Prog, d9-17OH-Prog and d4-cortisol were purchased from Cambridge Isotope Laboratories (Maryland, USA).

Plasmids

The plasmids expressing human 3βHSD2 (pCDNA6-hHSD3β2-V5), CYP17A1 (pIRES-hCYP17A1-V5-X-hCYPB5-6HIS), and CYP21A2 (pCDNA6-hCYP21A2-V5) were generous gifts from Prof Wiebke Arlt (Institute of Metabolism and Systems Research, University of Birmingham, UK). Plasmid DNA was purified using the NucleoBond® Xtra Maxi kit (Machery-Nagel GmbH, Germany) according to the manufacturer's instructions.

Cell culture

The human H295R adrenocortical carcinoma cell line was a generous gift from Prof William E. Rainey (University of Michigan, Medical School, Molecular and Integrative Physiology, USA), and was cultured as previously described [47]. The COS-1 monkey kidney cell line was purchased from the American Type Culture Collection (ATCC) and cultured as previously described [12]. To ensure that only mycoplasma-negative cells were used in experiments, cell cultures were regularly tested for mycoplasma infection using Hoechst staining [48].

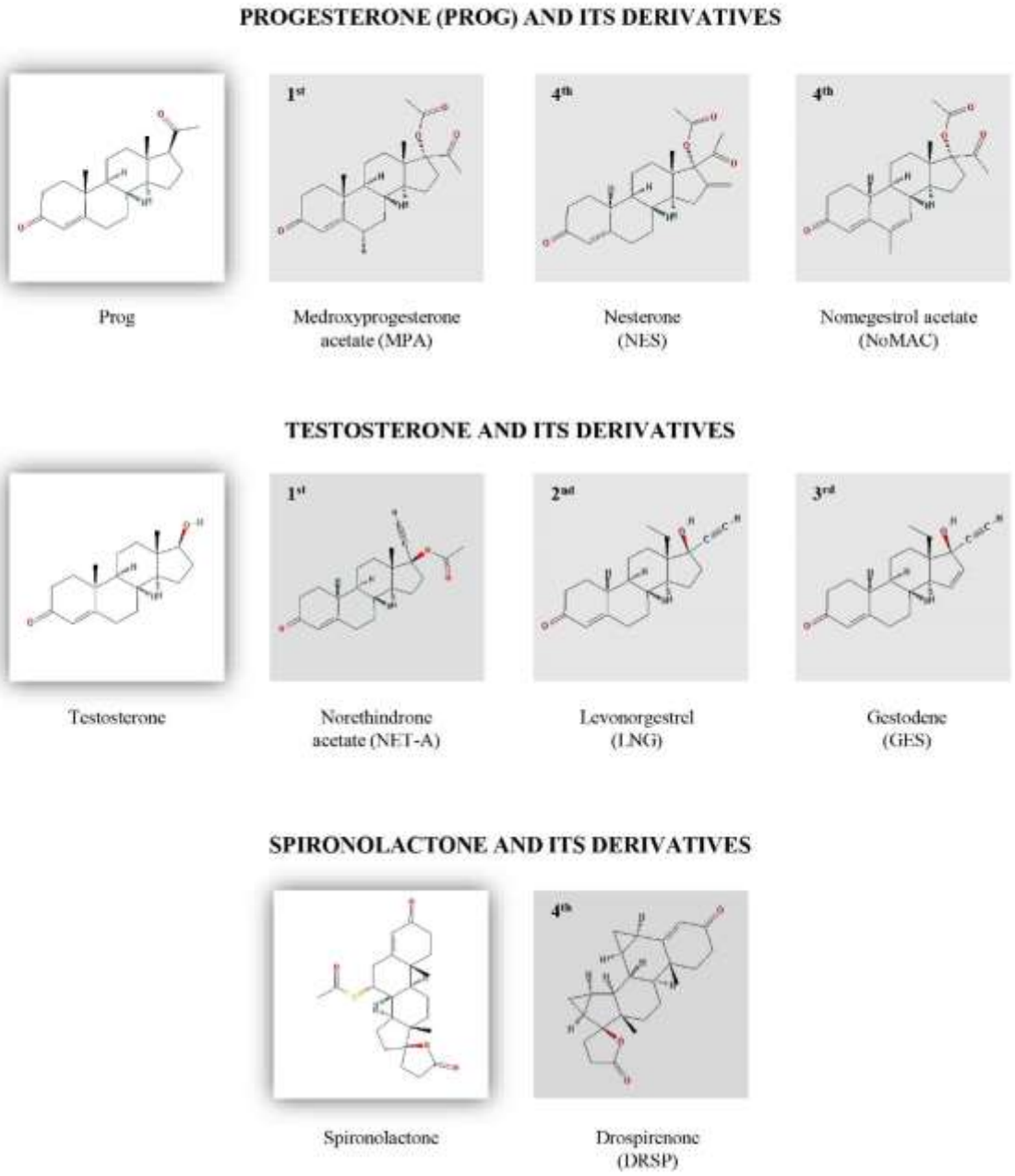

Fig 2. Chemical structures of the endogenous steroids progesterone (Prog) and testosterone, the synthetic MR antagonist spironolactone, and the progestins used in this study : Medroxyprogesterone acetate (MPA), nestorone (NES), nomegestrol acetate (NoMAC), norethisterone/norethindrone acetate (NET-A), levonorgestrel (LNG), gestodene (GES) and drospirenone (DRSP). The inserts (1st, 2nd, 3rd and 4th) denote the four consecutive generations of progestins.

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Steroid biosynthesis and progestin metabolism in the H295R cell line

H295R cells were seeded into 12-well plates at 4 x 10^5 cells per well, and two days later treated with DMSO (vehicle control) or 1 μM MPA, NET-A, LNG, GES, NES, NoMAC or DRSP, in the absence and presence of 10 μM FSK. As a negative control, medium containing the test

compounds were added to 12-well plates (no cells) and incubated at 37°C in an atmosphere of 90% humidity and 5% CO_2. After 48 hours, the medium (500 μl) was removed and steroids extracted using a 10:1 volume of dichloromethane to culture medium as described previously [47]. Briefly, 15 ng of the internal standards, d2-testosterone, d4-cortisol, d9-Prog and d9-17OH-Prog, were added to the samples, vortexed for 10 minutes and centrifuged at 3 000 rpm for 5 minutes. The dichloromethane phase containing the steroids were transferred to clean test tubes and dried at 50°C under nitrogen. The dried steroid residue was resuspended in 200 μl 50% methanol, vortexed for 2 minutes and stored at -20°C prior to analysis by UPLC–MS/MS. The cells were washed with 1x PBS, lysed with passive lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) TRIS-phosphate-EDTA and 1.44 mM EDTA) and the total protein concentration determined using the Bradford protein assay method [49]. All experiments were performed in parallel under the same experimental conditions.

H295R cell viability

The colorimetric MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was used, and performed essentially as previously described in [50] with the following modifications. Briefly, H295R cells were plated into 96-well plates at a cell density of 1×10^4 cells per well, and treated for 48 hours with DMSO (vehicle control) or 1 μM test compound in the absence or presence of 10 μM FSK. Four hours prior to the end of the incubation period, the medium was aspirated and replaced with 150 μl DMEM/F12 supplemented with 0.1% cosmic calf serum (HyClone® Thermo Scientific Inc., USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, South Africa) and 0.01% gentamycin (Gibco, Paisley, UK) and 5 mg/ml of the MTT solution (Sigma-Aldrich, South Africa). At the end of the incubation period, the medium was aspirated and the crystals resuspended in 200 μl solubilisation solution (DMSO). The plates were covered with foil and incubated at room temperature for 5 minutes with agitation, followed by the absorbance measurement at 550 nm using a BioTek® PowerWave 340 spectophotometer.

Steroid conversion assays in transiently transfected COS-1 cells

COS-1 cells were seeded into 10 cm dishes at 2 x 10^6 cells per dish. On day 2, the cells were transiently transfected with 7.5 μg of the appropriate expression vector for human 3βHSD2 (pCDNA6-hHSD3β2-V5), CYP17A1 (pIRES-hCYP17A1-V5-X-hCYPB5-6HIS) or CYP21A2 (pCDNA6-hCYP21A2-V5), using the X-tremeGENE 9 DNA transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. After 24 hours, the cells were replated into 24-well plates at a density of 1×10^5 cells per well, and incubated for 72 hours. To assay for the inhibition of substrate conversion by the progestins, the cells were treated with the appropriate steroid substrate, 1 μM Preg (for 3βHSD2) or Prog (for CYP17A1 and CYP21A2) or 17OH-Prog (CYP21A2), in the absence or presence of 1 μM MPA, LNG, GES, NES, NoMAC or DRSP. The duration of hormone treatment was based on optimal substrate conversion assays in COS-1 cells. Following the optimal treatment time, 500 μl of the medium was removed, the steroids/progestins extracted and the samples prepared for UPLC-MS/MS analysis as described in above. The cells were washed with 1x PBS, lysed with passive lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) TRIS-phosphate-EDTA and 1.44 mM EDTA) and the total protein concentration determined using the Bradford protein assay method [49].

Kinetic analysis in transiently transfected COS-1 cells

COS-1 cells were seeded into 10 cm dishes at 2 x 10^6 cells per dish. On day 2, the cells were transiently transfected with 7.5 μg of the expression vector for human 3βHSD2 (pCDNA6-

hHSD3β2-V5) using the X-tremeGENE 9 DNA transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. After 24 hours, the cells were replated into 24-well plates at a density of 5×10^4 cells per well, and incubated for 48 hours. The cells were subsequently treated with Preg (0.5, 1, 2, 4 and 8 μM) in the absence or presence of 0.2 or 0.5 μM NES, NoMAC, DRSP or trilostane. The steroid containing media (500 μl) were removed at specific time intervals and the steroids extracted using a 3:1 volume of tert-Butyl methyl ether (MTBE) to culture medium as previously described [51]. Briefly, the samples were vortexed for 10 minutes, incubated at -80°C for 1–2 hours allowing the medium (aqueous phase) to freeze, whereafter the MTBE phase containing the steroids were transferred to clean test tubes and dried at 50°C under nitrogen. The dried steroid residue was resuspended in 200 μl 50% methanol, vortexed for 2 minutes and stored at -20°C prior to analysis by UPLC–MS/MS. The cells were washed with 1x PBS, lysed with passive lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) TRIS-phosphate-EDTA and 1.44 mM EDTA) and the total protein concentration determined using the Bradford protein assay method [49]. The NonlinearModelFit function of Mathematica (http://www.wolfram.com) was used to estimate the kinetic parameters for 3βHSD2 activity and the inhibition constants (K_i) for NES, NoMAC, DRSP and trilostane.

Separation and quantification of steroid metabolites and progestins using UPLC-MS/MS

Steroid metabolites and progestins were separated using a high strength silica (HSS) T3 column (2.1 mm × 50 mm, 1.8 μm) coupled to an ACQUITY UPLC (Waters, Milford, USA) as previously described [52]. The mobile phases consisted of (A) 1% formic acid and (B) 100% methanol. The injection volume of each sample was 5 μl and the steroid metabolites and progestins were eluted at a flow rate of 0.600 ml per minute using a linear gradient from 55% A to 75% B in 5 minutes. For the kinetic analysis, Preg and Prog were separated using a linear gradient from 40% A to 80% B in 1.5 min. A Xevo TQ or Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA) was used in multiple reaction monitoring (MRM) mode using an electrospray in the positive ionization mode (ESI+). The following settings were used: Capillary voltage of 3.5 kV, cone voltage 15–30 V, collision energy 4–20 eV, source temperature 140°C, desolvation temperature 400°C, desolvation gas 800 L/h and cone gas 50 L/h. The MassLynx version 4 software program was used for data collection and analysis.

Quantitative real-time PCR (qPCR)

H295R cells were seeded into 12-well plates at 1 x 10^5 cells per well, and two days later treated with DMSO (vehicle control) or 1 μM NES, NoMAC or DRSP for 6 hours. Total RNA was isolated using Tri-reagent (Sigma-Aldrich, South Africa) according to the manufacturer's instructions, and subsequently reversed transcribed using ImProm-II Reverse Transcription System cDNA synthesis kit (Promega). Real-time qPCR was performed by using the Roche LightCycler® 96 and KAPA SYBR FAST qPCR master mix. The mRNA expression of steroidogenic enzymes and the reference gene *GAPDH* was measured using the following primer sets: *CYP17A1* [53], 5-TGGCCCCATCTATTCTGTTCG-3′ (forward primer) and 5′ -TAGAGT TGCCATTTGAGGCCG-3′ (reverse primer); *3βHSD2* [54], 5′ -TGCCAGTCTTCATCTACAC CAG-3′ (forward primer) and 5′ -TTCCAGAGGCTCTTCTTCGTG-3′ (reverse primer); *GAPDH* [55], 5′ -TGAACGGGAAGCTCACTGG-3′ (forward primer) and 5′ -TCCACCAC CCTGTTGCTGTA-3′ . The relative transcript levels of the target genes were calculated using the method described by [56], and normalised to the relative transcript levels of *GAPDH*.

Data manipulation and statistical analysis

GraphPad Prism® software version 5 was used for data manipulations, graphical presentations and statistical analysis. One-way ANOVA with Dunnett's (compares all columns versus control column) post-test was used for statistical analysis. Statistically significant differences are indicated by either *, **, *** to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, whereas $p>0.05$ indicates no statistical significance (ns). The error bars represent the standard error of the mean (SEM) of at least two independent experiments. The kinetic parameters for 3βHSD2 activity and the inhibition constants (K_i) for NES, NoMAC, DRSP and trilostane were fitted by minimizing the sum of the squared differences between the data sets and the models, using the NonlinearModelFit function of Mathematica (http://www.wolfram.com). Data was fitted to three different inhibition mechanisms: a competitive inhibition mechanism (inhibitor binds only to the free enzyme), a non-competitive inhibition mechanism (inhibitor binds to both the free enzyme and the enzyme-substrate complex) and an uncompetitive inhibition mechanism (inhibitor binds only to the enzyme-substrate complex).

Results

NES and NoMAC modulate steroid production by the human H295R adrenocortical carcinoma cell line

To assess whether the progestins influence the biosynthesis of endogenous adrenal steroids, the human H295R adrenocortical carcinoma cell line was treated with DMSO or 1 μM MPA, NET-A, LNG, GES, NES, NoMAC or DRSP in the absence and presence of 10 μM forskolin (FSK) for 48 hours, prior to steroid analysis by UPLC–MS/MS. FSK mimics the stimulatory effects of adrenocorticotropic hormone (ACTH) [57] which increases the basal gene expression of endogenous steroidogenic enzymes, resulting in increased steroid production [32, 54]. Indeed, treatment with FSK resulted in a 4.29-fold increase in the total amount of steroids produced by the H295R cells (Fig 3 insert; S1 Table). Interestingly, results in Fig 3 show that total steroid output was not affected by the first- (MPA and NET-A), second- (LNG) or third- (GES) generation progestins, but differentially influenced by the fourth-generation progestins. NES, but not DRSP, significantly inhibited the steroidogenic output by the H295R cells under both basal and FSK-stimulated conditions. Notably, even though NoMAC appeared to inhibit the steroidogenic output under both these conditions, output inhibition under basal conditions was not statistically significant. MTT cell viability assays revealed that the inhibitory effects observed for NES and NoMAC were not due to a decrease in cell viability (S1 Fig).

