Progestins and breast cancer: Significance of progesterone receptor isoforms and their altered ratios

by

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Declaration

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Abstract

Progestins used in menopausal hormonal therapy have been associated with increased incidence of breast cancer. While these synthetic ligands were designed, in four consecutive generations, to mimic the activity of natural progesterone (P_4) via the progesterone receptor (PR), the precise mechanism whereby some progestins and/or their metabolites may cause an increase in breast cancer incidence is still mostly unknown. Whether the PR, existing as two isoforms, PR-A and PR-B, plays a role in mediating the effects of progestins on breast cancer is unclear. As the metabolism of a progestin can ultimately influence effects via the PR, ultra-high performance supercritical fluid chromatography-tandem mass spectrometry was used to investigate the metabolism of P_4 and selected progestins in three breast cancer cell lines in the first part of this thesis. Unlike P_4 that was rapidly metabolised in all three cell lines, promegestone (R5020), gestodene (GES) and nomegestrol acetate (NOMAC) were not metabolised, while only drospirenone (DRSP) was metabolised in the MDA-MB-231 and T47D cells. Additionally, we showed that P_4 metabolism occurred at a similar rate in the MDA-MB-231 and T47D cells, but faster than its metabolism in the MCF-7 BUS cells. In the second part of this study, transactivation and transrepression transcriptional assays showed that the activities of a selected panel of progestins from all four generations are not all similar to each other, P_4 or R5020, via PR-A and PR-B. For transactivation, most progestins were more efficacious via PR-B, but more potent via PR-A. We also showed that an increase in PR-A density and excess PR-A relative to PR-B, resulted in decreased efficacies of all progestins for transactivation. While an increase in PR-A density resulted in an increase in the activity of all progestins for transrepression, the activity of only a few progestins were influenced by excess expression of PR-A relative to PR-B. Realtime PCR showed progestin- and gene-specific regulation of endogenous genes known to play a role in breast cancer in T47D breast cancer cells. While the response of some progestins on the selected genes were PR-B mediated, some progestin effects were not mediated by either PR-A or PR-B. In the third part of this thesis, investigations into the effects of the progestins on proliferation, apoptosis, anchorage-independent growth, migration and invasion showed that these processes are differentially influenced by P_4 and the selected progestins, and that the responses are also differentially mediated by PR-A or PR-B. Excess expression of PR-A resulted in both positive and/or negative ligand-independent, as well as progestin-induced, effects on these cancer hallmarks. Taken together, the findings of this thesis emphasize the fact that progestins do not always mimic the activities of P_4 or each other. The results further highlight the complexity of progestin action via the PR, underscoring the importance of distinguishing progestin activities via PR-A and PR-B, and also considering the PR-A:PR-B ratio when investigating the mechanisms of progestins and the PR in breast cancer. Finally, our results suggest that a progestin such as medroxyprogesterone acetate (MPA) acting via PR-A and/or PR-B may indeed increase breast cancer risk, while others like DRSP may not.

Opsomming

Progestiene wat in menopausale hormoonterapie gebruik word, word geassosieer met 'n verhoogde risiko van borskanker. Alhoewel dié sintetiese ligande, wat in vier opeenvolgende generasies ontwerp is om die aktiwiteit van natuurlike progesteroon (P_4) via die progesteroonreseptor (PR) na te boots, is die presiese meganisme waardeur sommige progestiene en/of hul metaboliete 'n toename in borskanker kan veroorsaak nog meestal onbekend. Dit is egter onduidelik of die PR, wat bestaan uit twee isovorme, PR-A en PR-B, 'n rol speel in die bemiddeling van die effekte van progestiene op borskanker. Aangesien die metabolisme van 'n progestien ook effekte via die PR kan beïnvloed, was superkritiese vloeistofchromatografie en tandem massaspektrometrie in die eerste deel van hierdie proefskrif gebruik om die metabolisme van P_4 en geselekteerde progestiene in drie borskanker-sellyne te ondersoek. In teenstelling met P_4 wat vinnig in al drie sellyne gemetaboliseer is, is promegestoon (R5020), gestodene (GES) en nomegestrol asetaat (NOMAC) nie gemetaboliseer nie, terwyl slegs drospirenoon (DRSP) in die MDA-MB-231- en T47D-selle gemetaboliseer is. Verder het ons getoon dat die metabolisme van P_4 teen 'n soortgelyke tempo in beide MDA-MB-231- en T47D-selle plaasgevind het, maar vinniger as die metabolisme daarvan in die MCF-7 BUS-selle. In die tweede deel van hierdie studie het transkripsie-toetse vir transaktivering en transonderdrukking getoon dat die aktiwiteit van 'n geselekteerde paneel progestiene uit al vier generasies, verskillend is van mekaar, P_4 en R5020, via PR-A en PR-B. Die transaktivering van meeste progestiene was meer doeltreffend via PR-B, maar meer potent via PR-A. Ons het ook getoon dat 'n toename in PR-A digtheid en 'n oormaat PR-A in verhouding tot PR-B, gelei het tot verminderde doeltreffentheid van alle progestiene vir transaktivering. Terwyl 'n toename in PR-A digtheid gelei het tot 'n toename in die aktiwiteit van alle progestiene vir transonderdrukking, is die aktiwiteit van slegs enkele progestiene beïnvloed deur oormatige uitdrukking van PR-A relatief tot PR-B. Deur gebruik te maak van intydse PKR het ons getoon dat die regulering van endogeniese gene, wat 'n rol in borskanker speel, progestien- en geenspesifiek is in die T47D-borskankerselle,. Alhoewel die reaksie van sommige progestiene op die geselekteerde gene deur PR-B bemiddel is, word sommige progestien-effekte nie deur PR-A óf PR-B bemiddel nie. In die derde deel van hierdie proefskrif het ondersoeke na die effekte van progestiene op proliferasie, apoptose, anker-onafhanklike groei, migrasie en indringing getoon dat hierdie prosesse differensieel deur P_4 en die geselekteerde progestiene beïnvloed word, en dat die reaksies ook differensieel deur PR-A of PR-B bemiddel word. Oormatige uitdrukking van PR-A het gelei tot beide positiewe en/of negatiewe ligandonafhanklike, sowel as progestien-geïnduseerde, effekte op hierdie kanker kenmerke. In samevatting, die bevindings van hierdie proefskrif beklemtoon die feit dat progestiene nie altyd die aktiwiteite van P_4 of van mekaar naboots nie. Die resultate dui verder daarop dat progestien-aksie via die PR kompleks is, en beklemtoon die feit dat daar van progestien-aktiwiteite via PR-A en PR-B onderskei moet word, asook dat die PR-A:PR-B verhouding in ag geneem moet word wanneer progestien meganismes en die PR in borskanker ondersoek word. Ten slotte dui ons resultate daarop dat 'n progestien soos medroksieprogesteroonasetaat (MPA) wat via PR-A en/of PR-B optree, wel die risiko van borskanker kan verhoog, maar nie ander soos DRSP nie.

I would like to dedicate this thesis to Dr. Egon Vogt, the man who started it all,

and to the ladies that helped finish it, Ulla Pickard and Annette Cartwright…

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Alphabetical List of Abbreviations

$3\alpha HP_4$	3α-dihydroprogesterone
$3\alpha HSD2$	3α-hydroxysteroid dehydrogenase type 2
$5\alpha P_4$	5α-progesterone
$17\alpha OHP_4$	17-alpha hydroxyprogesterone
$17\beta HSD5$	17β-hydroxysteroid dehydrogenase type 5
$20\alpha HP_4$	3α-dihydroprogesterone
$20\alpha HSD$	20α-hydroxysteroid dehydrogenase
AF-1	activation factor-1
AF-2	activation factor-2
AF-3	activation factor-3
AKR1C1	20α-hydroxysteroid dehydrogenase
AKR1C2	3α-hydroxysteroid dehydrogenase type 2
AKR1C3	17β-hydroxysteroid dehydrogenase type 5
AP-1	activator protein-1
ANOVA	analysis of the variance
AR	androgen receptor
BAX	BCL-2-like protein 4
BCL-2	B-cell lymphoma 2
BEH	Bridged Ethylene Hybrid
C/EBPα	CAAT enhancer binding protein alpha

C/EBPβ	CAAT enhancer binding protein beta
CEE	conjugated equine estrogen
ChIP	chromatin immunoprecipitation
CMA	chlormadinone acetate
CPA	cyproterone acetate
CS	charcoal-stripped
CSC	cancer stem cell
C-terminal	carboxy-(COOH-) terminal
Ct	cycle threshold
CCND1	cyclin D1
CVD	cardiovascular disease
CYP3A4	cytochrome P450 3A4
DBD	DNA binding domain
DEPC	diethyl pyrocarbonate
DMEM	Dulbeco's Modified Eagle's Medium
DMPA	depot medroxyprogesterone acetate
DMSO	dimethyl sulfoxide
DRSP	drospirenone
E_2	17β-estradiol
EC_{50}	Effective concentration 50
ECL	enhanced chemiluminescence

EDTA	ethylenediaminetetra-acetic acid
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ESI	electrospray ionisation
ETG	etonogestrel
FAK	focal adhesion kinase
FBS	fetal bovine serum
FOXA1	Forkhead box A1
FOXO1	Forkhead box protein O1
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA-binding protein 3
GC/MS	gas chromatography and mass spectrophotometry
GES	gestodene
GR	glucocorticoid receptor
GRB2	growth factor receptor-bound protein 2
HCl	hydrochloric acid
hPR-A	human progesterone receptor isoform A
hPR-B	human progesterone receptor isoform B