Upon closer inspection of the effects of the progestins on basal and FSK-stimulated production of steroid intermediates and end products in the steroidogenic pathway (summarised in Tables 1 and 2), it is clear that the fourth-generation progestins, NES, NoMAC and DRSP, modulate the synthesis of numerous endogenous steroids. These progestins appeared to increase the basal and FSK-stimulated concentrations of Preg, the first metabolite in the steroidogenic pathway, while in most cases the concentrations of the Δ^4 C21 steroids Prog, 17OH-Prog, 16OH-Prog, DOC, and CORT were reduced by NES and NoMAC, but not DRSP (Tables 1 and 2). DRSP increased the basal production of DOC, but had no effect on the production of steroids from the mineralocorticoid pathway in the presence of FSK. However, similarly to NES and NoMAC, DRSP inhibited the basal and FSK-stimulated production of deoxycortisol. Like NES, but unlike NoMAC, DRSP lowered the basal concentration of the glucocorticoid cortisol. Furthermore, NES and NoMAC also tended to decrease the concentrations of the Δ^4 C19 androgen precursors A4 and 11OH-A4 as well as the Δ^4 C19 androgen testosterone. Although both NES and NoMAC increased the concentration of the Δ^5 C19

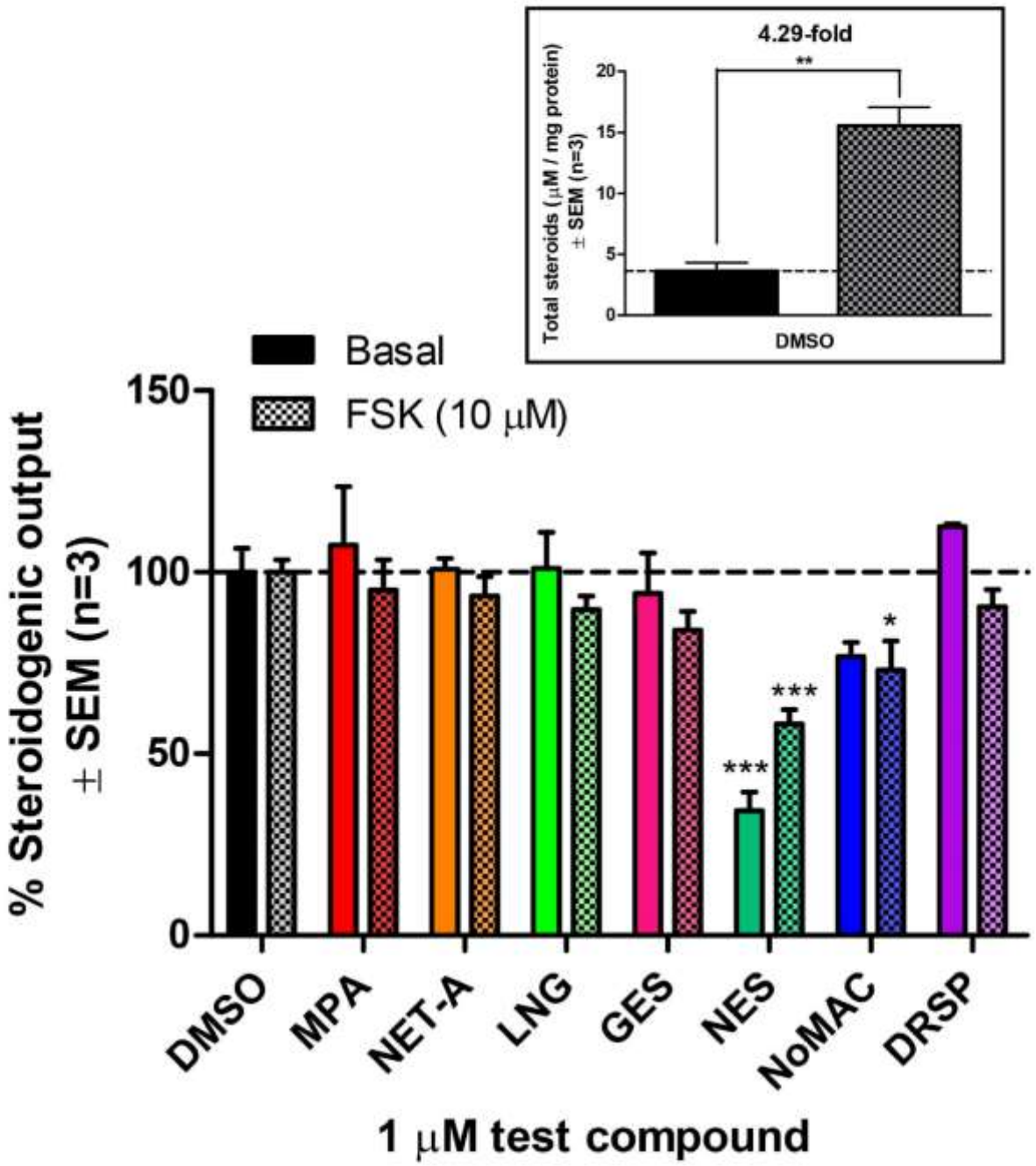

Fig 3. Effect of selected progestins on total steroid production by the human H295R adrenocortical carcinoma cell line under basal and FSK-stimulated conditions. Cells were incubated with DMSO (vehicle control) or 1 µM MPA, NET-A, LNG, GES, NES, NoMAC or DRSP, in the absence and presence of 10 µM FSK for 48 hours. Steroid metabolites were extracted from the cell culture medium and analyzed by UPLC–MS/MS. The concentrations of total steroid produced (µM) were normalized to protein concentration (mg/ml). The insert graph shows the total steroid production (µM/mg protein) in the absence of progestin treatment (DMSO) under basal and FSK-stimulated conditions. This total steroid production for both conditions was set as 100%, and the percentage change upon treatment with progestin relative to the vehicle control (DMSO) of each condition was plotted. Results shown are the average of three independent experiments with each condition performed in triplicate (± SEM).

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Table 1. Fold change in basal steroid production in response to selected progestins[a].

Steroid metabolite	MPA (1st)	NET-A (1st)	LNG (2nd)	GES (3rd)	NES (4th)	NoMAC (4th)	DRSP (4th)
Preg	-	-	-	-	↑ 2.87 ± 0.57 ***	↑ 2.17 ± 0.19 **	↑ 4.02 ± 0.33 ***
Prog	-	-	-	-	↓ 30.30 ± 0.02 ***	↓ 2.34 ± 0.12 *	↑ 11.11 ± 2.24 ***
17OH-Prog	-	-	-	-	↓ 8.33 ± 0.00 ***	-	↑ 6.32 ± 0.51 ***
16OH-Prog	-	-	-	-	↓ 14.18 ± 0.02 ***	↓ 3.41 ± 0.03 **	↑ 2.50 ± 0.68 *
DOC	-	-	-	-	↓ 7.68 ± 0.08 **	↓ 1.73 ± 0.07 ns	↑ 2.85 ± 0.30 ***
CORT	-	-	-	-	↓ 2.73 ± 0.26 *	↓ 1.43 ± 0.13 ns	↑ 1.58 ± 0.04 ns
11-DHC	-	-	-	-	↑ 2.53 ± 1.42 ns	↑ 1.49 ± 0.11 ns	↑ 2.07 ± 0.52 ns
Ald	-	-	-	-	-	-	-
Deoxycortisol	-	-	-	-	↓ 6.12 ± 0.01 ***	↓ 1.43 ± 0.07 **	↓ 1.81 ± 0.08 ***
Cortisol	-	-	-	-	↓ 2.97 ± 0.16 **	-	↓ 2.80 ± 0.05 **
Cortisone	-	-	-	-	↑ 5.33 ± 1.11 ***	-	↓ 1.82 ± 0.17 ns
DHEA	-	-	-	-	↑ 26.54 ± 5.50 ***	↑ 1.70 ± 0.30 ns	↑ 1.85 ± 0.08 ns
A4	-	-	-	-	↓ 7.12 ± 0.03 ***	-	↓ 1.75 ± 0.14 ns
11OH-A4	-	-	-	-	-	-	↓ 3.19 ± 0.19 *
Testosterone	-	-	-	-	↓ 3.77 ± 0.17 ***	↓ 1.58 ± 0.03 *	↓ 1.79 ± 0.04 *
Total steroid (μM)	-	-	-	-	**↓ 2.91 ± 0.05** **	-	-

[a]The human H295R cell line was treated with DMSO (vehicle control) or 1 μM MPA, NET-A, LNG, GES, NES, NoMAC or DRSP for 48 hours. Steroids were extracted and quantified by UPLC–MS/MS. The fold change ± SEM in response to progestin treatment relative to the vehicle control (DMSO), which was set as one, is indicated. (-) denotes no effect; 17OH-Preg, DHT, estrone and 17β-estradiol were below the limit of detection in the control samples and thus fold changes in the levels of these steroids in the presence of progestins could not be determined.
Statistically significant differences are indicated by either *, **, *** to indicate p<0.05, p<0.01 or p<0.001, respectively.

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adrenal androgen precursor DHEA under basal conditions, this increase was not significant in the case of NoMAC. Lastly, DRSP displayed similar inhibitory effects to that of NES and NoMAC on the production of the Δ^4 C19 androgen precursors and androgens. Interestingly, progestins from the first three generations had no effect on the synthesis of end products under both basal and FSK-stimulated conditions (Tables 1 and 2), but modulated the synthesis of some intermediates in the presence of FSK (Table 2). For example, MPA increased the synthesis of the androgen precursors, A4 and 11OH-A4, while GES and LNG inhibited A4 and 11-DHC, respectively.

We subsequently investigated whether NES, NoMAC and DRSP are metabolized in the H295R cell line and observed a significant reduction in the concentration of NES after the 48 hour incubation period suggesting that this progestin is metabolized by the cells. It should however be noted that more than 50% of this progestin was still unmetabolized after the incubation period. Conversely, NoMAC and DRSP were not metabolized (Fig 4). This result suggests that the observed effects in the presence of NoMAC and DRSP on steroid biosynthesis are due to the progestins themselves, while the effects observed for NES may be attibuted to NES itself and/or its metabolites.

NES and NoMAC inhibit the activity of 3βHSD2, while 3βHSD2 and CYP17A1 activities are inhibited by DRSP

Due to the modulation of steroidogenesis by NES, NoMAC and DRSP observed in the H295R cell line we next determined whether the activity of specific steroidogenic enzymes could be influenced by these three progestins. The increased production of Δ^5 steroids coupled to the decrease in Δ^4 steroid concentrations observed in most cases, suggested that the progestins

Table 2. Fold change in FSK-stimulated steroid production in response to selected progestins[b].

Steroid metabolite	MPA (1st)	NET-A (1st)	LNG (2nd)	GES (3rd)	NES (4th)	NoMAC (4th)	DRSP (4th)
Preg	-	-	-	-	↑ 3.35 ± 0.35 ***	↑ 1.47 ± 0.06 ns	↑ 1.28 ± 0.11 ns
Prog	-	-	-	-	↓ 12.55 ± 0.03 ***	↓ 1.63 ± 0.08 *	↑ 3.16 ± 0.74 ***
17OH-Prog	-	-	-	-	↓ 3.64 ± 0.03 **	-	↑ 2.35 ± 0.23 ***
16OH-Prog	-	-	-	-	↓ 35.71 ± 0.01 ***	↓ 2.28 ± 0.03 *	-
DOC	-	-	-	-	↓ 34.88 ± 0.01 ***	↓ 3.02 ± 0.02 ***	-
CORT	-	-	-	-	↓ 6.25 ± 0.03 ***	↓ 1.59 ± 0.12 ns	-
11-DHC	-	-	↓ 1.83 ± 0.13 *	-	-	-	-
Ald	-	-	-	-	↓ 2.22 ± 0.19 ns	-	-
Deoxycortisol	-	-	-	-	↓ 10.98 ± 0.02 ***	↓ 1.78 ± 0.03 *	↓ 1.44 ± 0.03 *
Cortisol	-	-	-	-	↓ 1.71 ± 0.16 ns	-	↓ 2.22 ± 0.17 ns
Cortisone	-	-	-	-	↑ 2.98 ± 0.93 *	↑ 2.08 ± 0.35 ns	↓ 1.73 ± 0.11 ns
DHEA	-	-	-	-	-	-	↓ 2.19 ± 0.13 *
A4	↑ 1.29 ± 0.08 **	-	-	↓ 1.38 ± 0.00 **	↓ 13.33 ± 0.03 ***	↓ 1.43 ± 0.06 **	↓ 1.92 ± 0.05 ***
11OH-A4	↑ 1.61 ± 0.00 ***	-	-	-	↓ 2.43 ± 0.03 ***	↑ 1.77 ± 0.10 ***	↓ 2.34± 0.04 ***
Testosterone	-	-	-	-	↓ 18.29 ± 0.04 ***	↓ 1.49 ± 0.02 *	↓ 1.65 ± 0.14 *
Total steroid (μM)	-	-	-	-	**↓ 1.72 ± 0.04 ** **	**↓ 1.37 ± 0.08 ***	-

[b]The human H295R cell line was treated with DMSO (vehicle control) or 1 μM MPA, NET-A, LNG, GES, NES, NoMAC or DRSP in the presence of FSK for 48 hours.

Steroids were extracted and quantified by UPLC–MS/MS. The fold change ± SEM in response to progestin treatment relative to the vehicle control (DMSO), which was set as one, is indicated. (-) denotes no effect; 17OH-Preg, DHT, estrone and 17β-estradiol were below the limit of detection in the control samples and thus fold changes in the levels of these steroids in the presence of progestins could not be determined.

Statistically significant differences are indicated by either *, **, *** to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

doi:10.1371/journal.pone.0164170.t002

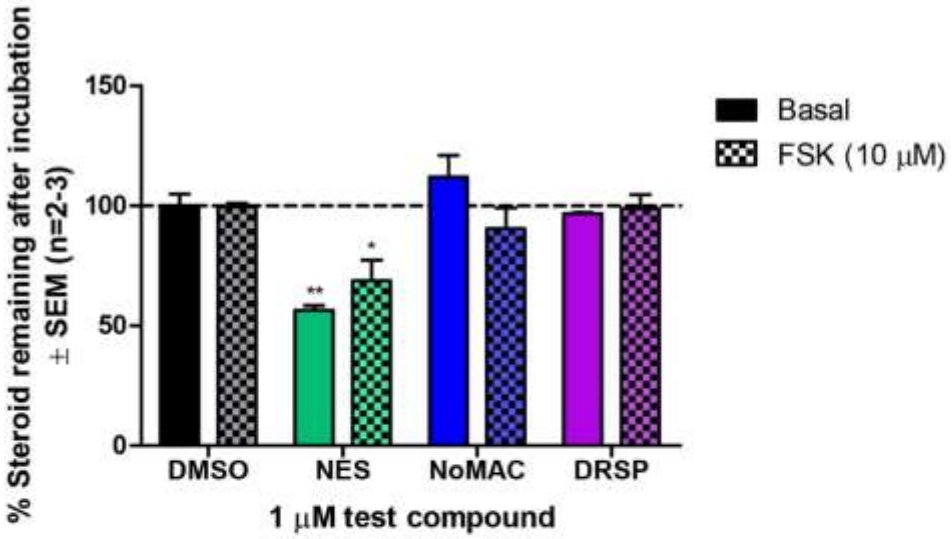

Fig 4. NES is metabolized by the H295R cells under both basal and FSK-stimulated conditions. H295R cells were treated with DMSO or 1 μM NES, NoMAC or DRSP in the absence and presence of 10 μM FSK for 48 hours. Medium containing the test compounds (no cells) was added to the wells of a 12-well plate as a negative control for metabolism. Steroids were extracted and analyzed by UPLC–MS/MS. The amount of progestin present in the medium after incubation with the cells was expressed as a % relative to the amount of progestin in the negative control for metabolism, which was set as 100%. Result shown is the average of at least two independent experiments with each condition performed in triplicate (± SEM).

doi:10.1371/journal.pone.0164170.g004

may be modulating the activity and/or expression of 3βHSD2. We also investigated the ability of the progestins to modulate the activity of CYP17A1 and cytochrome P450 21-hydroxylase (CYP21A2), for which the natural progestogen, Prog, is a substrate. Non-steroidogenic COS-1 cells were transiently transfected with the cDNA expression vectors for the human 3βHSD2, CYP17A1 and CYP21A2 enzymes, respectively, followed by treatment with the appropriate steroid substrate in the absence (DMSO) or presence of 1 μM MPA, LNG, GES, NES, NoMAC or DRSP. In addition to the fourth-generation progestins, one progestin from the earlier generations was included. Effects on the activity of 3βHSD2 was assessed using Preg as substrate (Fig 5A), while Prog was used as substrate to examine the effects on the activity of CYP17A1 (Fig 5B). Prog and 17OH-Prog were both used as substrates for investigating the effects on the activity of CYP21A2 (Fig 5C and 5D). As shown in Fig 5A, NES (97.44 ± 2.56%), NoMAC (84.52 ± 4.35%) and DRSP (79.77 ± 5.69%) significantly inhibited the activity of 3βHSD2. Interestingly, DRSP was the only progestin that inhibited the activity of CYP17A1 (55.20 ± 16.50%), while none of the progestins inhibited CYP21A2 activity (Fig 5C and 5D). None of the first-, second- or third-generation progestins affected the activity of the above-mentioned enzymes.

Having shown that NES, NoMAC and DRSP abrogate the ability of 3βHSD2 to convert Preg to Prog (Fig 5A), we next determined the K_i values of these inhibitors as well as that of the well-known 3βHSD inhibitor trilostane, serving as a positive control [58]. COS-1 cells were transiently transfected with the cDNA expression vector for the human 3βHSD2, followed by treatment with Preg in the absence or presence NES, NoMAC, DRSP or trilostane. In the absence of inhibitor, a K_m of 0.85 ± 0.05 μM and V_{max} of 31.1 ± 0.7 nmol/min/mg were obtained. The fits for all inhibitory mechanisms are shown in S2 Fig, while the results in Fig 6 show the fits with the mechanisms best describing the data. These fits resulted in a K_i value of 9.5 ± 0.96 nM for NES (with a non-competitive mechanism), 29 ± 7.1 nM for NoMAC (with a competitive mechanism), 232 ± 38 nM for DRSP (with a non-competitive mechanism) and 31.3 ± 5.5 nM for trilostane (with an uncompetitive mechanism). We subsequently used these K_i values to predict the 3βHSD2 activity when 1 μM of the inhibitor and 1 μM of the substrate are used (S3 Fig, dashed green line). We show that the residual activities predicted for NES (0.16 ± 0.03 nmol/min/mg), NoMAC (1.04 ± 0.30 nmol/min/mg) and DRSP (3.16 ± 0.87 nmol/min/mg) correlate with the experimental data (NES, 0.43 ± 0.01 nmol/min/mg; NoMAC, 2.62 ± 0.11 nmol/min/mg; DRSP, 3.39 ± 0.19 nmol/min/mg) obtained from Fig 5A, thus validating the determined K_i values.

Despite our observation that NES, NoMAC and DRSP inhibit the activity of 3βHSD2, and that DRSP also inhibits the activity of CYP17A1, it is possible that the modulation of steroidogenesis seen in H295R cells could also be due to the progestins altering the expression levels of these enzymes. We therefore used real-time qPCR to investigate the effect of NES, NoMAC and DRSP on the mRNA levels of *3βHSD2* and *CYP17A1* in H295R cells. The cells were treated with DMSO (vehicle control) or 1 μM NES, NoMAC or DRSP for 6 hours, followed by real-time qPCR analysis for the expression of *3βHSD2* and *CYP17A1*, respectively. The results in Fig 7A shows that none of the fourth-generation progestins inhibited the mRNA expression of the *3βHSD2* gene. Furthermore, our results show that neither NES nor DRSP inhibited the mRNA expression of *CYP17A1* (Fig 7B). Surprisingly, we show that NoMAC upregulated the mRNA expression of the *CYP17A1* gene.

Discussion

Progestins are classified into four generations and are widely used in endocrine therapies by pre- and post-menopausal women. To date, only a few studies have investigated the effects of

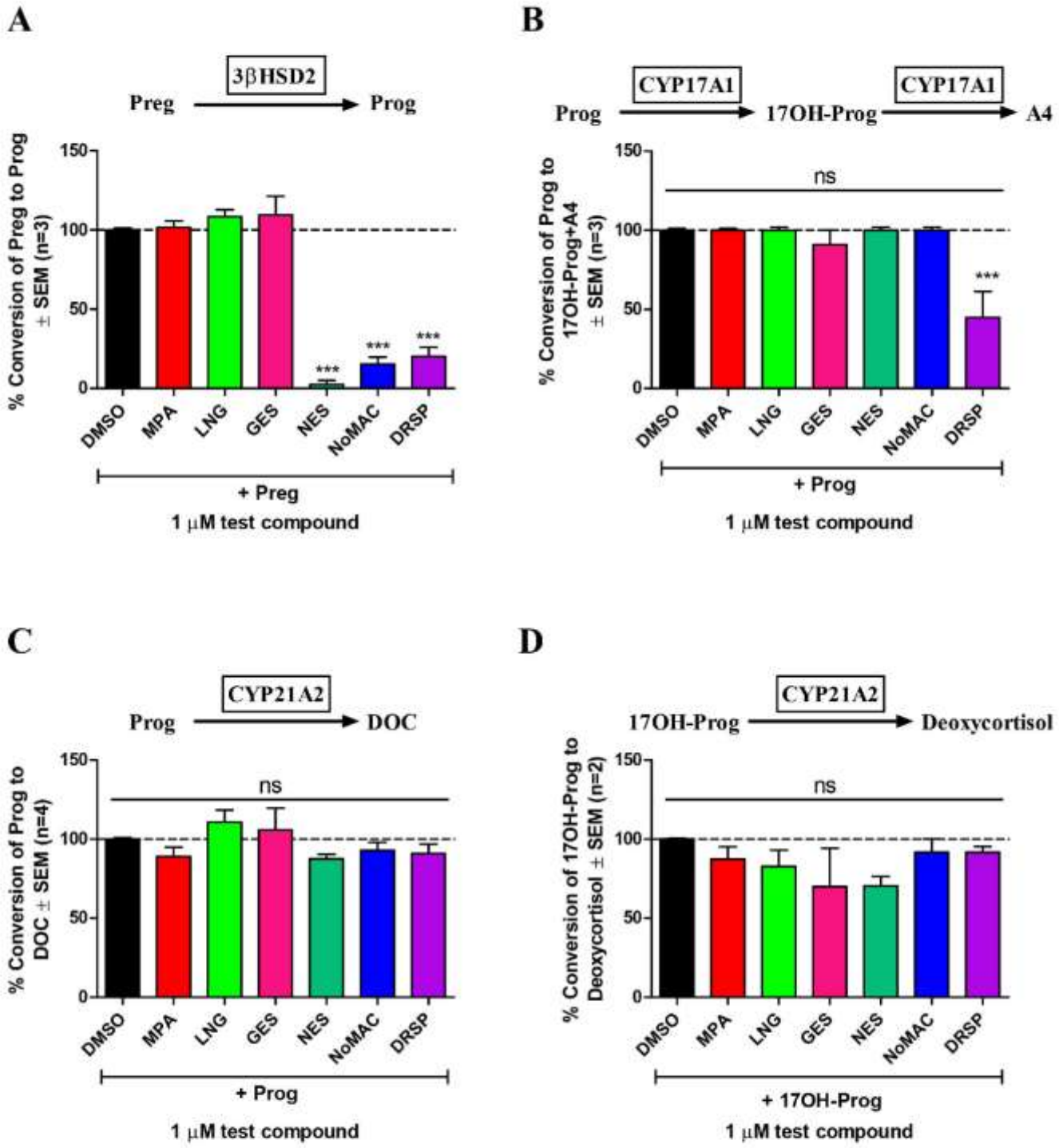

Fig 5. NES, NoMAC and DRSP inhibit the activity of human 3βHSD2, while only DRSP inhibits CYP17A1 activity. COS-1 cells were transiently transfected with plasmids expressing human (A) 3βHSD2 (pCDNA6-hHSD3β2-V5), (B) CYP17A1 (pIRES-hCYP17A1-V5-X-hCYPB5-6HIS) or (C and D) CYP21A2 (pCDNA6-hCYP21A2-V5), respectively. Cells were subsequently treated with 1 μM Preg (A) or Prog (B and C) or 17OH-Prog (D), in the absence (DMSO) and presence of 1 μM MPA, LNG, GES, NES, NoMAC or DRSP for 20 minutes (A), 4 hours (B) or 90 minutes (C and D), respectively. The steroid metabolites produced by the cells in the medium were extracted and analyzed by UPLC–MS/MS. The concentration of the steroids produced by the cells was normalized to the total protein concentration using the Bradford protein assay method. The % conversion of substrate to

product was plotted, with the substrate only response (DMSO) set as 100% and everything else relative to that. Results shown are the average of at least two independent experiments with each condition performed in triplicate (± SEM).

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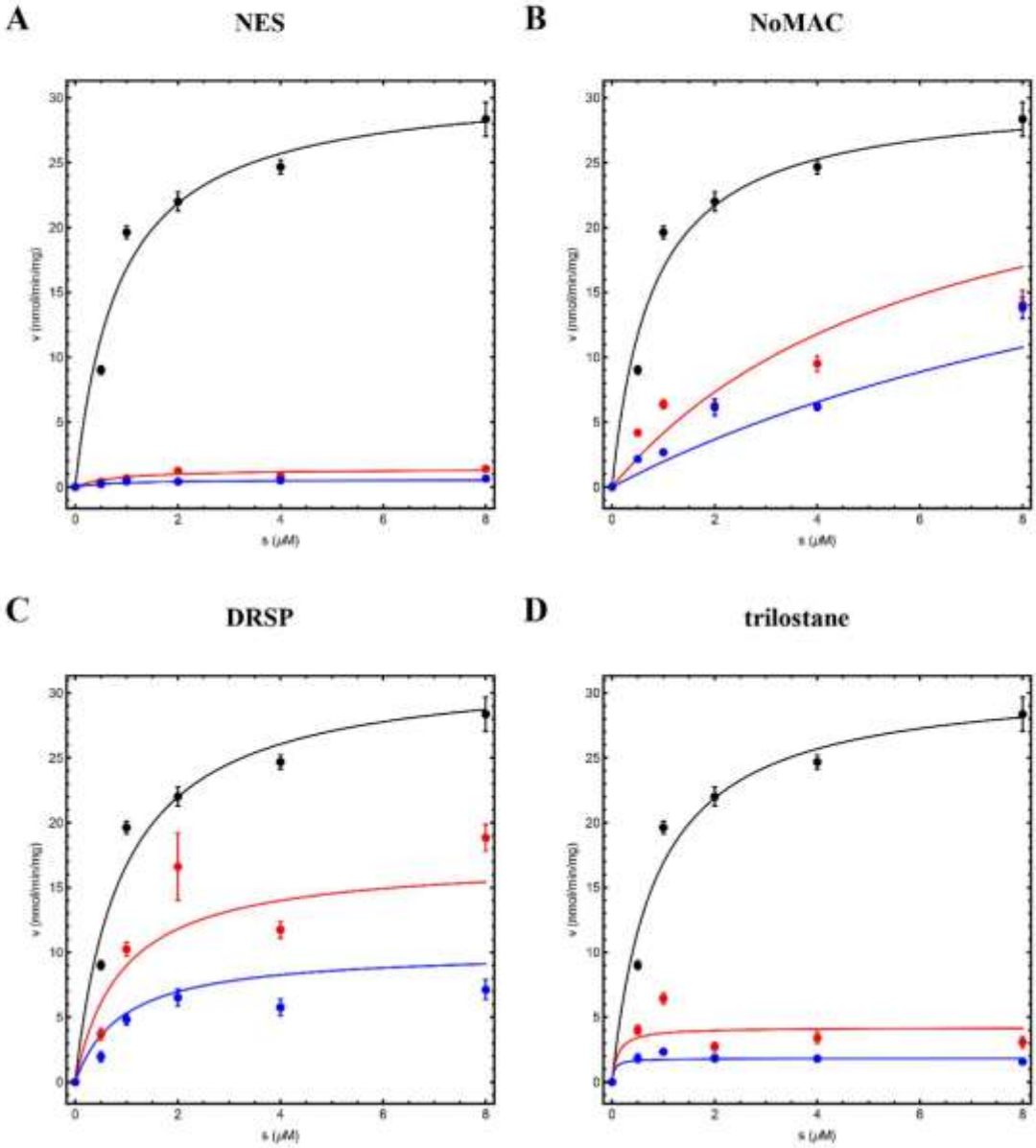
these compounds on the biosynthesis of endogenous steroids, and studies directly comparing the effects of different progestins in the same model system are lacking. To the best of our knowledge, the present study is the first to directly compare the effects of select progestins from all four generations on the production of both intermediates and end products of the steroidogenic pathway (Fig 1) in the human H295R adrenocortical carcinoma cell line. Surprisingly, although earlier studies have shown that progestins from the first-, second- and fourth-generation reduce the production of some endogenous steroids, we show that only the fourth-generation progestins NES, NoMAC and DRSP modulate the biosynthesis of endogenous steroids in H295R cells. Cell viability assays showed that these changes were not due to changes in cell viability (S1 Fig). The general trend observed was that these progestins decreased the concentrations of steroids in the glucocorticoid and androgen pathways, while the production of steroids in the progestogen and mineralocorticoid pathways were decreased by NES and NoMAC, and increased by DRSP. The observation that NES and NoMAC elicited mostly similar effects on steroidogenesis, but different to that of DRSP, may be due to the fact that NES and NoMAC are structurally similar (reviewed in [3]), while DRSP has a unique structure derived from the MR antagonist spironolactone [59, 60]. Furthermore, we found that NES, but not NoMAC and DRSP, is metabolized in the H295R cells (Fig 4), suggesting that the effects of NoMAC and DRSP are due to the progestins themselves, while the effect of NES may be due to NES itself, its metabolites or a combination thereof. The identification of the metabolites in the H295R cells was however, beyond the scope of the current study.