HRP	horseradish peroxidase
ID	inhibitory domain
IRS2	insulin receptor substrate-2
K	rate constant
Ki67	antigen Ki-67
KLF5	krueppel-like factor 5
LBD	ligand binding domain
LH	luteinizing hormone
LNG	levonorgestrel
Luc	luciferase
MAPK	mitogen-activated protein kinase
MHT	menopausal hormone therapy
MLL2	trithorax-group histone methyl transferase
MPA	medroxyprogesterone acetate
mPR	membrane PR
MR	mineralocorticoid receptor
MRM	multiple reaction monitoring
MTBE	methyl tert-butyl ether
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MS/MS	tandem mass spectrometry
MWS	million women study

NES	nestorone
NET	norethisterone
NET-A	norethisterone-acetate
NET-EN	norethisterone-enanthate
NFκB	nuclear factor kappa B
NOMAC	nomegestrol acetate
NRIP1	nuclear receptor interacting protein 1
NSC	non-silencing control
NTD	amino-(N)-terminal domain
Org2058	16α-ethyl-21-hydroxy-19-norprogesterone
P$_4$	progesterone
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol myristate acetate
PPD	polyproline domain
PR	progesterone receptor
PR-A	progesterone receptor isoform A
PR-B	progesterone receptor isoform B
PR-C	progesterone receptor isoform C

PRE(s)	progesterone response element(s)
qPCR	quantitative polymerase chain reaction
RLU	relative light units
R5020	promegestone
RU468	mifepristone
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
siRNA	short interfering ribonucleic acid
SMRT2	nuclear receptor co-repressor 2
SOX4	Sex-determining region Y-related high mobility group box 4
Sp1	Specific protein 1
SRD5A	5α-reductase
SRC-1	steroid receptor co-activator-1
SRC-2	steroid receptor co-activator-2
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifiers
t½	half-life
TAE	Tris-acetate-EDTA
TAT	tyrosine amino transferase
TBS	Tris buffered saline
TBST	Tris buffered saline Tween-20

TGFβ1	transforming growth factor beta 1
UHPSFC	ultra-high performance supercritical fluid chromatography
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
UT	untransfected
VEGF	vascular endothelial growth factor
WHI	Women's Health Initiative
WISDOM	Women's international study of long-duration estrogen after menopause
ZK98299	onapristone

Thesis Outline

This thesis comprises of five chapters and four addendums. Chapter 1 provides the background to the study, while Chapter 5 is a concluding discussion. Chapters 2, 3 and 4 are the results chapters and contain a brief introduction of the particular study, the study aims, materials and methods used, while reporting and discussing the results obtained. References for all the chapters are provided in one section following Chapter 5. Although the collective terms "we" and "our" are used in this dissertation, all experiments and data analyses were performed by the candidate.

Chapter 1: Literature Review. This chapter provides an overview of the current literature on progestins and the progesterone receptor isoforms, PR-A and PR-B, with specific emphasis on their roles in breast cancer.

Chapter 2: Investigating progestogen metabolism in breast cancer cell lines. This chapter contains the results of a study investigating the metabolism of P_4 and the progestins R5020, GES, NOMAC and DRSP in the MDA-MB-231, T47D and MCF-7 BUS breast cancer cell lines, as well as the rate of P_4 metabolism across these three cell lines. The candidate also investigated the metabolism of MPA, NET, LNG and NES in these breast cancer cell lines which were published in 2019 (Addendum A).

Chapter 3: Evaluating the role of the progesterone receptor isoforms and ratios on the regulation of gene expression by progestogens. This chapter includes the results of a study directly comparing the activity of P_4 and a selected panel of progestins from all four generations via human PR-A and PR-B exogenously expressed in the MDA-MB-231 cell line, for both transactivation and transrepression. Additionally, this chapter reports on the findings of a study comparing the effects of these ligands on the expression of endogenous genes in the T47D

breast cancer cell line endogenously expressing PR-A and PR-B. Also included are the results of investigations into the influence of PR-A density and excess PR-A expression relative to PR-B on gene expression by the progestogens

Chapter 4: A direct comparison of progesterone and progestin effects on hallmarks of breast cancer and the influence of the progesterone receptor isoform ratios. This chapter explored the effects of P_4 and the selected progestins on selected hallmarks of cancer, specifically cell proliferation, apoptosis, anchorage-independent growth, migration and invasion. All the investigations were conducted in the MDA-MB-231 breast cancer cell line exogenously expressing either PR-A or PR-B, or different ratios of PR-A:PR-B. Proliferation and apoptosis experiments were also performed in the T47D breast cancer cell line endogenously expressing both isoforms, in the absence and presence of exogenously expressed PR-A.

Chapter 5: Concluding Discussion and Future Prospectives. In this final chapter, the results of this study are summarised and conclusions are drawn from the findings presented in this thesis. This chapter also outlines perspectives for future investigations.

Addendum A contains publications that the candidate contributed to and lists local and international conference outputs.

Addendums B, C and D includes supplementary results which are not included but referred to in Chapters 2, 3 and 4, respectively.

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Chapter 1

Literature Review

1.1. Introduction

Progestogens are ligands that bind to and activate the progesterone receptor (PR) and include the endogenous ovarian sex steroid, progesterone (P_4), as well as progestins (synthetic progestogens) (Stanczyk *et al.*, 2013). Progestins are used in a number of clinical applications, but most often in contraception and menopausal hormone therapy (MHT). Alarmingly, a number of clinical and epidemiological studies have shown that the use of some progestins in MHT is associated with an increased risk of invasive breast cancer (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; Chlebowski *et al.*, 2013; Manson *et al.*, 2013; Cancer, 2019). This has caused much confusion amongst clinicians and menopausal women, resulting in a decline in the use of MHT [(Crawford *et al.*, 2019); reviewed in (Chlebowski *et al.*, 2020)]. Although MHT is often considered as a generic treatment, these preparations can differ in the dosage, route of administration, as well as the estrogen and progestogen component [reviewed in (Perkins *et al.*, 2018)]. The details of the preparations, particularly that of the "much blamed" progestins, are critical given that progestins differ in their structures and possibly functions, and that only a few progestins have been evaluated for breast cancer risk. Thus, while some progestins may increase breast cancer risk, it is plausible that others may not.

The mechanism whereby some progestins evidently increase breast cancer risk is still largely unknown. Given that progestins are PR ligands and that the PR may also be involved in breast cancer pathogenesis, it is likely that a mechanism requiring the PR may be involved. The role of the PR is, however, not that straightforward as some studies implicate the PR in breast cancer development and progression (Hopp *et al.*, 2004; McGowan *et al.*, 2004; Pathiraja *et al.*, 2011; Giulianelli *et al.*, 2012; Daniel *et al.*, 2015; McFall *et al.*, 2015, 2018; Singhal *et al.*, 2018; Lamb *et al.*, 2018; Truong *et al.*, 2019; Horwitz and Sartorius, 2020; Rosati *et al.*, 2020;

Sathyamoorthy and Lange, 2020), while other studies show that the PR may have an inhibitory effect on breast cancer cell proliferation when co-expressed with either the estrogen receptor (ER) alpha (ERα) (Mohammed *et al.*, 2015), or the glucocorticoid receptor (GR) (Ogara *et al.*, 2019). An added complexity in the understanding of PR mechanisms in breast cancer, is the fact that it exists as two structurally distinct, functional isoforms, PR-A and PR-B (Kastner *et al.*, 1990). Notably, studies investigating the role of the PR in breast cancer often do not distinguish between the PR isoforms. Differentiating between the two isoforms is important as they are known to elicit both similar and different activities (Conneely *et al.*, 2000, 2003; Richer *et al.*, 2002; Faivre and Lange, 2007; Leo and Lin, 2008; Bellance *et al.*, 2013; Brisken, 2013; McFall *et al.*, 2015; Diep *et al.*, 2016a; Singhal *et al.*, 2018; Truong *et al.*, 2019). The PR isoforms are proposed to regulate the expression of genes either as a monomer, PR-A/PR-A and PR-B/PR-B homodimers, or as PR-A/PR-B heterodimers. The majority of PR-regulated genes are regulated specifically by PR-B, while PR-A regulates only a small portion of unique genes. Furthermore, PR-A and PR-B also similarly regulate a distinct set of genes, which is also influenced by the PR-A:PR-B ratio [(Graham *et al.*, 2005; Singhal *et al.*, 2018) reviewed in (Jacobsen and Horwitz, 2012)]. In breast cancer, the expression levels of PR-A and PR-B are often disrupted, with PR-A generally expressed at a higher level than PR-B (Graham *et al.*, 1995, 2005; Graham and Clarke, 2002; Mote *et al.*, 2002, 2015; Hopp *et al.*, 2004; Rojas *et al.*, 2017; Lamb *et al.*, 2018; McFall *et al.*, 2018; Singhal *et al.*, 2018). The resulting effects of this disruption on PR signalling mechanisms, and whether it will influence the activity of different PR ligands such as progestins, is mostly unexplored. It is probable that the response of breast cancer cells to progestins will be determined by a number of factors, including the ratio of PR-A to PR-B, monomer or dimerization state of these isoforms as well as the structure of the progestin. The aim of this review is to reveal the known differences between selected progestins used in MHT, as well as any reported activities in terms of breast cancer, with a particular focus

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on what has been reported for some progestins in terms of their action via the individual PR isoforms, as well as the function of PR-A and PR-B and the consequence of their dysregulated ratios in breast cancer.