To understand the mechanism whereby the fourth-generation progestins modulate adrenal steroid biosynthesis, we investigated the effects of the progestins on the activity and/or expression of 3βHSD2, CYP17A1 and CYP21A2. 3βHSD2 was investigated as NES and NoMAC tended to increase the concentrations of the Δ^5 C21 steroid Preg (Tables 1 and 2) and the Δ^5 C19 steroid DHEA (Table 1), while the production of several Δ^4 C21 (Prog, 17OH-Prog, 16OH-Prog, DOC, CORT, deoxycortisol and cortisol) and Δ^4 C19 (A4, 11OH-A4 and testosterone) steroids were decreased. DRSP also increased the production of Preg and decreased the production of A4, 11OH-A4 and testosterone (Δ^4 C19 steroids), while differentially affecting the production of Δ^4 C21 steroids, suggesting inhibition of additional steroidogenic enzymes. Moreover, as progestins were designed to mimic Prog, and considering that Prog is a substrate for both CYP17A1 and CYP21A2, the possibility that progestins modulate the activities and/or expression of these enzymes could not be excluded.

In COS-1 cells transfected to constitutively express human 3βHSD2 (Fig 5A), CYP17A1 (Fig 5B) and CYP21A2 (Fig 5C and 5D), respectively, we show that NES and NoMAC had no effect on the activity of CYP17A1 or CYP21A2, but that these progestins significantly inhibited the activity of 3βHSD2. DRSP also had no effect on the activity of CYP21A2 (Fig 5C and 5D), but inhibited the activities of both 3βHSD2 (Fig 5A) and CYP17A1 (Fig 5B). The inhibition of 3βHSD2 in COS-1 cells by DRSP correlates with the observed increase in the concentration of Preg and decrease in the concentrations of the Δ^4 C19 steroids observed in the H295R cells, while the accumulation of Prog, 16OH-Prog and 17OH-Prog in the H295R cells is likely due to a bottleneck caused by the simultaneous inhibition of 3βHSD2 and CYP17A1. Discrepancies between the inhibition observed in COS-1 cells and the results observed in the H295R cells may further be explained by the once-off addition of substrate in the case of the assays performed in COS-1 cells, which is in contrast to the H295R cells which continuously produce steroids and also express multiple enzymes which may compete for binding to the same substrate.



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	Ligand				
	Preg	NES	NoMAC	DRSP	trilostane
K_i / K_m (nM) ± SEM	850 ± 50	9.5 ± 0.96	29 ± 7.1	232 ± 38	31.3 ± 5.5

Fig 6. Inhibition of 3βHSD2 activity by NES, NoMAC, DRSP and trilostane. COS-1 cells were transiently transfected with a plasmid expressing human 3βHSD2 (pCDNA6-hHSD3β2-V5), and subsequently treated with increasing concentrations (0.5, 1, 2, 4 and 8 μM) of Preg (substrate) in the presence of 0.0, 0.2 or 0.5 μM (A) NES, (B) NoMAC, (C) DRSP or (D) trilostane. The conversion of Preg to Prog was analyzed using UPLC-MS/MS. Michaelis-Menten plots are shown in the absence (black symbols and lines) and presence of 0.2 μM (red symbols and lines) and 0.5 μM (blue symbols

and lines) of NES, NoMAC, DRSP and trilostane. A K_m of 0.85 ± 0.05 μM and V_{max} of 31.1 ± 0.7 nmol/min/mg were obtained in the absence of inhibitor. Three inhibitory mechanisms were fitted (S2 Fig) and the best fit mechanism is shown. Each data point represents the mean ± SE of one experiment performed in duplicate. These results were validated by a model predicting 3βHSD2 activity in an independent experiment (S3 Fig, dashed green line).

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Notably, our real-time qPCR results show that neither NES, NoMAC nor DRSP inhibit the mRNA expression of *3βHSD2* in H295R cells (Fig 7A), and that DRSP has no significant effect on *CYP17A1* gene expression (Fig 7B). It was interesting to note that although *CYP17A1* mRNA expression was increased in the presence of NoMAC, this did not translate to an observed increase in activity. While the possibility that NES, NoMAC and DRSP modulate the protein levels of these steroidogenic enzymes cannot be excluded, our COS-1 data confirms enzyme inhibition of 3βHSD2. Subsequent kinetic studies suggest that the K_i values determined for these progestins are similar to that of the well-known 3βHSD2 inhibitor trilostane. Although the mechanism of inhibition that best fitted the data (Fig 6) suggest that trilostane, unlike NES, NoMAC and DRSP, is an uncompetitive inhibitor of 3βHSD2, it should be noted that a similar fit was also obtained with the non-competitive mechanism (S2 Fig). Trilostane has previously been reported to inhibit the activity of 3βHSD2 via a non-competitive mechanism [61]. The fitted data suggest that NES and DRSP are non-competitive inhibitors of 3βHSD2, while NoMAC is a competitive inhibitor (Fig 6). It is noteworthy that the K_i values determined for the fourth-generation progestins in this study were validated by their ability to independently predict the inhibition of 3βHSD2 activity in the presence of 1 μM substrate and inhibitor (S3 Fig).

To our knowledge, our study is the first to show that NES and/or its metabolites, NoMAC and DRSP differentially suppress adrenal steroid biosynthesis and that this inhibition in the production of steroid hormones in the H295R cells are in line with the inhibition of human

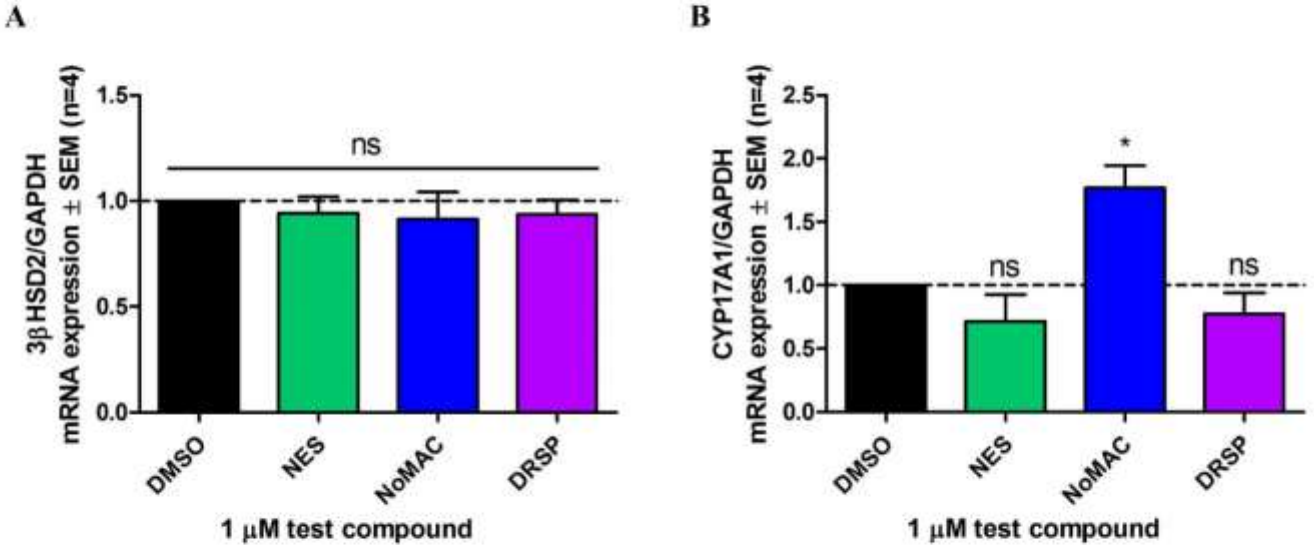

Fig 7. NES, NoMAC and DRSP do not inhibit the mRNA expression of *3βHSD2* and *CYP17A1* in the human H295R adrenocortical carcinoma cell line. The H295R cell line was incubated with DMSO (vehicle control) or 1 μM NES, NoMAC or DRSP for 6 hours. Total RNA was isolated, reversed transcribed to cDNA and real-time qPCR performed to determine the relative mRNA expression levels of (A) *3βHSD2* and (B) *CYP17A1*. *GAPDH* was used as the reference gene. Results shown are the average of four independent experiments with each condition performed in duplicate (± SEM).

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3βHSD2 activity in the COS-1 cells. Despite the fact that other studies did not investigate the effects of these fourth-generation progestins on the activity and/or mRNA expression of 3βHSD2, effects have been reported for first- and second-generation progestins [34, 37–39]. Our results are in agreement with the findings that MPA [37], as well as NET and LNG [38] have no effects on the activity of rat ovarian 3βHSD. Conversely, using a yeast expression system, Lee et al. have previously shown an inhibition of 3βHSD2 by MPA and determined a K_i of 3 μM [34]. Despite this relatively high K_i treatment of breast cancer patients with high doses of MPA (serum concentrations of 0.14–1.7 μM) have previously been shown to decrease the serum levels of cortisol, A4, DHEA-S and testosterone [24–27, 62, 63]. While we did not observe inhibition with 1 μM MPA in our test system, we show potent inhibition of 3βHSD2 by NES, NoMAC and DRSP. Considering their potent K_i values, which are in the nanomolar range and an order of magnitude lower than the K_i determined for MPA by Lee and co-workers (1999), it is likely that NES, NoMAC and DRSP modulate steroid levels *in vivo*. Furthermore, it is important to note that these validated K_i values fall within the serum ranges reported for the contraceptive usage of NES (0.086–27.3 nM), NoMAC (3–33 nM) and DRSP (26.7–253 nM), further highlighting the potential of these progestins to modulate steroid levels *in vivo* [64–71]. Indeed, results showing decreased concentrations of mineralocorticoids and glucocorticoids in the presence of NES and NoMAC, likely by the inhibition of 3βHSD2, suggest that the use of these fourth-generation progestins may be beneficial for women suffering from metabolic syndromes and/or CVDs caused by glucocorticoid and mineralocorticoid excess [21, 68–70].

Furthermore, it has previously been shown that when DRSP was combined with ethinyl estradiol in a combined oral contraceptive and administered to hyperandrogenic women diagnosed with polycystic ovary syndrome (PCOS), serum concentrations of total and free testosterone, A4 and DHEA-S were decreased [28]. Although the authors did not investigate the effect of DRSP on the activity of any steroidogenic enzyme, they suggested that the decrease may be due to inhibition of the 17α-hydroxylase and 17,20-lyase activities of CYP17A1, as they found a decease in the ratio of 17OH-Prog/Prog and A4/17OH-Prog. Our study in the COS-1 cells directly investigating the inhibition of CYP17A1, showed that DRSP does indeed inhibit the activty of this enzyme, and also the activity of 3βHSD2. While previous studies showed that MPA inhibits the 17α-hydroxylase activity of rat ovarian CYP17A1 [37], our results are in agreement with others showing that MPA has no effect on the activity of the human CYP17A1 [34]. Taken together, the inhibition of androgen production observed with the fourth-generation progestins in our study, but not earlier generation progestins like MPA, suggest that the use of the fourth-generation progestins may have better therapeutic benefits for women with hyperandrogenism associated disorders such as PCOS than the earlier generations. Although a number of different progestins are used in the treatment of PCOS, the degree of androgenicity of the progestin is an important consideration. The fact that NES, NoMAC and DRSP do not display any androgenic properties, while the selected earlier generation progestins used in this study do [3], further supports the preferential use of the fourth-generation progestins to treat PCOS.

The implications of decreased androgen production in other disorders or diseases such as breast cancer, however, are not straightforward. For example, as epidemiological and case-control studies indicate an association between elevated concentrations of androgens and increased risk of developing breast cancer [72–75], decreased androgen production may be advantageous in terms of androgen receptor (AR)-positive breast cancers. Conversely, the observed decrease of androgens may be detrimental as androgens and the AR have been proposed to have protective roles in breast cancer (reviewed in [76, 77]). This complexity is further highlighted by the fact that the use of both an androgenic progestin (MPA) and a non-

androgenic progestin (NoMAC) used in HRT were shown to be associated with an increased risk of developing breast cancer in postmenopausal women [78].

Conclusion

In summary, all three of the fourth-generation progestins investigated in this study had effects on steroidogenesis, with effects observed with NES and NoMAC being mostly similar, while those observed for DRSP often differed. The results showing that NES, NoMAC and DRSP inhibit 3βHSD2 activity, while DRSP inhibits the activities of both 3βHSD2 and CYP17A1 in the COS-1 cells, correlate to the changes observed in the biosynthesis of steroid hormones in the H295R cell line. Although the concentration (1 μM) of the progestins used in this study are supraphysiological, the K_i values determined for the inhibition of 3βHSD2 fall within the serum ranges reported for the contraceptive usage of NES, NoMAC and DRSP, supporting the likelihood that these progestins affect adrenal steroidogenesis *in vivo*. The findings of our study further highlight the fact that, although progestins are all designed to mimic the biological activity of Prog, relatively minor differences in their structures may cause profound alterations in their biochemical activity.

Supporting Information

S1 Fig. Viability of basal and forskolin (FSK)-stimulated H295R cells in the presence of different generation progestins. Cells were incubated for 48 hours with DMSO (vehicle control) or 1 μM MPA, NET-A, LNG, GES, NES, NoMAC or DRSP in the absence or presence of 10 μM FSK. Cell viability was measured using the MTT assay and results are expressed as fold proliferation relative to DMSO = 1. Results shown are the average of three independent experiments (±SEM) performed in triplicate.
(TIF)

S2 Fig. Michaelis-Menten plots of 3βHSD activity in the absence or presence of NES, NoMAC, DRSP and trilostane. COS-1 cells were transiently transfected with a plasmid expressing human 3βHSD2 (pCDNA6-hHSD3β2-V5), and subsequently treated with Preg (0.5, 1, 2, 4 and 8 μM) in the presence of 0.0, 0.2 or 0.5 μM NES, NoMAC, DRSP or trilostane. The conversion of Preg to Prog was analyzed using UPLC-MS/MS. Three inhibitory mechanisms were fitted to the data sets: competitive, non-competitive and uncompetitive, using the rate equations shown in the figure. Confidence intervals (95%) for the fits are indicated in the plots with grey fillings. Each data point represents the mean ± SE of at least duplicate experiments.
(TIF)

S3 Fig. Predicted Michaelis-Menten plots of 3βHSD2 in the presence of 1 μM NES, NoMAC and DRSP. Michaelis-Menten plots were predicted (dashed green line) based on the data presented in Fig 6. The predicted V_{max} in the presence of 1 μM NES (0.16 ± 0.03 nmol/min/mg), NoMAC (1.04 ± 0.30 nmol/min/mg) and DRSP (3.16 ± 0.87 nmol/min/mg) correlates with the residual activities determined experimentally (NES, 0.43 ± 0.01 nmol/min/mg; NoMAC, 2.62 ± 0.11 nmol/min/mg; DRSP, 3.39 ± 0.19 nmol/min/mg) as shown in Fig 5A.
(TIF)

S1 Table. Basal and FSK-stimulated production of steroid metabolites in the human adrenal H295R cell line.
(PDF)

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Conceptualization: RLdT JLS KS DA.

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Formal analysis: RLdT MP JLS KS DA.

Funding acquisition: RLdT KS DA.

Investigation: RLdT MP JLS.

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Resources: RLdT KS DA.

Visualization: RLdT MP JLS KS DA.

Writing – original draft: RLdT KS DA.

Writing – review & editing: RLdT MP JLS KS DA.

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RESEARCH ARTICLE

11-Ketotestosterone and 11-Ketodihydrotestosterone in Castration Resistant Prostate Cancer: Potent Androgens Which Can No Longer Be Ignored

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Abstract

Dihydrotestosterone (DHT) is regarded as the most potent natural androgen and is implicated in the development and progression of castration resistant prostate cancer (CRPC). Under castrate conditions, DHT is produced from the metabolism of the adrenal androgen precursors, DHEA and androstenedione. Recent studies have shown that the adrenal steroid 11β-hydroxyandrostenedione (11OHA4) serves as the precursor to the androgens 11-ketotestosterone (11KT) and 11-ketodihydrotestosterone (11KDHT). In this study we comprehensively assess the androgenic activity of 11KT and 11KDHT. This is the first study, to our knowledge, to show that 11KT and 11KDHT, like T and DHT, are potent and efficacious agonists of the human androgen receptor (AR) and induced both the expression of representative AR-regulated genes as well as cellular proliferation in the androgen dependent prostate cancer cell lines, LNCaP and VCaP. Proteomic analysis revealed that 11KDHT regulated the expression of more AR-regulated proteins than DHT in VCaP cells, while *in vitro* conversion assays showed that 11KT and 11KDHT are metabolized at a significantly lower rate in both LNCaP and VCaP cells when compared to T and DHT, respectively. Our findings show that 11KT and 11KDHT are *bona fide* androgens capable of inducing androgen-dependant gene expression and cell growth, and that these steroids have the potential to remain active longer than T and DHT due to the decreased rate at which they are metabolised. Collectively, our data demonstrates that 11KT and 11KDHT likely play a vital, but overlooked, role in the development and progression of CRPC.

Introduction

Prostate cancer (PCa) is the second most common cancer among men worldwide [1] with androgen deprivation therapy (ADT) being the first line treatment for advanced PCa since androgen signalling is essential for normal and malignant growth of prostate tissue. This

treatment, which almost entirely eliminates circulating levels of testosterone (T), is initially effective. However, most men experience only short term regression (2–3 years), with nearly all patients developing the more aggressive castration-resistant PCa (CRPC) which is associated with poor survival rates [2].

The majority of evidence suggests that CRPC develops as a result of the reactivation of androgen receptor (AR) signalling despite castrate levels of T (≤50 ng/dL) [3–5]. The AR and AR-regulated genes are expressed in most clinical cases of CRPC demonstrating that the AR axis is reactivated and drives tumour growth [4,5]. Mechanisms proposed to be responsible for the continued AR activation include up-regulation of AR expression and/or gain-of-function mutations of the AR[6].

Recent clinical trials demonstrating beneficial clinical outcomes after treatment with the AR antagonist enzalutamide [7] and the CYP17A1 inhibitor abiraterone [8–10] have highlighted the continued androgen dependency of CRPC. Studies have confirmed that the adrenal androgen precursors, dehydroepiandrosterone (DHEA) and androstenedione (A4), serve as the source of intratumoral androgen production under castrate conditions [11–15]. The potent androgen, 5α-dihydrotestosterone (DHT), is produced by the alternate 5α-dione pathway, which bypasses T, to produce DHT via 5α-androstanedione (5α-dione) [12–15].

In addition to DHEA and A4, the human adrenal gland produces substantial amounts of the inactive C19 steroid 11β-hydroxyandrostenedione (11OHA4) [16–18] via the cytochrome P450 11β-hydroxylase (CYP11B1) catalysed hydroxylation of A4 [19,20]. 11OHA4 is one of the most abundant C19 steroid produced by the human adrenal, both before and after adrenocorticotrophic hormone (ACTH) treatment [17]. A recent study by our laboratory identified a novel pathway for 11OHA4 metabolism in androgen dependent prostate cancer cells, which leads to the production of the androgens 11-ketotestosterone (11KT) and 11keto-5α-dihydrotestosterone (11KDHT) (Fig 1). We showed that at the concentration of 1 nM, 11KT and 11KDHT have androgenic properties comparable to T and DHT, respectively [21]. However, further work is needed to characterize these androgens.

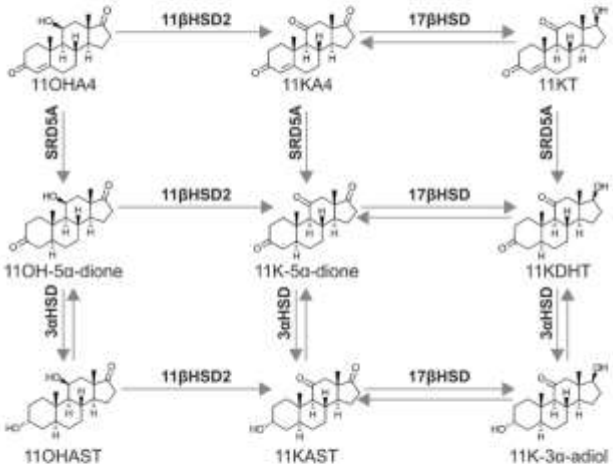

Fig 1. Biosynthesis of 11KT and 11KDHT from the adrenal androgen precursor 11OHA4. Enzymes: 11βHSD2, 11β-hydroxysteroid dehydrogenase; 17βHSD2, 17β-hydroxysteroid dehydrogenase; SRD5A1, steroid 5α-reductase type 1; 3αHSD2, 3α-hydroxysteroid dehydrogenase. Steroids: 11OHA4, 11β-hydroxyandrostenedione; 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OH-5α-dione, 11OH-5α-androstanedione; 11K-5α-dione, 11-keto-5α-androstanedione; 11KDHT, 11-ketodihydrotestosterone; 11OHAST, 11β-hydroxyandrosterone; 11KAST, 11-ketoadrenosterone; 11K-3α-adiol, 11-keto-5α-androstane-3α,17β-diol.

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The aim of this study was therefore to compare the androgenic properties of 11KT and 11KDHT to that of T and DHT. Competitive whole cell binding assays revealed that 11KT and 11KDHT bind to the human AR with affinities similar to that of T and DHT. Transactivation assays on a synthetic androgen response element (ARE) demonstrated that the relative agonist potencies and efficacies of 11KT and 11KDHT are comparable to that of T and DHT, respectively. Moreover, we showed that 11KT and 11KDHT treatment of two androgen dependent prostate cancer cell lines, LNCaP and VCaP, result in the regulation of endogenous AR-regulated genes at both the mRNA and protein level, and also drive cellular proliferation. Finally, we demonstrate that 11KT and 11KDHT are metabolised at a lower rate than T and DHT in both LNCaP and VCaP cells and as a result are likely able to exert prolonged androgenic effects. These findings confirm that both 11KT and 11KDHT are *bona fide* androgens and suggest that the 11OHA4 pathway may be a potential role player in the development and progression of CRPC.

Materials and Methods

Steroids

11KT, 11KDHT and mibolerone (Mib) were purchased from Steraloids. T and DHT were purchased from Sigma-Aldrich, while [H^3]-Mib (76.8 Ci/mmol) was purchased from Perkin Elmer. All steroids were dissolved in absolute ethanol and added to the culturing medium at a final concentration of no more than 0.1% ethanol. Cortisol-9, 11, 12, 12-d4 (cortisol-d9), testosterone-1, 2-d2 (T-d2), progesterone-2, 2, 4, 6, 6, 17α, 21, 21, 21-d9 (P4-d9) and 4-pregnen-17α-ol-3,20-dione-2, 2, 4, 6, 6, 21, 21, 21-d8 (17OHP4-d8) were purchased from Cambridge Isotope Laboratories. Gestodene (GES) and drospirenone (DRSP) were purchased from Sigma-Aldrich.

Plasmid constructs

The reporter construct for selective androgen response elements (AREs), 4xSC ARE1.2 [22] and the plasmid expressing the human androgen receptor, pSVARo [23] were obtained from Prof. F. Claessens (University of Leuven, Belgium).

Whole cell binding assays

COS-1 cells were purchased from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin. Cultures were maintained in 75 cm^2 culture flasks (Greiner Bio-One International) at 37°C, in an atmosphere of 90% humidity and 5% CO_2. Competitive whole cell binding assays were performed in COS-1 cells as previously described by Africander *et al.* [24]. Using the dissociation constant (K_d) for Mib reported in Africander *et al.* [24], the K_i values for DHT, 11KDHT, T and 11KT were determined from heterologous displacement curves using the equation by Swillens [25] which takes ligand depletion into account.

Luciferase reporter assays

COS-1 cells were seeded into 10 cm^2 dishes at a density of 2 x 10^6 cells per dish. Following a 24 hour incubation, cells were co-transfected with 9 μg luciferase reporter construct (4*xSC* ARE1.2) and 0.9 μg hAR expression construct (pSVARo) using XtremeGene HP transfection reagent (Roche). Cells were incubated for 24 hours and subsequently replated into 24-well Corning® CELLBIND® surface plates (Corning, NY, USA) at a density of 1 X 10^5 cells per

well. The following day, cells were treated with increasing concentrations of DHT, T, 11KDHT or 11KT in serum-free DMEM. Cells were lysed and analysed as previously described [26], with the exception that the protein concentration in the lysate was determined by the Pierce BCA method (Pierce Chemical).

Prostate cancer cell lines

LNCaP cells, purchased from the European Collection of Cell Cultures (ECACC), were cultured in RPMI-1640 media supplemented with 10% FCS and 1% penicillin-streptomycin and at all stages cultured using Corning® CELLBIND® surface plates. VCaP cells were purchased from the American Type Culture Collection (ATCC) and were cultured in DMEM supplemented with 10% FCS, 1% sodium pyruvate and 1% penicillin-streptomycin in 75 cm^2 culture flasks (Greiner Bio-One International). LNCaP and VCaP cells were authenticated by the ECACC and ATCC, respectively, using the PCR of short tandem repeat sequences within chromosomal microsatellite DNA (STR-PCR). Both cell lines were cultured at 37°C, in an atmosphere of 90% humidity and 5% CO_2 and were passaged for fewer than 6 months from the time of resuscitation.

RNA isolation and qPCR

LNCaP and VCaP cell lines were plated at a density of 4 X 10^5 cells per well into 12-well plates. The following day, cells were treated for 24 hours with 1 or 10 nM of the appropriate steroid in media supplemented with 10% CS-FCS. The AR inhibitor bicalutamide (1 μM) (Sigma-Aldrich) was included as a negative control. Total RNA was isolated using a Direct-zol™ RNA MiniPrep kit (Zymo Research) and cDNA subsequently synthesized using a Transcription First Strand cDNA synthesis kit (Roche). qPCR was performed using a LightCycler 96 instrument and the KAPA SYBR® FAST qPCR Master Mix for LightCycler® (KAPA Biosystems). Primer sequences were as follows, *KLK3* [27]: 5′ -AGGCCTTCCCTGTACACCAA-3′ (forward) 5′ -GTCTTGGCCTGGTCATTTCC-3′ (reverse), *FKBP5* [28]: 5′ -GAATACACCAAAG CTGTTGA-3′ (forward) and 5′ -CTCTTCCTTGGCATCCT-3′ (reverse), *TMPRSS2* [28]: 5′ -CTGCCAAGGTGCTTCTC-3′ (forward) and 5′ -TTAGCCGTCTGCCCTC-3′ (reverse). Reference genes: *PBGD* [28]: 5′ -CATGTCTGGTAACGGCAATG-3′ (forward) and 5′ GTACG AGGCTTTCAATGTTG-3′ (reverse), *ALAS* [29]: 5′ -TTCCACAGGAGCCAGCATAC-3′ (forward) and 5′ -GGACCTTGGCCTTAGCAGTT-3′ (reverse). PCR efficiency exceeded 90% for all primer sets.

Protein quantification by LC-MS

Sample preparation. VCaP cells were plated at a density of 4 X 10^6 cells per 10 cm dishes and incubated with media supplemented with 10% CS-FCS for 48 hours prior to treatment with 1 nM DHT, T, 11KDHT, 11KT or a vehicle control. After 48 hours, cells were collected, washed with PBS three times, weighed, and stored at -80°C until use.

Cells were thawed and proteins extracted using an extraction buffer containing; 100 mM NaCl, 2 mM EDTA, 6 M guanidine-HCl, 1% octylgluco-mano-pyranoside (OGP) and 5 mM tris-carboxyethyl phosphine (TCEP) in 100 mM triethylammonium bicarbonate (TEAB) (pH = 8). After centrifugation, the remaining pellet was resuspended in a second extraction buffer containing; 1% taurocholic acid and 1 M NaCl in 100 mM TEAB (pH = 8). The supernatants from each extraction were pooled and an overnight acetone precipitation was performed. Thereafter, the samples underwent centrifugation and remaining supernatants were treated with 5% phosphotungstic acid. The pellets were air dried and dissolved in 100 mM TEAB containing 4 M guanidine-HCl and 1% OGP. Protein concentrations were determined spectrophotometrically.

Samples were reduced using 50 mM TCEP in 100 mM TEAB followed by the modification of reduced cystein residues using methyl methanethiosulfonate (MMTS). Thereafter, the samples were diluted with 100 mM TEAB and proteins digested using trypsin. After being dried and resuspended in 2% acetonitrile containing 0.1% formic acid, the samples were desalted using C18 stage tips.