1.2. Progestogens

1.2.1. Classification

Progestogens are compounds with progestational activity, which refers to the fact these compounds cause the transformation of a proliferative endometrium to a secretory endometrium in preparation for pregnancy (Stanczyk *et al.*, 2013). The term progestogen describes both natural P_4 and synthetic progestogens (progestins). Although it is appreciated by some that there are differences between P_4 and the progestins (Carroll *et al.*, 2017; Africander and Louw-du Toit, 2020; Horwitz and Sartorius, 2020), the terms progestogen and progestin are often still used interchangeably. Progestins are synthetic ligands designed to mimic the activity of the naturally occurring hormone P_4. Given that progestins are man-made ligands and that some often display effects opposite to P_4, progestins should not be referred to as P_4. Similarly, P_4 should not be referred to as a natural progestin, as a progestin by definition is synthetic. We will thus refer to a progestogen when discussing both natural P_4 and progestins (synthetic), and progestins only when referring to synthetic PR ligands.

1.2.2. Progesterone (P_4)

P_4 is synthesized and secreted mainly by the ovaries of pre-menopausal women, but also the placenta, during pregnancy, as well as the adrenal glands and adipose tissue in post-menopausal women (Norman and Litwack, 1987; Graham and Clarke, 1997; Payne and Hales, 2004; Tuckey, 2005; Miller and Auchus, 2011; Schumacher *et al.*, 2012; Capper *et al.*, 2016). It plays a vital role in normal mammary gland development, sexual differentiation, menstruation,

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maintenance of pregnancy and sexual behaviour, as well as having non-reproductive functions in the brain, bone, cardiovascular and nervous systems (Graham and Clarke, 1997; Geller *et al.*, 1999; Li *et al.*, 2004; Scarpin *et al.*, 2009; Acharya *et al.*, 2017). During the follicular phase of the menstrual cycle, P_4 levels are low and increase after ovulation in preparation for pregnancy, stimulating the proliferative endometrium to become a secretory endometrium (luteal phase) that aids in implantation of a follicle [reviewed in (Graham and Clarke, 1997)]. Lack of follicle implantation results in decreased P_4 levels causing menstruation and endometrial repair. In contrast, follicle implantation results in the maintenance of high P_4 levels, thereby inhibiting ovulation (Graham and Clarke, 1997; Mendelson, 2009). The latter physiological actions of P_4 were exploited for the design of progestins for contraceptive use [reviewed in (Hapgood *et al.*, 2013)].

1.2.3. Progestins

Progestins have many therapeutic applications in female reproductive medicine and were designed with the aim of eliciting effects similar to that of P_4, but with a higher bioavailability and longer half-life than P_4 (Speroff and Darney, 1996; Hapgood *et al.*, 2004; Gellersen *et al.*, 2009; Stanczyk *et al.*, 2013). Although progestins are used for multiple gynaecological treatments, they are used mostly in contraception and MHT. In contraception, progestins not only transform the endometrium from a proliferative to a secretory state, but also inhibit ovulation by inhibiting follicle stimulating hormone (FSH) and luteinizing hormone (LH) released from the pituitary gland, and prevent sperm penetration through the thickening of the cervical mucus (Kuhl, 1990; Sitruk-Ware, 2004; Sitruk-Ware and Nath, 2010; Africander *et al.*, 2011; Benagiano *et al.*, 2014). Contraceptive treatments are prescribed as either progestin-only, or a progestin in combination with an estrogen for better control of the menstrual cycle [reviewed in (Hapgood *et al.*, 2018). While estrogen is prescribed to alleviate the symptoms

associated with the cessation of estrogen production by the ovary (Johnson, 1998; Greendale *et al.*, 1999), the progestin is added to MHT to inhibit the proliferative effects of estrogen on the uterine epithelium of women with an intact uterus [reviewed in (Perkins *et al.*, 2018; Africander and Louw-du Toit, 2020; Horwitz and Sartorius, 2020)]. Several progestins are clinically available and vary from P_4, as well as each other, in terms of chemical structure and in some cases biological function (Stanczyk, 2003; Sitruk-Ware, 2004). Progestins are classified according to four successive generations, with most structurally related to either P_4 or testosterone, and only one, the fourth generation progestin drospirenone (DRSP), being derived from the mineralocorticoid receptor (MR) antagonist, spironolactone [reviewed in (Africander *et al.*, 2011; Stanczyk *et al.*, 2013; Schindler, 2014; Louw-du Toit, Storbeck, *et al.*, 2017; Hapgood *et al.*, 2018; Africander and Louw-du Toit, 2020)]. While the progestins related to testosterone are referred to as 19-nortestosterone derivatives, the progestins related to P_4 are further subdivided into 17α-hydroxyprogesterone (17-OHP$_4$) derivatives which contain a methyl group at carbon 10, and the 19-norprogesterone derivatives which lack this group (Sitruk-Ware, 2004; Stanczyk *et al.*, 2013). The first generation progestin MPA is an example of a 17-OHP$_4$ derivative, while the fourth generation progestins, nestorone (NES) and nomegestrol acetate (NOMAC), are both 19-norprogesterone derivatives. Examples of 19-nortestosterone derivatives include the first generation progestin norethisterone (NET), second generation levonorgestrel (LNG) and third generation gestodene (GES) (Sitruk-Ware, 2004; Stanczyk *et al.*, 2013). The 19-nortestosterone derivatives NET and LNG, do exhibit some androgenic activity, while the third generation progestin GES, a derivative of LNG, was designed to decrease this androgenic activity (Stanczyk and Archer, 2014). Fourth generation progestins such as NES, NOMAC and DRSP, were subsequently designed to have a more similar activity to P_4 than the earlier generations (Africander *et al.*, 2011; Louw-du Toit *et al.*, 2017a).

1.2.4. Molecular mechanism of P$_4$ and progestins.

Both P$_4$ and the progestins elicit their progestogenic effects by activating the PR [reviewed in (Graham and Clarke, 1997; Africander *et al.*, 2011)]. The PR can be found in many different tissues including the female genital tract, breast, adipose tissue, brain and pituitary gland (Graham and Clarke, 1997; Conneely *et al.*, 2003). It is a ligand-activated transcription factor belonging to the family of steroid receptors including the GR, MR, androgen receptor (AR) and ER subtypes [reviewed in (Grimm *et al.*, 2016)]. Two functional PR isoforms, PR-A and PR-B, are transcribed from the same gene under the control of distinct promoters (Kastner *et al.*, 1990). While both isoforms can bind P$_4$ and are involved in the development of the female reproductive system [reviewed in (Graham and Clarke, 1997)], PR-B has been shown to be more important for mammary gland development, while PR-A is more important for uterine development as well as maintenance of ovarian and uterine function (Lydon *et al.*, 1995; Conneely *et al.*, 2003; Mulac-Jericevic *et al.*, 2003; Lanari *et al.*, 2012). While these isoforms are expressed at similar levels in normal human breast tissue, the PR isoform ratio becomes dysregulated in the cancerous breast (Mote *et al.*, 2002). Notably, the ratio of PR-A and PR-B expression can differ across the various tissue types (Asavasupreechar *et al.*, 2020). For example, whereas PR-A predominates over PR-B in most cases of breast cancer, PR-B is the dominant isoform in uterine and ovarian cancer. This dominance of one isoform over another has also recently been shown in other normal tissues, such as the kidney, where PR-A is predominant and the liver where PR-B is predominant (Asavasupreechar *et al.*, 2020).

Like all steroid receptors, both PR isoforms consist of multiple domains, namely an amino-(N)-terminal domain (NTD), a hinge region (H), a highly conserved DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) (Figure 1.1). PR-B consists of 933 amino acids resulting in a protein of ~ 114 kDa, whereas PR-A is a smaller ~ 94 kDa protein

as it lacks the 164 amino acids found in the NTD of PR-B (Kastner *et al.*, 1990). A third smaller (~ 60 kDa) PR isoform, called PR-C, has also been identified. PR-C lacks the NTD and as it also lacks a large part of the DBD, it does not bind to DNA and is thus transcriptionally inactive (Wei *et al.*, 1990; Wei and Miner, 1994). The NTD of the PR is a highly dynamic structure with conformational flexibility, allowing multiple conformations to occur until the most stable arrangement of the NTD and co-regulators is obtained [reviewed in (Grimm *et al.*, 2016)]. A polyproline domain (PPD) is found within the NTD and is essential for the non-genomic activity of the PR isoforms (Boonyaratanakornkit *et al.*, 2001, 2017). The DBD and LBD both play important roles in dimerization and nuclear localization of the PR isoforms, whereas the DBD is essential for DNA binding specificity (Connaghan-Jones *et al.*, 2007; Africander *et al.*, 2011; Grimm *et al.*, 2016). PR-A and PR-B have two activation function (AF) domains, AF-1 located in the NTD and AF-2 in the LBD, both serving as platforms for interactions with co-regulators, such as steroid receptor coactivator-1 (SRC-1) (Africander *et al.*, 2011; Hill *et al.*, 2012; Kumar *et al.*, 2013; Grimm *et al.*, 2016; Woo *et al.*, 2019). While AF-2 is ligand-dependent and important for the induction of transcriptional activity, AF-1 is ligand-independent and important in protein-protein interactions. Specific to PR-B, is an additional activation domain, AF-3, present in the NTD (Sartorius *et al.*, 1994). This domain plays an important modulatory role in the activity of ligand-bound PR-B, making it a stronger activator of transcription than PR-A (Meyer *et al.*, 1990; Savouret *et al.*, 1990; Sartorius *et al.*, 1994; Jennifer K. Richer *et al.*, 2002; Tung *et al.*, 2006; Faivre and Lange, 2007; Lanari *et al.*, 2012). In contrast, PR-A is reportedly more transcriptionally active in the absence of ligand (Jacobsen *et al.*, 2002). Moreover, PR-A can repress the activity of PR-B, as well as that of other steroid receptors such as the GR, MR, AR and ER (Tung *et al.*, 1993; Vegeto *et al.*, 1993; Chalbos and Galtier, 1994; McDonnell *et al.*, 1994; Giangrande *et al.*, 2000; Abdel-Hafiz *et al.*, 2002; Patel *et al.*, 2018). The fact that PR-A can inhibit the activity of other steroid receptors may be

supported by the presence of an active N-terminal inhibitory domain (ID) on PR-A that is masked on PR-B (Tung *et al.*, 1993; Vegeto *et al.*, 1993; Chalbos and Galtier, 1994; McDonnell *et al.*, 1994; Wen *et al.*, 1994).