LC-MS. Liquid chromatography was performed using a Thermo Scientific Ultimate 3000 RSLC equipped with a C18 trap column and a C18 analytical column. Samples were analysed using a Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer equipped with a Nanospray Flex ionization source. The raw files generated were imported into Proteome Discoverer v1.4 (Thermo Scientific) and processed using the Mascot and SequestHT algorithms. Peptide validation was performed using the percolator node set to search against a decoy database with a strict false discovery rate (FDR) of 1%. Additional analyses were performed using the X! Tandem Sledgehammer algorithm. Output files from the three algorithms were combined and analysed using Scaffold (Proteomesoftware).

Proliferation assays

LNCaP cells were plated at 2 X 10^4 cells per well in Corning® CELLBIND® 96-well surface plates, while VCaP cells were plated at 4 X 10^4 cells per well in 96-well plates (Greiner Bio-One International). The cells were incubated for 72 h after which the media was replaced with media supplemented with 10% CS-FCS and the cells incubated for a further 24 h. Steroids were subsequently added to obtain final concentrations of 0.1, 1 and 10 nM. LNCaP cells were incubated for 7 days following the addition of steroid, while VCaP cells were incubated for 10 days. Cell growth was subsequently assessed using a resazurin assay [30,31].

Steroid metabolite analysis

Sample preparation. LNCaP and VCaP cells were plated at 2 X 10^5 cells per well in 12-well Corning® CellBIND® surface plates. After 24 h, the media was replaced with media supplemented with 10% CS-FCS and incubated for an additional 24 h. Cells were treated with 100 nM (DHT, 11KDHT) or 10 nM (T, 11KT) as well as a vehicle control and 1 ml aliquots taken at specific time intervals (6, 12, 24, 48 and 72 h). An internal standard mix containing 15 ng cortisol-d4, 1.5 ng T-d2, 15 ng 17OHP4-d9, 15 ng P4-d9, 12.4 ng GES and 14.7 ng DRSP were added to samples prior to extraction. Samples were subsequently extracted with 3 ml methyl tertiary butyl ether (MTBE). After vortexing for 10 min and centrifugation for 5 min, the aqueous layer was frozen at -80°C and the organic layer transferred to a clean test tube. Steroids were dried under a stream of nitrogen and resuspended in 50% methanol prior to analysis by UPC^2- MS/MS.

UPC^2-MS/MS. Steroids were analysed by ultra-performance convergence chromatography-tandem mass spectrometry (UPC^2-MS/MS). Steroid metabolites were separated using an Acquity UPC^2 system (Waters Corporation, Milford, USA) with an Acquity UPC^2 BEH 2-EP column (3 mm X 100 mm, 1.7 μm particle size). The mobile phase consisted of liquid CO_2 modified with methanol. Separation was achieved using a 4 minute linear gradient from 2% to 9.5% methanol at a constant flow rate of 2.0 $mL.min^{-1}$. The column temperature and automated back pressure regulator (ABPR) were set to 60°C and 2000 psi, respectively. The steroids were quantified using a Waters Xevo triple quadrupole mass spectrometer (Waters, Milford, USA). Steroids were measured in multiple reaction monitoring (MRM) mode using electrospray in the positive ionization mode (ESI+). Calibration curves were constructed by using weighted (1/x2) linear least squares regression. Data was collected with MassLynx (version 4.1) software (Waters, Milford, USA).

Statistical analysis

The Graph Pad Prism® software (Version 6) was used for data manipulations, graphical representations and statistical analysis. Non-linear regression and one site competition were used in whole cell binding assays, while non-linear regression and sigmoidal dose response were used in transactivation experiments. The effect of steroid treatment on mRNA expression and cellular proliferation were analysed using a Student's t tests and one-way ANOVA followed by Dunnett's multiple comparisons test, respectively. All steroid treatments were compared to the vehicle control. Statistically significant differences are indicated by *, ** or *** for $p<0.05$, $p<0.01$ or $p<0.001$, respectively. The effect of steroid treatment on protein expression was analysed in Scaffold (Proteomesoftware) using a Student's t test. Statistically significant differences are indicated by * for $p<0.05$.

Results

11KT and 11KDHT bind to the AR with affinities similar to that of T and DHT

The results indicate that 11KT (K_i = 80.8 nM) and 11KDHT (K_i = 20.4 nM) bind to the human AR with affinities similar to that of T (K_i = 34.3 nM) and DHT (K_i = 22.7 nM) (Fig 2A and 2D). The affinity of these ligands for the AR was approximately 100-fold lower than that of the synthetic androgen, Mib (K_d = 0.38 nM).

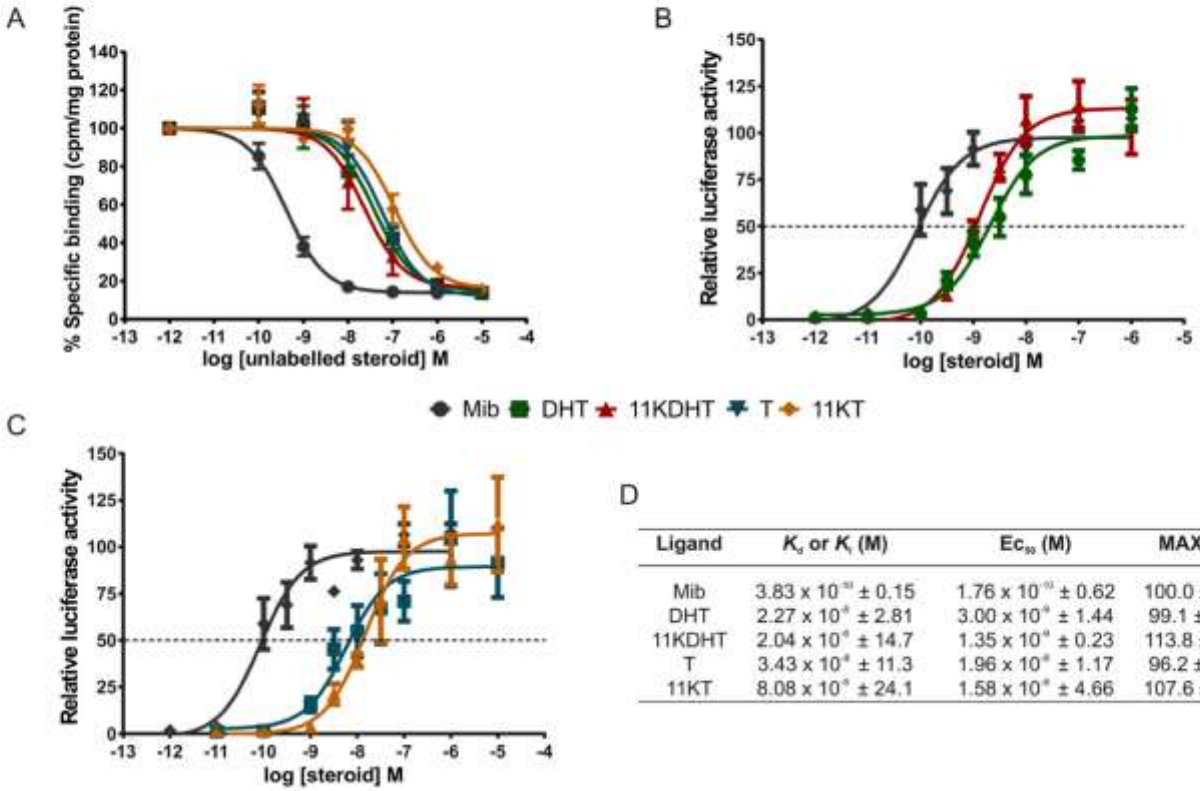
11KT and 11KDHT are full AR agonists

In light of the observation that 11KT and 11KDHT bind to the AR with affinities similar to that of T and DHT, and our previous finding that 11KT and 11KDHT act as AR agonists at a physiologically relevant concentration of 1 nM [21], we set out to determine the relative agonist potency and efficacy of 11KT and 11KDHT for transactivation. Using COS-1 cells transiently transfected with a human AR expression vector and a selective-AR androgen response element (ARE) driven luciferase reporter construct, we show that 11KT and 11KDHT display similar maximal induction ($p > 0.05$) as Mib and DHT, confirming that they are both full AR agonists (Fig 2). Of note, the potency determined for 11KDHT (1.3 nM) was statistically equal to that of DHT (3.0 nM) which is considered to be the most potent natural androgen (Fig 2C and 2D). Moreover, the efficacies of 11KDHT (113.84%) and DHT (99.14%) were not statistically different. Similarly potency and efficacy of 11KT (15.8 nM; 107.59) were not statistically different to that of T (19.6 nM; 96.21) (Fig 2B and 2D).

11KT and 11KDHT induce AR-regulated gene expression

mRNA expression. In order to investigate if 11KT and 11KDHT demonstrate androgenic activity on endogenous AR-regulated genes, the mRNA expression levels of *KLK3* (NM_001030047), *FKBP5* (NM_001145775) and *TMPRSS2* (NM_001135099) were assessed in two androgen dependent prostate cancer cell lines, LNCaP and VCaP. LNCaP cells express a mutated AR (T877A) [32], while VCaP cells express the wild type AR. A twenty-four hour time point was chosen as significant upregulation of *KLK3* and *TMPRSS2* has previously been demonstrated at this time point in both LNCaP and VCaP cells [33,34].

Treatment with 1 or 10 nM 11KDHT resulted in the significant upregulation of *KLK3*, *TMPRSS2* and *FKBP5* in both LNCaP (Fig 3) and VCaP (Fig 4) cells, with the exception of *KLK3* at 1 nM in LNCaP cells ($p = 0.0529$) (Fig 3). Consistent with a previous study [28], we show that all three endogenous AR-regulated genes were significantly upregulated by DHT in both cell lines, with the exception of *FKBP5* at 1 nM in LNCaP cells ($p = 0.0579$) (Fig 3). Interestingly,



D

Ligand	K_d or K_i (M)	Ec_{50} (M)	MAX (%)
Mib	$3.83 \times 10^{-10} \pm 0.15$	$1.76 \times 10^{-10} \pm 0.62$	100.0 ± 0.00
DHT	$2.27 \times 10^{-9} \pm 2.81$	$3.00 \times 10^{-9} \pm 1.44$	99.1 ± 0.16
11KDHT	$2.04 \times 10^{-9} \pm 14.7$	$1.35 \times 10^{-9} \pm 0.23$	113.8 ± 12.8
T	$3.43 \times 10^{-9} \pm 11.3$	$1.96 \times 10^{-9} \pm 1.17$	96.2 ± 20.9
11KT	$8.08 \times 10^{-9} \pm 24.1$	$1.58 \times 10^{-9} \pm 4.66$	107.6 ± 20.0

Fig 2. Binding of DHT, T, 11KDHT and 11KT to the human AR (A) and transactivation via an ARE (B and C). Binding affinities, agonist potencies and efficacies of DHT, 11KDHT, T and 11KT relative to the synthetic AR agonist mibolerone are summarised in (D). Whole cell binding assays (A) were conducted in COS-1 cells transiently transfected with pSVARo. Cells were incubated with 0.2 nM [^{3}H]-Mib in the absence and presence of increasing concentrations of either unlabelled Mib, DHT, 11KDHT, T and 11KT for 16 hours. Results are plotted as % specific binding where the total specific binding of [^{3}H]-Mib only is set to 100% and binding of unlabelled steroid is set as a % binding relative to that. Whole cell binding results are shown as means ± SEM of three independent experiments performed in triplicate. Transactivation assays (B and C) where performed in COS-1 cells transiently transfected with the pSVARo expression vector and the 4xSC ARE1.2-luc reporter. Agonist activity was measured by incubating cells in the presence of increasing concentrations of either Mib, DHT, T, 11KDHT or 11KT for 24 h. Induction is shown as % luciferase activity expressed in relative light units (rlu's), with the maximal response of Mib (10^{-5} M) set to 100%. Luciferase assays are shown as means ± SEM of six independent experiments performed in quadruplicate.

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treatment with T did not upregulate the mRNA expression of either *KLK3*, *TMPRSS2* or *FKBP5* in LNCaP cells at either test concentration, but significantly upregulated their expression at both test concentrations in VCaP cells (Fig 4). In contrast, 11KT treatment resulted in the upregulation of all of the AR-regulated genes in LNCaP cells at both 1 and 10 nM.

While 1 nM 11KT only significantly upregulated the expression of *KLK3* in VCaP cells, 10 nM 11KT upregulated all three AR-regulated genes significantly. This data confirms that both 11KDHT and 11KT are androgenic and are able to regulate the mRNA expression of endogenous AR-regulated genes. We confirmed that any observed changes in gene expression were due to AR activation by including a control for each test compound in the presence of the competitive AR inhibitor, bicalutamide. No significant changes in gene expression were observed upon steroid treatment in these samples with the exception of *KLK3* in LNCaP cells treated with 10 nM 11KDHT (Figs 3 and 4). Finally, it is worth noting that in LNCaP cells the induction of *KLK3*, *TMPRSS2* and *FKBP5* expression by 11KDHT and 11KT tended to result in a greater fold induction than that observed for DHT or T, respectively (Fig 3). The differences