Figure 1.1. Structural domains of the human PR isoforms. Simple representation of the structural domains of PR-B, PR-A and PR-C. N-terminal domain – NTD; DNA binding domain – DBD; ligand binding domain – LBD; inhibitory domain – ID; Activation function (AF) 1, 2 and 3; hinge region – H; polyproline domain – PPD; [Adapted from (Diep *et al.*, 2015) including information from (Boonyaratanakornkit *et al.*, 2001; Cork *et al.*, 2008)].

The differences in isoform structure lead to different conformations upon agonist or antagonist binding, which in turn may cause isoform-specific co-factor recruitment and therefore isoform-specific regulation of genes (Tetel *et al.*, 1999; Giangrande *et al.*, 2000; Conneely *et al.*, 2003; Brinton *et al.*, 2008; Scarpin *et al.*, 2009; Singhal *et al.*, 2018). An agonist is generally defined as a ligand that induces a similar response to that of the cognate ligand, while an antagonist is a ligand that binds to a receptor and inhibits the agonist-induced response [reviewed in (Africander *et al.*, 2011)]. Indeed, it has been shown that in the presence of the PR antagonists like mifepristone (RU486) or onapristone (ZK98299), the co-repressor nuclear receptor co-repressor 2 (SMRT2) has a higher affinity for liganded PR-A, compared to liganded PR-B, and that this association is facilitated through the ID which is exposed on PR-A (Giangrande *et al.*, 2000). In contrast, in the presence of the PR agonist R5020, PR-B has a higher affinity than

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PR-A for the co-activators SRC-1 and SRC-2 (Giangrande *et al.*, 2000; Molenda-Figueira *et al.*, 2008).

Although progestins were designed to act via the PR, some progestins also interact with other members of the steroid receptor family which include the GR, MR, ERα and AR. For example, MPA can interact with the GR (Koubovec *et al.*, 2005; Ronacher *et al.*, 2009), MR (Africander *et al.*, 2013; Louw-du Toit *et al.*, 2020) and AR (Africander *et al.*, 2014; Louw-du Toit *et al.*, 2017b). Many other progestins have also been characterized in terms of their binding to and transcriptional activity via the GR (Koubovec *et al.*, 2005; Ronacher *et al.*, 2009), MR (Africander *et al.*, 2013; Louw-du Toit *et al.*, 2020), AR (Africander *et al.*, 2014; Louw-du Toit *et al.*, 2017b) and ERα (Louw-du Toit *et al.*, 2017b). These transcriptional activities of a selected panel of progestins relevant to this thesis are summarized in Tables 1.1-1.2. Similar studies characterising these progestins in terms of efficacy, the maximal response a ligand can elicit, and potency, the concentration at which half the maximal response is elicited, via the PR are limited (Markiewicz and Gurpide, 1994; Tegley *et al.*, 1998; Lim *et al.*, 1999; Attardi *et al.*, 2002b; Austin *et al.*, 2002; Madauss *et al.*, 2004; Bray *et al.*, 2005; Zhang *et al.*, 2005; Sasagawa *et al.*, 2008; Abdel-Hafiz *et al.*, 2009; Escande *et al.*, 2009; Attardi *et al.*, 2010; Stanczyk *et al.*, 2013; Bain *et al.*, 2015; Kumar *et al.*, 2017), and available studies rarely distinguish between PR-A and PR-B (Lim *et al.*, 1999), or directly compare multiple progestins in the same model system (Markiewicz and Gurpide, 1994; Attardi *et al.*, 2002b, 2010; Jeffrey D. Bray *et al.*, 2005; Sasagawa *et al.*, 2008a; Bain *et al.*, 2015; Kumar *et al.*, 2017). The available studies characterising progestin activity use multiple model systems which have different expression levels of most steroid receptors. Furthermore, the PR isoform usually investigated is either PR-B or not specified. Comparing the results of different progestins across studies may thus not give a true representation of the activity of these progestins when acting via PR-A or PR-B.

Table 1.1. Activity of progesterone (P$_4$) and structurally related progestins via steroid receptors.

Progestogen (Abbreviation)	Description/ Generation	MR		GR		AR		ERα[a]	
		Mineralocorticoid	Anti–mineralocorticoid	Glucocorticoid	Anti-glucocorticoid	Androgenic	Anti-androgenic	Estrogenic	Anti-estrogenic
Progesterone (P$_4$)	Natural	+	+	+	n/d	±	-	-	-
Promegestone (R5020)	PR-specific agonist	-	n/d	+	n/d	n/d	-	n/d	n/d
Medroxyprogesterone acetate (MPA)	1st	-	+	+	n/d	±	-	-	-
Nestorone (NES)	4th	-	+	n/d	n/d	-	±	-	-
Nomegestrol acetate (NOMAC)	4th	-	+	n/d	n/d	-	-	-	-

[a]None of the progestogens display activity via ERβ. +, confirmed agonist or antagonist activity; -, no activity; n/d, not determined; ±, inconsistent in literature. Abbreviations: GR – glucocorticoid receptor; MR – mineralocorticoid receptor; AR – androgen receptor; ERα - estrogen receptor alpha. Activity was determined *in vitro* using an array of different assays. (Agarwal and Paillard, 1979; Tegley *et al.*, 1998; Attardi *et al.*, 2002a, 2010; Austin *et al.*, 2002; Koubovec *et al.*, 2005; Sasagawa *et al.*, 2008; Ronacher *et al.*, 2009; Africander *et al.*, 2011, 2013, 2014; Stanczyk *et al.*, 2013; Hapgood *et al.*, 2013, 2018; Bain *et al.*, 2015; Kumar *et al.*, 2017; Louw-du Toit *et al.*, 2017b; Louw-du Toit *et al.*, 2020).

Table 1.2. Activity of progestins structurally related to testosterone or spironolactone.

Progestogen (Abbreviation)	Description/ Generation	MR		GR		AR		ERα [a]	
		Mineralocorticoid	Anti-mineralocorticoid	Glucocorticoid	Anti-glucocorticoid	Androgenic	Anti-androgenic	Estrogenic	Anti-estrogenic
Testosterone and structurally related progestins									
Norethisterone (NET)	1st	-	+	-	+	+	n/d	+	-
Levonorgestrel (LNG)	2nd	-	+	n/d	n/d	+	n/d	+	-
Gestodene (GES)	3rd	-	+	±	n/d	±	n/d	+	-
Spironolactone and the structurally related drospirenone									
Drospirenone (DRSP)	4th	+	+	n/d	n/d	-	+	-	-

[a] None of the progestogens display activity via ERβ. +, confirmed agonist or antagonist activity; -, no activity; n/d, not determined; ±, inconsistent in literature. Abbreviations: GR – glucocorticoid receptor; MR – mineralocorticoid receptor; AR – androgen receptor; ERα - estrogen receptor alpha. Activity was determined *in vitro* using an array of different assays (Attardi *et al.*, 2002a, 2010; Austin *et al.*, 2002; Koubovec *et al.*, 2005; Sasagawa *et al.*, 2008; Ronacher *et al.*, 2009; Africander *et al.*, 2011, 2013, 2014; Stanczyk *et al.*, 2013; Hapgood *et al.*, 2013, 2018; Bain *et al.*, 2015; Kumar *et al.*, 2017; Louw-du Toit *et al.*, 2017b; Louw-du Toit *et al.*, 2020).

Moreover, the various model systems used, may endogenously express different levels of steroidogenic enzymes, resulting in differential progestin metabolism which may confound the their reported activities. Notably, together with our collaborators, we have shown that a selected panel of progestins were differentially metabolised in nine commonly used laboratory cell line model systems (Skosana *et al.*, 2019). For instance, it was shown that MPA and NET are metabolised in the T47D cells, while NES is metabolised in MCF-7 BUS cells (Skosana *et al.*, 2019), indicating that progestins can be differently metabolised in various model systems. This suggests that the responses seen in one model system may in actual fact be due to the parent compound and/or its metabolites. Previous pharmacological characterisations of progestin activity should be interpreted with caution if metabolism was not taken into consideration. Since some metabolites of P_4 have been linked to breast cancer progression [reviewed in (Wiebe, 2006)], the metabolites of these progestins will also need to be considered. There is very little information regarding the formation and activity of the metabolites of different progestins [reviewed in (Bick *et al.*, 2021)], or how they influence breast cancer [reviewed in (Trabert *et al.*, 2020a)]. Indeed, one *in vitro* study isolated 3 metabolites of MPA (6β-, 2β-, and 1β-hydroxy-MPA) from human liver microsomes which were determined to be metabolised via cytochrome P450 3A4 (CYP3A4) (Zhang *et al.*, 2008). Additionally, some metabolites have been identified for NET, LNG, GES and NES, and it has been shown that some of these can bind and activate the PR, AR or ER (Stanczyk and Roy, 1990; Lemus *et al.*, 2001; Stanczyk, 2003; Kuhl, 2005; Stanczyk *et al.*, 2013; Kumar *et al.*, 2017). While it is possible that positive or negative effects of the progestins are elicited via steroid receptors other than the PR, recent evidence for MPA has shown that, despite the fact that MPA can bind and activate multiple steroid receptors, the majority of its effects were elicited by the PR (Moore *et al.*, 2020).