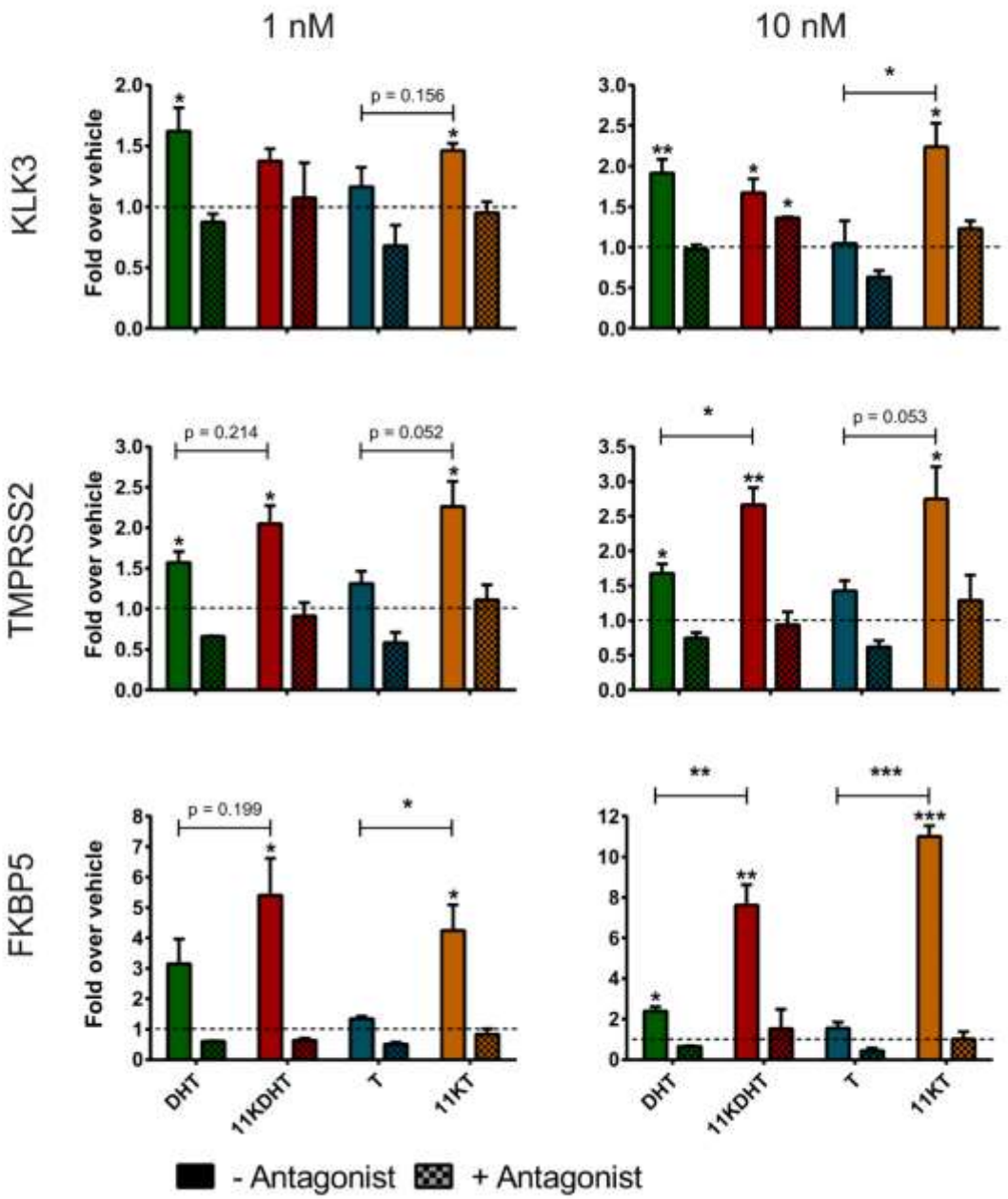

Fig 3. Induction of AR-regulated gene expression in LNCaP cells by DHT, 11KDHT and 11KT. Cells were incubated with CS-FCS-supplemented media for 24 hours prior to treatment with 1 or 10 nM steroid for an additional 24 hours prior to analysis by qPCR. Gene expression was calculated relative to the geometric mean of the reference genes *ALAS* and *PBGD*. Fold change over vehicle was calculated using the method described by Pfaffl et al [48]. Results are shown as means ± SEM of three independent experiments performed in triplicate. Data from individual experiments was all normalized using log transformation and mean-centering prior to analysis [49].

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between 10 nM DHT and 11KDHT were statistically significant for both *TMPRSS2* and *FKBP5*. Interestingly, differences between T and 11KT induced expression were statistically significant for *KLK3* (10 nM treatment) and *FKBP5* (1 and 10 nM treatments), and approached significance for *TMPRSS2* (p = 0.052, 1 nM treatment; p = 0.053, 10 nM treatment).

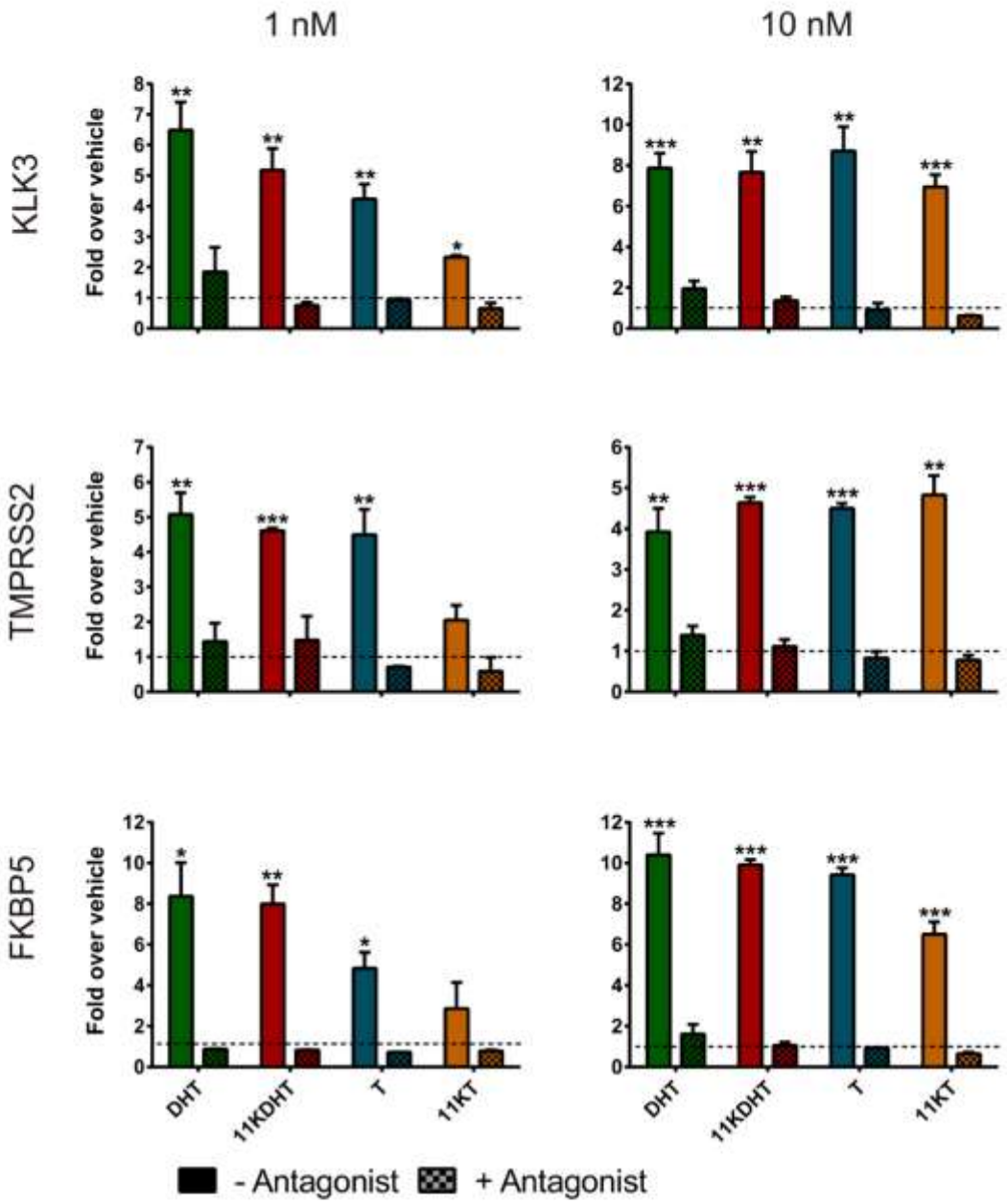

Fig 4. Induction of AR-regulated gene expression in VCaP cells by DHT, 11KDHT, T and 11KT. Cells were incubated with CS-FCS supplemented media for 24 hours prior to treatment with 1 or 10 nM steroid for an additional 24 hours prior to analysis by qPCR. Gene expression was calculated relative to the geometric mean of the reference genes *ALAS* and *PBGD* and are expressed as the fold change over the vehicle control. Results are shown as means ± SEM of three independent experiments performed in triplicate. Data from individual experiments was all normalized using log transformation and mean-centering prior to analysis [49].

doi:10.1371/journal.pone.0159867.g004

Protein expression. The qPCR results described above showed that a robust response to androgen treatment was achieved in VCaP cells. Considering that VCaP cells are an accepted cell model for CRPC [35], while LNCaP cells are not [36], we selected VCaP cells for proteomic analysis. Cells were treated with 1 nM DHT, 11KDHT, T or 11KT, proteins were extracted and

analysed using LC-MS. A total of 1439 proteins were identified which were common to all treatments. We subsequently determined the fold change in protein expression (relative to the vehicle control) for twenty-seven known AR-regulated proteins (Table 1). Of these, the expression of twenty-two selected proteins (ABCE1, ACACA, ACLY, ACSL3, BCAM, COPA, CPT1A, DCXR, FKBP5, GSK3A, IDI1, MCM7, NAMPT, PDIA3, PDIA4, PSA, PSMD2, PSMD3, RAB7A, RDH11, RPN1 and UGDH) are known to be upregulated by androgens. The number of these proteins which were significantly upregulated by steroid treatment were 3/22 for DHT, 13/22 for 11KDHT, 19/22 for T and 17/22 for 11KT. Notably, FKBP5 was significantly upregulated by DHT (2.5-fold), 11KDHT (4.1-fold) and T (3.4-fold), but not 11KT. This result correlates with the qPCR data reported above (Fig 4). The remaining five proteins (AIM1, HNRPL, LIMA1, NONO and TOP1) are known to be downregulated by androgens. Four out of five of these proteins were downregulated by DHT, 5/5 by 11KDHT, 4/5 by T and 3/5 by 11KT.

Table 1. Regulation of AR-regulated proteins by DHT, 11KDHT, T and 11KT in VCaP cells. Cells were incubated with CS-FCS supplemented media for 48 hours prior to treatment with 1 nM steroid. Proteins were subsequently identified using mass spectrometry. Fold changes were calculated relative to the vehicle control. Statistically significant changes are indicated (P<0.05). Results are representative of three independent experiments.

ID	Protein	Fold change				References
		DHT	11K DHT	T	11KT	
Upregulated by androgens						
ABCE1	ATP-binding cassette sub-family E member 1	2.5	3.1	4.8*	2.8*	[50]
ACACA	Acetyl-CoA carboxylase 1	1.6	2.2	2.8*	2.0*	[50]
ACLY	ATP-citrate synthase	2.5	3.7*	4.6*	4.4*	[50]
ACSL3	Long-chain-fatty-acid—CoA ligase 3	2.0	5.4*	4.0*	2.3*	[50]
BCAM	Basal cell adhesion molecule	1.1	2.3*	2.4*	1.6*	[50]
COPA	Coatomer subunit alpha	1.4	1.8	2.4*	1.9*	[51]
CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform	2.0	4.2*	11*	7.5*	[52]
DCXR	L-xylulose reductase	1.3	1.4	2.1*	1.7*	[51]
FKBP5	Peptidyl-prolyl cis-trans isomerase	2.5*	4.1*	3.4*	1.7	[50]
GSK3A	Glycogen synthase kinase-3 alpha	10*	3.7	_	13*	[50]
IDI1	Isopentenyl-diphosphate Delta-isomerase 1	2.5	4.2	6.8*	6.6*	[50]
MCM7	DNA replication licensing factor MCM7	0.8	2*	2.0*	2.2*	[50]
NAMPT	Nicotinamide phosphoribosyltransferase	2.5	2.5*	2.7*	2.3*	[50]
PDIA3	Protein disulfide-isomerase A3	1.0	1.4*	1.0	1.0	[50]
PDIA4	Protein disulfide-isomerase A4	1.1	1.5*	1.2	1.0	[50]
PSA	Puromycin-sensitive aminopeptidase	1.4	2.5*	3.8*	3.1*	[53]
PSMD2	26S proteasome non-ATPase regulatory subunit 2	2*	1.8	1.9*	1.5*	[54]
PSMD3	26S proteasome non-ATPase regulatory subunit 3	1.1	1.3	1.8*	1.6*	[51]
RAB7A	Ras-related protein Rab-7a	1.1	1.9*	1.9*	1.2	[50]
RDH11	Retinol dehydrogenase 11	3.3	6.2*	5.5*	2.9*	[50]
RPN1	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 1	1.3	2.5*	2.2*	1.6*	[50]
UGDH	UDP-glucose 6-dehydrogenase	1.4	1.3	1.6*	1.2	[53]
Downregulated by androgens						
AIM1	Absent in melanoma 1 protein	0.3*	0.4*	0.6	0.8	[50]
HNRPL	Heterogeneous nuclear ribonucleoprotein L	0.6*	0.6*	0.7*	_	[51]
LIMA1	LIM domain and actin-binding protein 1	0.5	0.4*	0.4*	0.5	[50]
NONO	Non-POU domain-containing octamer-binding protein	0.8*	_	0.8*	0.8*	[51]
TOP1	DNA topoisomerase 1	0.6*	_	0.6*	_	[51]

*p<0.05

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11KT and 11KDHT induce androgen dependent cellular proliferation

In order to assess the contribution of the novel steroids to the promotion of cell growth, the LNCaP and VCaP cell lines were treated with 0.1, 1 or 10 nM DHT, 11KDHT, T or 11KT (Fig 5). At 0.1 nM, all test steroids significantly induced LNCaP cell growth (DHT, 1.4-fold; 11KDHT, 1.6-fold; T, 1.7-fold and 11KT, 2.0-fold). At 1 and 10 nM, however, DHT and T no longer induced significant growth. This finding was not unexpected since androgen-induced cell growth is biphasic in LNCaP cells [37,38]. In contrast, both 11KDHT and 11KT induced significant cell proliferation, at both 1 nM (11KDHT, 1.8-fold; 11KT, 1.8-fold) and 10 nM (11KDHT, 2.2-fold; 11KT, 3.0-fold). In VCaP cells, all test steroids resulted in a significant increase in cellular proliferation at both 0.1 nM (DHT, 1.4-fold; 11KDHT, 1.5-fold; T, 1.5-fold; 11KT, 1.5-fold) and 1 nM (DHT, 2.1-fold; 11KDHT, 2.0-fold; T, 1.7-fold; 11KT, 1.9-fold). At 10 nM all steroid treatments appeared to stimulate cell growth, though this was only found to be significant for T and 11KT (T, 1.8-fold; 11KT, 1.8-fold).