1.3. PR mechanisms

PR signalling pathways are either activated through genomic (Aagaard *et al.*, 2011) or non-genomic mechanisms (Carnevale *et al.*, 2007; Kariagina *et al.*, 2008; Boonyaratanakornkit *et al.*, 2017). While genomic mechanisms entail translocation of a ligand bound receptor to the nucleus, a process that can span several hours (Griekspoor *et al.*, 2007; Aagaard *et al.*, 2011), non-genomic mechanisms entail the rapid activation of signalling pathways and include the liganded cytoplasmic PR directly interacting with membrane- associated c-Src tyrosine kinase, as well as activation of a membrane PR (mPR) (Boonyaratanakornkit *et al.*, 2017). We will focus on the genomic mechanism in this chapter. Notably, the literature on PR mechanisms do not always distinguish between PR-A and PR-B. In the following sections, the precise role of a specific PR isoform will be stated if known, if not clear, or if applicable to both isoforms, the term PR will be used.

1.3.1. Genomic mechanism

Generally, in the absence of ligand, PR-B is distributed between the cytoplasm and the nucleus, while PR-A is predominantly found in the nucleus (Lim *et al.*, 1999; Boonyaratanakornkit *et al.*, 2017). Upon ligand binding, the PRs are phosphorylated and undergo a conformational change leading to the dissociation from heat shock proteins and translocation of the cytoplasmic PR to the nucleus (Griekspoor *et al.*, 2007). Within the nucleus the PRs will move to distinct nuclear foci (Arnett-Mansfield *et al.*, 2007) and either bind to specific palindromic progesterone response elements (PREs) in the promoter region of target genes (Aagaard *et al.*, 2011) to activate gene transcription (transactivation) or tether to DNA-bound transcription factors to repress gene transcription (transrepression) (Figure 1.2) (Kalkhoven *et al.*, 1996; Santos *et al.*, 2011).

Figure 1.2. Classical mechanism of transactivation and transrepression by the PR. Natural P$_4$ or progestins bind to the PR. In the nucleus, the PR binds to a PRE within the promoter of a target gene causing transcription of the gene in a process called transactivation (indicated by green arrow). For transrepression, the PR monomer tethers to a transcription factor, leading to an inhibition of transcription (indicated by the red stop line). Chromatin effects and additional regulatory mechanisms that may be involved are not indicated [Minor adaption from (Hapgood *et al.*, 2018)].

For transactivation, PR dimers bind to DNA, which can be either PR-A/PR-A or PR-B/PR-B homodimers or PR-A/PR-B heterodimers (Scarpin *et al.*, 2009). Notably, it has been shown that the PR does not have to dimerize to cause activation of transcription, but can also bind as a monomer to single or multiple PRE half sites to upregulate transcription (Heneghan *et al.*, 2005; Jacobsen *et al.*, 2009; Jacobsen and Horwitz, 2012; Ballaré *et al.*, 2013). It has been suggested that the monomer assembly of the PR onto DNA is more thermodynamically efficient than a pre-formed dimer (Connaghan-Jones *et al.*, 2007). As the presence of classical

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palindromic PREs across promoters of endogenous genes appear to be scarce (Jacobsen *et al.*, 2009), the suggestion of monomer assembly may not be unfounded.

Like the ER and AR that need pioneer factors, such as Forkhead box A1 (FOXA1), to facilitate chromatin remodelling [reviewed in (Zaret and Carroll, 2011; Beato *et al.*, 2020)], the PR has been reported to be facilitated by FOXA1 and CAAT enhancer binding protein (C/EBP)α. However, it has also been observed that the PR can interact with chromatin without prior nucleosome displacement [reviewed in (Beato *et al.*, 2020)]. Various co-regulators (co-activators and co-repressors) have been reported to be recruited to the PR, with the type of co-regulator recruited dependent on various factors. Interestingly, it has been shown that a specific PR isoform can be recruited to the genome, and that this is facilitated by accessory co-regulators specific to each isoform (Singhal *et al.*, 2018). Indeed PR-A has been shown to associate with co-regulators such as signal transducer and activator of transcription 1 (STAT1), growth factor receptor-bound protein 2 (GRB2) and nuclear receptor interacting protein 1 (NRIP)1, while only PR-B is associated with krueppel-like factor 5 (KLF5) and trithorax-group histone methyl transferase (MLL2) (Singhal *et al.*, 2018).

Studies investigating gene regulation by the PR, focusing on the classical transrepression mechanisms are scarce. Although best described for the GR, this mechanism has been shown for other steroid receptors including the PR, AR, MR and ER [(Kalkhoven *et al.*, 1996; Louw-du Toit *et al.*, 2017b; Louw-du Toit *et al.*, 2020), reviewed in (Adcock, 2000; Pascual and Glass, 2006; De Bosscher and Haegeman, 2009; Santos *et al.*, 2011)]. For the PR, it has been shown that it can form a complex with other transcription factors, such as nuclear factor κB (NFκB) or activator protein 1 (AP-1) bound to their *cis*-regulatory elements on the DNA of target genes, thereby repressing transcription of those genes (Kalkhoven *et al.*, 1996; Proietti *et al.*, 2018). To our knowledge, only one study has investigated isoform-specific

transrepression of gene expression and showed similar repression activity via PR-A and PR-B in response to the PR agonist Org2058 (Kalkhoven *et al.*, 1996). Interestingly, repression of gene expression by the PR has also been shown on a PRE in a process facilitated by various co-repressor complexes [(Izzo *et al.*, 2014; Nacht *et al.*, 2016; Walter *et al.*, 2017); reviewed in (Proietti *et al.*, 2018)]. For example, both the liganded-PR-A and PR-B have been shown to downregulate gene expression of GATA-binding protein 3 (GATA3), a gene involved in tumour suppression, through a PRE in the proximal promoter sequence (Izzo *et al.*, 2014). According to the study by Nacht and colleagues, the PREs responsible for repression are located closer to the transcription start site than PREs involved in transactivation, as the latter are located closer to the enhancer regions of the investigated genes involved in proliferation of the T47D breast cancer cells (Nacht *et al.*, 2016).

1.3.2. Post-translational modifications of the PR

Differences in the biological activity of PR-A and PR-B are not only attributed to their structural differences, but also to isoform-specific regulation of similar, but distinct sets of genes, which may in fact be determined by post-translational modifications of the PR. The PR isoforms have multiple sites where post-translational modifications, such as phosphorylation, ubiquitination, acetylation, methylation and SUMOylation, can occur (Figure 1.3). These modifications have been shown to modify receptor function, target gene specificity, as well as co-factor interactions, and can influence the stability and degradation of the PRs, as well as hormone sensitivity and nuclear translocation of the unliganded and liganded receptor [reviewed in (Abdel-Hafiz and Horwitz, 2014; Diep *et al.*, 2016a; Grimm *et al.*, 2016; Dwyer *et al.*, 2020)]. As the phosphorylation and SUMOylation of the PR are frequently discussed in the literature, this will be the focus of this next section.

There are fourteen phosphorylation sites that have been identified on the PR (Figure 1.3), the majority of which occur on serine (S) resides in the NTD, with six being specific to PR-B (S20, S25, S81, S102, S130, S162) [reviewed in (Abdel-Hafiz and Horwitz, 2014)]. Phosphorylation of S345, S400 and S676 residues common to both isoforms, has been associated with reduced transcriptional activity, directing regulation of PR growth-promoting genes, as well as enhanced ligand-independent regulation (Abdel-Hafiz and Horwitz, 2014). Interestingly, phosphorylation of S294 and S400, has been shown to be a possible mechanism behind PR-mediated gene repression [(Nacht *et al.*, 2016) reviewed in (Proietti *et al.*, 2018)]. Besides the six residues found only in the NTD of PR-B, there is minimal evidence of PR isoform-specific phosphorylation. However, it has been shown that the phosphorylation of S294 in the NTD, common to both isoforms, occurs to a greater extent on PR-B than on PR-A. For PR-B, S294 is associated with progestogen sensitivity, nuclear translocation of unliganded PR-B, and receptor degradation (Lange *et al.*, 2000; Daniel *et al.*, 2010; Abdel-Hafiz and Horwitz, 2014). Furthermore, it has been shown that PR-B, phosphorylated at S294, causes upregulation of genes associated with proliferation and cell survival, such as c-myc, and B-cell lymphoma 2 and cyclin D1 [reviewed in (Knutson and Lange, 2014)]. Phosphorylation of S294 on PR-A, however, is associated with increased cancer stem cell (CSC) like behaviours (Truong *et al.*, 2019). This highlights that the divergent roles of the PR isoforms may in part be due to post-translational modifications. Additionally, it has also been shown that phosphorylation at S294 marks the PR isoforms for ubiquitination and subsequent degradation by the 26S proteasome (Lange *et al.*, 2000; Salghetti *et al.*, 2001; Shen *et al.*, 2001). Another post-translational modification that has been shown to be substantially involved in PR-A and PR-B activity is SUMOylation. This is the ligand-dependent process of adding small ubiquitin-related modifiers (SUMO) to the PR at residue K388. Although the SUMOylated PR is very stable, this modification causes the transcriptional activity of PR-A and PR-B to be diminished

Figure 1.3. Reported post-translational modification sites of the PR isoforms. The PRs can undergo a number of post-translational modifications such as phosphorylation, acetylation, SUMOylation, mono-methylation and O-GlcAcylation. The residues and modifications involved are denoted as follows: S – serine; K – lysine; P – phosphorylation; Ac – acetylation; M – methylation. N-terminal domain – NTD; DNA binding domain – DBD; ligand binding domain – LBD; inhibitory domain – ID; Activation function 1 -AF-1; Activation function 2 - AF-2; Activation function 3 -AF-3; hinge region – H; aa, amino acid; polyproline domain – PPD; ubiquitination - U. [Adapted from (Diep *et al.*, 2015) including information from (Boonyaratanakornkit *et al.*, 2001; Abdel-Hafiz and Horwitz, 2014; Grimm *et al.*, 2016)]

(Abdel-Hafiz *et al.*, 2002, 2018; Knutson *et al.*, 2012; Abdel-Hafiz and Horwitz, 2014). Evidence indicates that phosphorylation of the PR at residue S294 can block SUMOylation at K388 in T47D breast cancer cells expressing both PR isoforms, in turn influencing promoter-selective transcription (Knutson *et al.*, 2017). This has also been shown to subsequently lead to rapid turnover of the receptor and transcriptional hyperactivity in a ligand-independent manner (Shen *et al.*, 2001; Diep *et al.*, 2015). This transcriptionally hyperactive state of the PR has been associated with the regulation of genes involved in cell proliferation and survival (Daniel and Lange, 2009; Knutson *et al.*, 2017), as well as endocrine resistance and CSC biology (Knutson *et al.*, 2012, 2017). The latter results suggest that the suppression of PR SUMOylation, in response to S294 phosphorylation, may be a mechanism whereby the PR

contributes to breast cancer progression. It is noteworthy that, at least one study has indicated that PR phosphorylation of S294 does not influence SUMOylation of the PR (Abdel-Hafiz *et al.*, 2009). Interestingly, it has recently been suggested that the phosphorylation of PR-A at S294 is associated with a more metastatic cancer phenotype (Truong *et al.*, 2019). Considering that progestins are PR ligands implicated in increased breast cancer incidence (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; Chlebowski *et al.*, 2013; Manson *et al.*, 2013; Collaborative Group on Hormonal Factors in Breast Cancer, 2019), the focus of the next section will be on the association of progestins used in MHT with increased breast cancer risk.

1.4. Clinical evidence for an association between progestogens and breast cancer

Although increased risk of developing breast cancer with the use of MHT was already recognized over 30 years ago (Bergkvist *et al.*, 1989), alarm was only raised by the findings of the USA Women's Health Initiative (WHI) study in 2002, where the observed negative side-effects were considered so severe that the trial was terminated two years earlier than planned (Rossouw *et al.*, 2002). The findings showed that MHT consisting of conjugated equine estrogen (CEE) and a progestin (MPA) increased risk of invasive breast cancer in healthy post-menopausal women. Although the use of CEE only also showed an increased risk of stroke, no increase in breast cancer risk or cardiovascular disease (CVD) was observed (Rossouw *et al.*, 2002), thus implicating the progestin (MPA) component in breast cancer and CVD side-effects. Although subsequent studies have shown an increased risk of breast cancer also with estrogen only MHT, the risks are greater with combined estrogen and progestin MHT [reviewed in (Africander and Louw-du Toit, 2020). These findings have been further corroborated in a recent meta-analysis, indicating an increased risk of breast cancer associated with MPA, but also NET-A and LNG (Collaborative Group on Hormonal Factors in Breast Cancer, 2019).

Progestins as a class have been associated with increasing breast cancer risk, yet only eight progestins (R5020, MPA, NET, NET-A, LNG, CPA, CMA and NOMAC) out of the vast number of progestins that are clinically available, have been investigated for breast cancer risks (Africander and Louw-du Toit, 2020). This highlights the need for comparative investigations of multiple progestins for a more definitive answer on whether all progestins contribute to breast cancer risk. While large scale clinical trials comparing multiple progestins in parallel is an enormous and challenging goal, investigations at the molecular level would be a good place to start.

Following the much publicized results of the WHI trial, multiple other clinical trials and observational studies have also linked progestins used in MHT to increased risk of developing breast cancer, although the results to date have been contentious (Waters *et al.*, 2002; Hulley *et al.*, 2002; Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; Greenspan *et al.*, 2005; Veerus *et al.*, 2006; Tierney *et al.*, 2009; Schierbeck *et al.*, 2012; Marjoribanks *et al.*, 2017; Collaborative Group on Hormonal Factors in Breast Cancer, 2019). Similar findings to the WHI were reported by the Women's international study of long-duration estrogen after menopause (WISDOM) clinical trial, linking CEE-MPA treatment with increased risk of developing invasive breast cancer (Vickers *et al.*, 2007). The Million Women Study (MWS) in the UK showed that the use of estrogen-progestin combined treatments containing MPA, NET and LNG all increased the risk of developing invasive breast cancer (Million Women Study Collaborators, 2003), while the French E3N-EPIC cohort study showed the same for R5020, MPA, NET-A, NOMAC, chlormadinone acetate (CMA) and cyproterone acetate (CPA) (Fournier *et al.*, 2005). However, one cannot ignore the fact that there have been studies that show no associated risk with progestins such as NET-A and LNG (Schierbeck *et al.*, 2012; Lundström *et al.*, 2020). Understanding the possible link between progestins in breast cancer is not straightforward, highlighted by the fact that MPA and its analogue megestrol

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acetate have previously been used to treat breast cancer, albeit at concentrations higher than that used in MHT and contraception [reviewed in (Lim *et al.*, 2016)].

Whether P_4 is also associated with increased risk of breast cancer is controversial. While most studies have shown that CEE or estradiol (E_2) in combination with micronized P_4, or dydrogesterone (the structural isomer of P_4) is not associated with increased breast cancer risk [reviewed in (Africander and Louw-du Toit, 2020)], some studies have shown risk. For example, it has been shown that the use of oral E_2 or CEE in combination with micronized P_4, or P_4-only containing vaginal gels increased the risk of breast cancer [reviewed in (Greendale *et al.*, 1999; Marjoribanks *et al.*, 2017)]. A very recent review by Trabert and colleagues very elegantly summarizes the evidence in support of P_4 increasing breast cancer risk and highlights the possible involvement of the ratio of P_4 metabolites (Trabert *et al.*, 2020). However, a subsequent study in post-menopausal women has shown that the ratio of the P_4 metabolites, 5α-dihydroprogesterone (5αP_4) and 3α-dihydroprogesterone (3αHP_4), are not associated with an increase in breast cancer (Trabert *et al.*, 2020b). Taken together, P_4 and only eight progestins used in MHT, have been investigated in terms of breast cancer risk, and strikingly, only one is a fourth generation progestin (NOMAC). As highlighted in a recent commentary (Bluming, 2021), the two main clinical studies that have implicated progestins in increasing breast cancer risk (WHI and MWS) had many shortfalls, thus questioning the actual conclusions of an increased risk of breast cancer with progestin use. Nonetheless, results from these clinical trials have resulted in a negative bias towards progestins as a class. Given the vast number of progestins available for clinical use, and the small number assessed for breast cancer risk, this bias may be unfounded for some progestins.

1.5. Experimental evidence for the role of progestogens and the PR in breast cancer.

Given that the PR belongs to the steroid receptor family of ligand activated transcription factors, it is not surprising that most studies investigate the ligand-dependent effects of the PR. However, it has been shown that both PR-A and PR-B can elicit ligand-independent effects on gene transcription, influence the activity of other steroid receptors and other signalling pathways (Jacobsen *et al.*, 2002, 2005; Khan *et al.*, 2012; Bellance *et al.*, 2013; Vicent *et al.*, 2013; Daniel *et al.*, 2015). We will however, focus mostly on the ligand-dependent effects of the PR isoforms in this review.

The mechanisms underlying the association between some progestins and breast cancer risk are still largely unknown. Furthermore, it has been postulated that these progestins may not in fact be "cancer causers" but aid in the activation of already present tumours and CSC [reviewed in (Horwitz and Sartorius, 2020)]. Although a number of *in vitro* studies have started investigating these mechanisms, results from these studies are often conflicting, and most studies do not investigate multiple progestins in parallel, or distinguish actions via different steroid receptors and/or their isoforms. A recent study by Moore and colleagues using a microarray approach in human ZR-75-1 breast cancer cells showed that although MPA can elicit effects through the GR and AR, the effects were predominantly via PR (Moore *et al.*, 2020). However, the authors did not assess the individual role of PR-A and PR-B. This result is rather illuminating considering that, for progestins such as MPA that can act via multiple steroid receptors, it is generally considered that the adverse side-effects are via off-target receptors and not the PR. It would thus be interesting to determine whether this is also the case for other progestins, and to determine whether a specific PR isoform is dominant in these progestin-mediated responses. Here, we focus on studies investigating the effects of P_4 and

progestins in breast cancer and/or the role of the PR.

Few studies have investigated the effects of progestins on gene expression in breast cancer. While it has been shown that P_4 and R5020, acting via PR-A and/or PR-B, can regulate a plethora of genes associated with breast cancer development and progression (Jacobsen *et al.*, 2002; Jacobsen and Horwitz, 2012; Khan *et al.*, 2012), many studies do not distinguish between activities of the PR isoforms on gene expression. Moreover, studies investigating effects of progestins, mainly focus on the effects of R5020 and MPA. In fact, it has been shown that MPA can regulate genes involved in apoptosis, proliferation and cell metastasis. For example, MPA has been shown to increase the mRNA expression of the anti-apoptotic B-cell lymphoma 2 (BCL2) gene (Ory *et al.*, 2001), and also upregulate cyclin D1 (CCND1) and MYC mRNA expression (Giulianelli *et al.*, 2012; Wargon *et al.*, 2015). It has also been shown that MPA decreased the expression of the tumour suppressor gene, GATA3, via both PR-A and PR-B (Izzo *et al.*, 2014). It has also been reported that R5020 decreases the expression of urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR), and increases the expression of plasminogen activator inhibitor 1 (PAI-1), via either PR-A or PR-B, whereas the expression of β1-integrin was only decreased via PR-B (Bellance *et al.*, 2013). Recently, an elegant study conducted by Truong and colleagues showed a possible role for PR-A in CSC biology, showing isoform-dependent regulation of genes involved in CSC behaviours (Truong *et al.*, 2019). These authors also showed that PTGES, p21 and WNT4 were specifically upregulated via PR-A, and not PR-B, in response to R5020, while FOXO1, NOTCH1 and KLF4 were upregulated by both isoforms with PR-A having the more pronounced response (Truong *et al.*, 2019).