11KT and 11KDHT are metabolised slower that T and DHT, respectively

In order to elucidate the potential mechanism by which 11KDHT and 11KT induce greater fold change in endogenous AR-regulated gene expression than DHT and T, respectively, we investigated the rate at which these steroids are metabolised by LNCaP and VCaP cells. Androgen inactivation is achieved by metabolism via 3α-hydroxysteroid dehydrogenases (3αHSD), producing inactive steroids, and/or glucuronidation catalysed by uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes [39]. LNCaP cells are known to express high levels of UGTs, while VCaP cells express lower levels of these enzymes [40,41]. UPC^2-MS/MS was employed to measure the metabolism of 10 nM T and 11KT, and 100 nM DHT and 11KDHT. A 10-fold

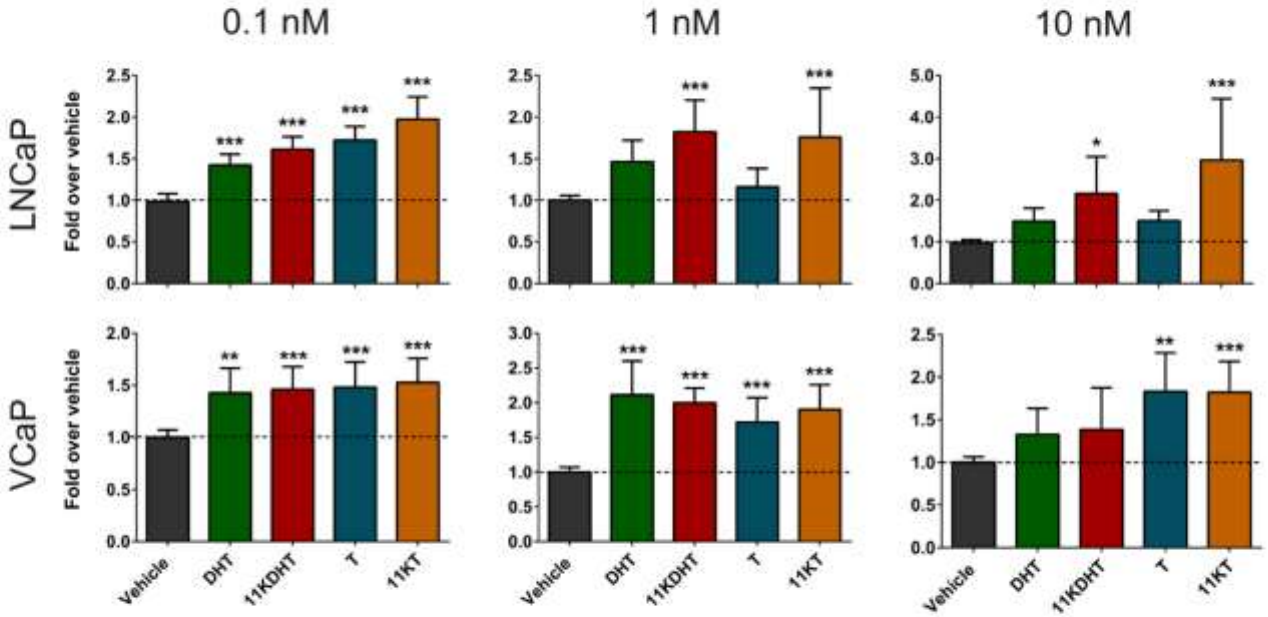

Fig 5. Induction of cell proliferation in LNCaP and VCaP cells by DHT, 11KDHT, T and 11KT. Cells were incubated with media supplemented with CS-FCS for 24 hours prior to treatment with 0.1, 1 or 10 nM steroids. Resazurin assays were carried out on day 7 (LNCaP) or day 10 (VCaP) after treatment. Results are shown as means ± SEM of three independent experiments with eight replicates each.

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higher concentration of DHT and 11KDHT were chosen due to the poor ionisation of these steroids during analysis.

The results indicated a significant difference in the metabolism of 11KDHT and 11KT when compared to equivalent amounts of DHT and T, respectively (Fig 6). In LNCaP, 84% of the DHT substrate is metabolised in 6 hours, while only 42% of the 11KDHT is metabolised during the same period. Similarly 90% of the T is metabolised in 12 hours, while only 37% of the 11KT is metabolised.

A slower metabolic rate was observed in the VCaP cells. For example, DHT and T were depleted in 24 hours in LNCaP cells, while the complete metabolism of DHT and T by VCaP cells was only achieved after 48 hours. Nevertheless, the same trend was observed between the respective steroids. DHT was metabolised significantly faster (63% in 12 hours) than the same concentration of 11KDHT (17% in 12 hours). While DHT was fully metabolised after 48 hours, 21% of the 11KDHT remained detectable after 72 hours. The difference between T and 11KT metabolism in VCaP cells was also significant with only 30% of the 11KT being metabolised in 24 hours, while 81% of the T had been metabolised. T was depleted after 48 hours, while 42% of the 11KT remained after 72 hours.

Discussion

CRPC is generally considered an androgen dependent condition [7–10,42]. There is overwhelming evidence indicating that the disease is able to survive castrate levels of T partly due

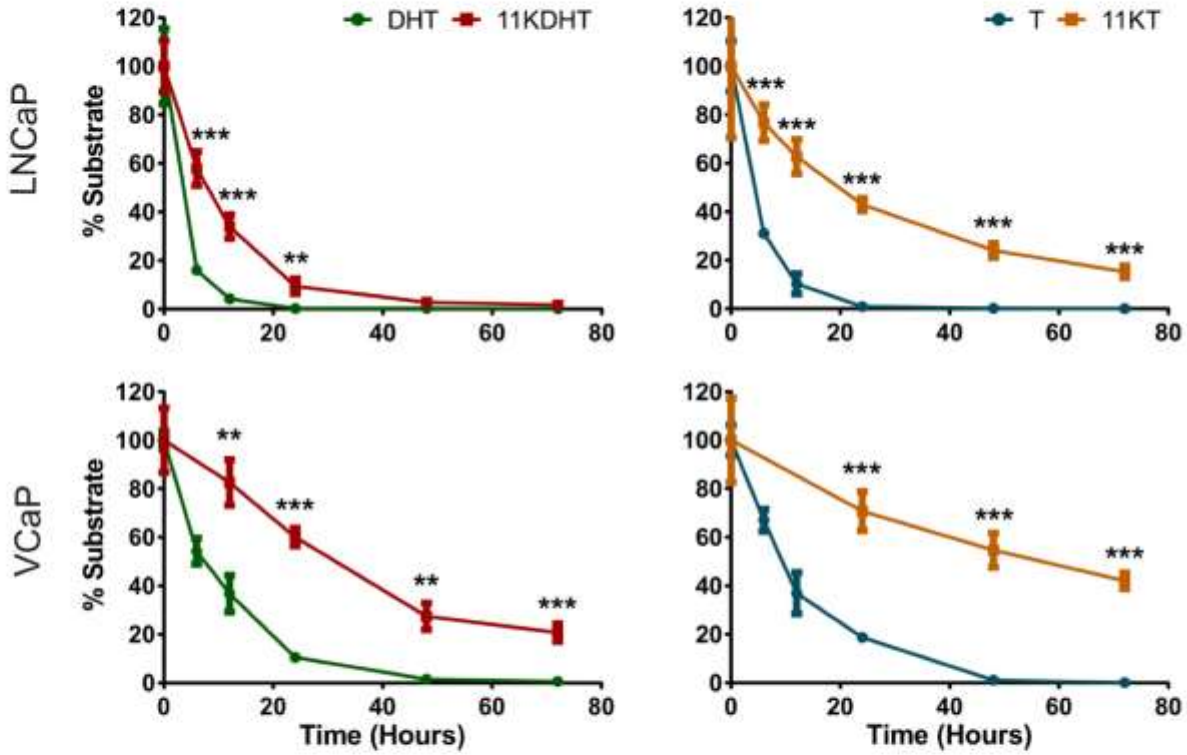

Fig 6. Metabolism of DHT, 11KDHT, T and 11KT by LNCaP and VCaP cells. Steroids were analysed by ultra-performance convergence chromatography-mass spectrometry (UPC^2-MS/MS). Results are representative of two independent experiments performed in triplicate.

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to the availability of the adrenal androgen precursors DHEA and A4, which function as the substrates for intratumoral DHT production via the alternate 5α-dione pathway [12–15,43]. While the contribution of this pathway to the pool of active androgens is undoubtedly significant, the contribution of androgens from alternate sources, such as 11OHA4, cannot be ignored, especially considering that the human adrenal produces significantly more 11OHA4 than A4 [17]. The aim of this study was therefore to determine how the androgenic activity of the 11OHA4 metabolites, 11KT and 11KDHT, compare to that of the established androgens, T and DHT.

We first established the apparent K_i values of these steroids for the human AR, as well as their relative potencies and efficacies for transactivation via an ARE. Data showed that these steroids bind to the same site as that of Mib, and that 11KDHT binds to the AR with a similar affinity to that of T and DHT. While it appeared that the affinity of 11KT for the receptor was lower than that of 11KDHT, the difference was not statistically significant (Fig 2A and 2D). When comparing the potency and efficacy of these steroids using an AR-selective ARE, we demonstrated that both 11KT and 11KDHT are potent, efficacious AR agonists. The potency and efficacies of 11KT were comparable to that of T (Fig 2B and 2D) as has also previously been suggested [17,44]. To date, this is the first study to report the potency and efficacy of 11KDHT, and we furthermore showed that 11KDHT and DHT, the most potent natural androgen in mammals, are equipotent on the synthetic ARE-containing promoter used in this study (Fig 2C and 2D). These findings showing that DHT and T are not the only potent natural androgens, have significant implications for androgen dependent cancers such as CRPC. We therefore investigated whether the observed androgenic activity translated into the ability of these steroids to regulate endogenous AR-regulated gene expression and cell growth in the androgen dependent prostate cancer cell lines LNCaP and VCaP.

The results showing that 11KDHT and 11KT upregulated the expression of the endogenous AR-regulated genes, *KLK3*, *TMPRSS2* and *FKBP5* (Figs 3 and 4), and induced cell growth (Fig 5) in both the androgen-dependent LNCaP and VCaP cells confirm the status of 11KT and 11KDHT as *bone fide* androgens. The inclusion of the AR antagonist, bicalutamide, confirmed that the upregulation of *KLK3*, *TMPRSS2* and *FKBP5* gene expression by these steroids was AR dependent in both cell lines. Interestingly, the induction of *KLK3*, *TMPRSS2* and *FKBP5* gene expression by 11KT and 11KDHT in LNCaP cells was greater than that observed for T and DHT, respectively (Fig 3), highlighting the previously undetermined ability of these steroids to drive endogenous AR-regulated gene expression in mammalian cell lines. Similarly 11KT and 11KDHT induced significant cell growth in LNCaP cells at concentrations of 1 and 10 nM, while T and DHT failed to induce growth at the same concentrations (Fig 5). Although the induction of LNCaP cell growth by DHT and the synthetic androgen R1881 has previously been shown to be biphasic [37,38], a similar trend was not observed for 11KT and 11KDHTand thus requires further investigation in future.

We hypothesised that differences in the expression of endogenous AR-regulated genes, as observed in LNCaP cells (Fig 3), may in part be due to differences in the rates at which these androgens are metabolised. Active androgens, such as DHT and T, are inactivated by glucuronosyltransferase (UGT) catalysed glucuronidation, thereby blunting androgen signalling [45]. In addition, 5α-reduced steroids, such as DHT, can be inactivated by the action of 3α-hydroxysteroid dehydrogenases (3αHSD) prior to glucuronidation [18]. While T and DHT are metabolised by UGTs and 3αHSDs the efficiency with which 11KT and 11KDHT can be metabolised was uncertain. We therefore measured the decrease in 11KT and 11KDHT concentrations over time in both LNCaP and VCaP cells, and show that T and DHT were metabolised by both cell lines at a significantly higher rate than 11KT and 11KDHT, respectively (Fig 6). In addition, the rate of metabolism was significantly higher in LNCaP cells for all the steroids.

This result correlates with the higher levels of UGT1A, UGT2B15 and UGT2B17 expressed in LNCaP cells as compared to VCaP cells [40,41]. The reduced rate of metabolism observed for 11KT and 11KDHT may allow these steroids to activate the androgen axis for a longer period than T and DHT, resulting in the increased expression of endogenous AR-regulated genes observed in the LNCaP cells (Fig 3).

The same trend was not, however, observed in VCaP cells when considering AR-regulated gene expression. Overall the upregulation of AR-regulated gene expression was greater in VCaP cells than LNCaP cells, which is not unexpected given both the lower rate of metabolism (Fig 6), due to reduced UGT expression [40,41], and the amplification of the AR gene in VCaP cells [46]. Proteomic analysis of AR-regulated protein expression did, however, reveal significant differences between DHT and 11KDHT treated cells. DHT exposure resulted in the significant regulation of only 7 out of the 27 AR-regulated proteins included in this study, while 11KDHT significantly regulated 18 of these proteins (Table 1). Since DHT and 11KDHT are equipotent and equally efficacious on a synthetic promoter (Fig 2C and 2D), the data suggests that the difference in protein expression may at least in part be due to differences in the rate of metabolism during the 48 hour induction period employed prior to proteomic analysis. T and 11KT resulted in the regulation of 23 and 20, proteins, respectively (Table 1). T and 11KT were also the only steroids to induce significant cell growth in VCaP cells at a concentration of 10 nM (Fig 5). While T and 11KT can be inactivated by glucuronidation, these steroids also serve as substrates for steroid 5α-reductase (SRD5A1), resulting in the production of the potent androgens DHT and 11KDHT. Like T and 11KT, the metabolic fate of DHT and 11KDHT is two-fold, however, in the case of DHT and 11KDHT both routes result in inactivation. These steroids are either glucuronidated directly or converted to the inactive metabolites 5α-androstane-3α,17β-diol (3α-adiol) and 11-keto-5α-androstane-3α,17β-diol (11K-3α-adiol) by 3αHSDs prior to glucuronidation [18].

In addition to the likely role played by 11KT and 11KDHT in CRPC, 11KT has recently been implicated in the androgen excess associated with classic 21-hydroxylase deficiency (21OHD). The levels of 11KT and other 11-oxygenated steroids were shown to be elevated significantly (3–4 fold) in patients with classic 21OHD when compared to healthy age matched controls. This finding, together with the observation that the routinely measured androgens, A4 and T (in woman), do not correlate well with the clinical evidence of androgen excess in 21OHD patients, led Turcu et al. [47] to propose that 11KT may be responsible for the androgen mediated effects associated with this condition.

Conclusion

This study provides comprehensive evidence that 11KT and 11KDHT are potent and efficacious AR agonists, capable of driving gene regulation, protein expression and cell growth in androgen-dependent prostate cancer cells. The most novel and significant finding is that DHT and 11KDHT are equipotent and are equally efficacious, which highlights the fact that DHT may not be the only potent natural androgen. Differences in the rate at which these androgens are metabolised, with 11KT and 11KDHT being metabolised at a significantly lower rate than T and DHT respectively, have significant implications for androgen-dependent conditions such as CRPC. These findings highlight that not only can 11KT and 11KDHT activate the androgen axis, and in so doing drive cell growth, these steroids have the potential to remain active longer than T and DHT. Taking only intratumoral levels of DHT and T into account, as is currently the case in therapeutic approaches, could therefore lead to a substantial underestimation of AR activation in CRPC. Future studies should therefore focus on determining the

physiological levels of 11KT and 11KDHT and assessing their contribution to CRPC as well as conditions resulting in androgen excess such as 21OHD.

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Author Contributions

Conceived and designed the experiments: EP DJA MV KS. Performed the experiments: EP MV MSP JQ. Analyzed the data: EP DJA MV MSP JQ KS. Contributed reagents/materials/analysis tools: DJA MV KS. Wrote the paper: EP DJA MSP KS.

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