In addition to investigations on gene expression, several studies have also investigated whether P_4 and progestins are involved in the development of breast cancer by investigating key tumour

cell behaviours (cancer hallmarks), including proliferation, apoptosis, migration and invasion. However, the results are often contradictory and the studies which focus on the PR seldom specify which isoform is involved (Groshong *et al.*, 1997; Formby and Wiley, 1999; Hyder *et al.*, 2001; Franke and Vermes, 2003; Seeger *et al.*, 2003b; Carvajal *et al.*, 2005; Werner *et al.*, 2005; Moore *et al.*, 2006; Liang *et al.*, 2007, 2010; Chen *et al.*, 2011; de la Mare *et al.*, 2014; Azeez *et al.*, 2015). For example, using the ER^+/PR^+ T47D, MCF-7, ZR-75 and BT-474 breast cancer cell lines, it has been shown that R5020 (Hissom and Moore, 1987), P_4 (Carvajal *et al.*, 2005; Liang *et al.*, 2007, 2010), MPA, NET-A, NET (Schoonen *et al.*, 1995; Franke and Vermes, 2003; Seeger *et al.*, 2003a; Saitoh *et al.*, 2005), LNG and GES (Catherino *et al.*, 1993; Schoonen *et al.*, 1995; Seeger *et al.*, 2003a) increase cell proliferation. Furthermore, MPA only and in the presence of E_2 or a combination of growth factors, also increase proliferation in normal breast explant tissue and the MCF-10A normal breast cell line, respectively (Krämer *et al.*, 2005; Eigeliene *et al.*, 2006). In contrast, other studies have shown that R5020, P_4, MPA and NET either have no influence, or an inhibitory effect, on cell proliferation in the aforementioned breast cancer cell lines (Horwitz and Freidenberg, 1985; Sutherland *et al.*, 1988; Musgrove *et al.*, 1991; Catherino *et al.*, 1993; Botella *et al.*, 1994; Schoonen *et al.*, 1995b; Groshong *et al.*, 1997; Formby and Wiley, 1999; Seeger *et al.*, 2003b; Franke and Vermes, 2003; Werner *et al.*, 2005; Moore *et al.*, 2006; Chen *et al.*, 2011; Azeez *et al.*, 2015). Similarly, LNG and GES have been reported to have no effect on the proliferation of MCF-7 cells (van der Berg et al., 1992), while NOMAC has been shown to have no effect or elicit anti-proliferative effects in T47D cells (Botella *et al.*, 1994; Desreux *et al.*, 2003). In terms of apoptosis, R5020, P_4 and MPA have been reported to elicit anti-apoptotic effects in the ER^+/PR^+ T47D, MCF-7, HCC1500 breast cancer cell lines (Ory *et al.*, 2001; Krämer *et al.*, 2005; Moore *et al.*, 2006), while other studies have shown that MPA, NET and NET-A are pro-apoptotic in HCC1500 and MCF-7 breast cancer cells (Krämer *et al.*, 2005; Chen *et al.*, 2011).

Although the above-mentioned studies used cell models expressing both the ER and the PR, a role for PR-A or PR-B was not indicated. However, it has been shown that P_4, MPA and NET-A decrease cell proliferation of T47D cells via PR-B but not PR-A (Petit *et al.*, 2009). This decrease in proliferation was associated with an increase in catalase activity, which increased hydrogen peroxide production, resulting in cell apoptosis and necrosis (Petit *et al.*, 2009). Furthermore, in response to P_4, MPA and NET, it has also been shown that PR-B causes a more profound increase in vascular endothelial growth factor (VEGF) expression than PR-A in T47D cells (Wu *et al.*, 2005). VEGF is a protein involved in vascular expansion and associated with tumour progression [reviewed in (Reeves *et al.*, 2009)]. These results suggest that PR-B may be driving tumour progression in these cell lines. Moreover, a recent study has suggested that PR-B may be associated with a more proliferative phenotype, as R5020 via PR-B caused an increase in anchorage-independent growth, while PR-A did not (Truong *et al.*, 2019). The same study also suggests that PR-A may be involved in CSC expansion, thus contributing to a more invasive phenotype. Whether similar activities are observed for PR-A and PR-B when bound to other progestins is unclear.

Few studies have investigated the effect of progestins on cell migration and invasion. Nonetheless, the available studies show that R5020, P_4, MPA, NES, and to a lesser extent DRSP, increase migration and invasion of T47D cells (Fu *et al.*, 2008a; Fu *et al.*, 2008b; Holley *et al.*, 2009). *In vivo* studies also show that MPA, NET and LNG promote the growth and metastasis of T47D and BT-474 human breast cancer cell xenografts in nude mice (Liang *et al.*, 2007, 2010). The latter was attributed to influencing expression of VEGF (Liang *et al.*, 2007, 2010; de la Mare *et al.*, 2014). While some of the above studies aimed to determine whether the PR is mediating these effects by using the PR antagonist mifepristone (RU486) (Fu *et al.*, 2008a; Fu *et al.*, 2008b), these results do not definitively point to a role of the PR as RU486 is also an antagonist of the GR (Ronacher *et al.*, 2009), AR (Africander *et al.*, 2014)

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and MR (Africander *et al.*, 2013). Nonetheless, there is some evidence pointing towards PR isoform-specific effects on cell migration and invasion. Specifically, R5020 and P_4 decreases the protein expression of the cell adhesion molecule, E-cadherin via PR-B and not PR-A in T47D cells stably expressing either PR-A or PR-B (Kariagina *et al.*, 2013). This result suggests a negative role for PR-B in breast cancer, as a loss of E-cadherin is associated with increased invasion of breast cancer (Padmanaban *et al.*, 2019). Cell migration was also inhibited in response to R5020, in the bi-inducible MDA-MB-231 cell line expressing only PR-B (Bellance *et al.*, 2013). The mechanism of inhibition was suggested to be through the PR-B-mediated regulation of the focal adhesion kinase (FAK)-dependent signalling pathway. In contrast, the activity of PR-A, in response to R5020 and P_4, has also been shown to promote invasiveness by inhibiting the E_2-induced suppression of invasion in breast cancer cells (McFall *et al.*, 2015, 2018). Lastly, R5020 via PR-B has also been reported to increase migration through the activation of insulin receptor substrate (IRS)-2 of the insulin-like growth factor system (Ibrahim *et al.*, 2008).

While various ideas have been brought forward trying to explain the role of P_4 and progestins in breast cancer, it is still vastly understudied. For P_4, it has been postulated that it can possibly contribute to breast tumour formation, because of the high P_4 levels during the luteal phase of the menstrual cycle frequently activating the PR through paracrine signalling (Brisken *et al.*, 2015). An alternate hypothesis is that P_4, but also progestins, do not cause breast cancer but rather reactivate the stem cell-like properties of CSC already present in the breast (Horwitz and Sartorius, 2008; Ogba *et al.*, 2014), suggesting that P_4 and progestins could differentially drive the progression of existing breast tumours [reviewed in (Horwitz and Sartorius, 2020)]. However, as only P_4, R5020 and MPA have been used in studies investigating the theory of occult tumours, this cannot be assumed for all progestins.

1.5.1. Progestins and dysregulated PR isoform ratios

While the PR isoforms are expressed at equivalent levels in non-cancerous breast tissue, the ratio of PR-A:PR-B becomes dysregulated in cancerous states [Rojas et al., 2017; Mote et al., 2002; Hopp et al., 2004]. While some tumours do co-express equal levels of the PR isoforms, a few tumours have been shown to overexpress PR-B relative to PR-A, with the majority of tumours overexpressing PR-A relative to PR-B (Graham *et al.*, 1995; Graham and Clarke, 2002; Mote *et al.*, 2002, 2015; Hopp *et al.*, 2004; Rojas *et al.*, 2017; Lamb *et al.*, 2018; McFall *et al.*, 2018; Singhal *et al.*, 2018). It has been postulated that the predominance in PR-A expression could be due to the increased activity of mitogen-activated protein kinase (MAPK) in breast cancer, with this increase in kinase activity leading to hyperactivity and increased turnover of PR-B (Shen *et al.*, 2001; Diep *et al.*, 2015). Although the alteration in isoform ratio occurs during early stages of breast cancer development (Mote *et al.*, 2002), the mechanism by which this occurs and the implications for overall PR signalling in breast cancer are actively being investigated.

It has been observed that when the PR-A:PR-B ratio is elevated, in response to the PR agonist Org2058, a small subset of genes gained progestin responsiveness, compared to when the ratio of PR-A:PR-B was low (Graham *et al.*, 2005). The authors suggested that this change in gene regulation was due to predominance of PR-A:PR-B heterodimers. Interestingly, these effects were not seen at a PR isoform ratio closer to 1, only at the high PR-A:PR-B ratio (Graham *et al.*, 2005). Given that the genes regulated at the high PR-A:PR-B ratio are associated with cell shape and adhesion, it is likely that the PR isoform ratio contributes to cancer cell metastasis. In support of this, it has recently been shown that differential gene expression patterns of genes associated with overall poor patient survival, occur in PR[+] patient tumours expressing a high PR-A:PR-B ratio (Singhal *et al.*, 2018). These recent results are in agreement a previous study

that implicated the overexpression of PR-A with endocrine resistance (Hopp *et al.*, 2004). Furthermore, a study by McGowan and colleagues co-culturing of T47D cells and bone marrow fibroblasts collected from bone marrow donors, showed that an elevated PR-A:PR-B ratio in T47D cells caused an increase in invasion to bone marrow fibroblasts in response to Org2058 (McGowan *et al.*, 2004). Similarly, the overexpression of PR-A relative to PR-B has also been implicated in contributing to metastatic characteristics and is associated with a more invasive phenotype (McFall *et al.*, 2015, 2018; Rosati *et al.*, 2020). Despite the negative implications of excess expression of PR-A relative to PR-B, it has also been shown that this elevation in PR-A, leads to an increase in both progestogen and anti-progestin responsiveness (McFall *et al.*, 2015, 2018; Wargon *et al.*, 2015; Rojas *et al.*, 2017; Rosati *et al.*, 2020). These authors suggested that in tumours expressing a high ratio of PR-A:PR-B, progestogen or anti-progestin therapy could be considered as a treatment strategy. In fact, there are currently a few clinical trials assessing the possible combination of either P_4 or anti-progestins in combination with standard endocrine therapies [reviewed in (Horwitz and Sartorius, 2020)]. However, a complete analysis of the activity of multiple progestin in the presence of a high PR-A:PR-B ratio is warranted to determine if some may be better than others.

1.6. Conclusion

Progestins are clinically used by millions of women in contraception and MHT, however, some of these synthetic ligands have been associated with an increased breast cancer incidence. Alarmingly, out of the large number of progestins available for clinical use, only a handful have actually been investigated for an increased risk of breast cancer. Considering that the structures and selectivities of the progestins differ, one cannot assume that all progestins would influence breast cancer risk in a similar manner. It is thus important to evaluate more progestins for their association with breast cancer risk in large scale clinical studies. In the absence of

such studies, studies investigating the effects of progestins in breast cancer at the cellular level are crucial, particularly studies that conduct parallel comparisons of multiple progestins. Given that progestins are PR ligands and that the PR has also been implicated in breast cancer development and progression, investigations into the role of the progestins via the PR are vital. Results from available studies of these progestins at the molecular level are contradictory, and the role of the PR is mostly unclear. Moreover, the majority of studies investigating the PR in breast cancer do not focus on the contributions of the individual isoforms, which is an oversight as it is known that they are involved in different signalling mechanisms. Surprisingly, there is a gap in the literature with respect to the binding affinities and relative agonist potencies and efficacies of different progestins via the individual PR isoforms for both transactivation and transrepression. Similarly, studies specifically investigating the regulation of endogenous genes involved in breast cancer development and progression by multiple progestins, and the role of a specific PR isoform, are also lacking. The few studies that do investigate the progestin and/or PR mechanism seldom consider progestin metabolism in their cell models. This is important as differential metabolism of progestins may influence the interpretation of these results. An added complexity in understanding the role of progestins and the PR is breast cancer, is the fact that PR-A is upregulated relative to PR-B in most breast cancer tumours. Although both negative and positive outcomes have been associated with the excess PR-A:PR-B, this evidence has been limited to only P_4, R5020 and MPA. Further studies in the presence of other progestins are warranted. A further complexity is the fact that both PR isoforms can crosstalk with other steroid receptors in breast cancer, a topic that we did not elaborate on in this chapter. Further insights into the role of the individual PR isoforms in mediating the effects of specific progestins on breast cancer development, as well as the underlying mechanisms, are needed to provide us with a clearer and more comprehensive understanding of progestin action. Such studies are essential so that women and clinicians can make informed choices as to which progestin to use in reproductive medicine, while limiting risks of breast cancer.

Hypothesis and Aims

From the literature it is clear that P_4 and all progestins differ in structure and may display differential biological effects. It is also clear that the PR isoforms have distinct functions in different tissues. However, the transcriptional activities of P_4 and the progestins via the individual PR isoforms are unclear. Similarly, the role of progestogens and the PR isoforms in breast cancer are also vague. In light of this, the primary hypothesis of this dissertation is that the progestins will have differential activities compared to each other and P_4, via PR-A and PR-B on gene regulation and specific cancer hallmarks. As most breast cancers express excess PR-A relative to PR-B, we also hypothesized that the density of PR-A and the co-expression of PR-A and PR-B at various PR-A:PR-B ratios, would influence the effects of P_4 and the progestins. As metabolism of progestins in a specific cell model could partly explain the differential activities of progestins, we also hypothesised that the progestins would be differentially metabolised across different breast cancer cell lines.

The overall aims of this project were three-fold:

Firstly, the metabolism of P_4 and selected progestins (R5020, GES, NOMAC and DRSP) in MDA-MB-231, T47D and MCF-7 BUS breast cancer cell lines was determined using ultra-high performance supercritical fluid chromatography – tandem mass spectrometry (UHPSFC-MS/MS) analysis. Additionally, the half-life ($t\frac{1}{2}$) of P_4 was also evaluated in the three cell lines.

Secondly, the relative agonist efficacies and potencies of P_4 and selected progestins from all four generations (R5020, MPA, NET, LNG, GES, NES, NOMAC and DRSP) were directly compared in MDA-MB-231 cells exogenously expressing either PR-A, PR-B or co-expressing PR-A and PR-B at a 1:1, 2:1 and 5:1 ratio, on PRE- and NFκB-containing-reporters. Using

T47D cells that endogenously express both PR-A and PR-B, the effects of the progestogens were assessed on the regulation of genes involved in breast cancer development and progression (GATA3, SOX4, TGFβ1, FOXO1, Ki67 and BAX). Moreover, we investigated the role of the PR isoforms, as well as the influence of the excess expression of PR-A, on the regulation of the selected genes in response to the progestogens.

Thirdly, the effects of P_4 and the selected progestins were assessed and directly compared in parallel, on hallmarks of cancer. Specifically, using the MDA-MB-231 cells exogenously expressing either PR-A, PR-B or co-expressing PR-A and PR-B at various PR-A:PR-B ratios, we directly compared the effects of P_4 and the progestins on proliferation, apoptosis, anchorage-independent growth, migration and invasion. The effects of P_4 and the progestins on growth and survival of the T47D cells endogenously expressing PR-A and PR-B were also assessed, as well as the role of the PR isoforms and influence of overexpression of PR-A.

Chapter 2

Investigating progestogen metabolism in breast cancer cell lines

2.1. Background and aims

Progestins are highly effective as contraceptives and in MHT, however, their use has been associated with a number of adverse effects (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; Li *et al.*, 2012; Chlebowski *et al.*, 2013; Manson *et al.*, 2013; Hapgood *et al.*, 2018; Lopez-Pier *et al.*, 2018; Graafland *et al.*, 2020). For this reason, many researchers are investigating the mechanisms of progestins at the cellular level. *In vitro* studies evaluating the relative biological activities of progestins mainly utilise mammalian cell lines as model systems (Richer *et al.*, 1998; Lim *et al.*, 1999; Zhang *et al.*, 2000; Shen *et al.*, 2001; Attardi *et al.*, 2002b, 2010; Grossmann *et al.*, 2004; Wang *et al.*, 2007; Sasagawa *et al.*, 2008b; Africander *et al.*, 2013; Louw-du Toit *et al.*, 2017b). Specific concentrations of steroids are used in these experiments and it is generally assumed that these concentrations remain constant throughout the course of the experiment. However, should the progestins be metabolised in the selected model system, the interpretation of results of experiments that are reliant on concentration, e.g. dose-response analyses, may be confounded. In such experiments, neglecting to determine whether progestins are metabolised may result in the reporting of inaccurate efficacies and potencies of progestins. Moreover, should metabolism occur in a selected model system, the possibility that observed biological activities are due to progestin metabolites, or a combination of metabolites and the parent compound cannot be excluded. Analysis of results would also be confounded should the cognate ligands for a steroid receptor be metabolised, as these ligands are often used as comparative positive controls. Indeed, it is well-known that P_4, the natural ligand for the PR, is metabolised *in vitro* and *in vivo*, with several metabolites identified in cell lines of both the normal and cancerous breast (reviewed in Wiebe, 2006), while the synthetic PR ligand, R5020, is generally considered non-metabolizable (O'Connell *et al.*, 2011; Morrill *et al.*, 2012; Ting *et al.*, 2015). However, together with our collaborators, we have recently investigated the metabolism of a number of

progestins commonly used in, or developed for, alternate forms of contraception i.e. MPA, NET, LNG, etonogestrel (ETG) and NES (Skosana *et al.*, 2019) (Addendum A1), in nine commonly used mammalian cell lines. This study showed that the metabolism of the selected panel of progestins, at 24 hours, is cell- and progestin-specific. For example, MPA and NET were metabolised in the T47D breast cancer cell line but not in the MDA-MB-231 or MCF-7 BUS breast cancer cell lines, while NES was only metabolised in the MCF-7 BUS cell line (Skosana *et al.*, 2019). In contrast, natural P_4 was significantly metabolised in all of the cell lines investigated.

The overall aim of this PhD study was to compare the activities of selected progestins from different generations to each other and P_4 in breast cancer cell lines. These progestins include first generation MPA and NET, second generation LNG, third generation GES and the fourth generation progestins NES, NOMAC and DRSP. The PR-specific ligand R5020 (Kobayashi *et al.*, 2010; Diep *et al.*, 2013) was also included as a reference ligand as its progestational activity is superior to that of P_4 (Stanczyk *et al.*, 2013). Considering that the metabolism of R5020, GES, NOMAC and DRSP were not investigated in Skosana *et al.*, 2019, this chapter examined their metabolism in two breast cancer cell lines employed in this dissertation (Chapter 3 and 4) and another commonly used in our laboratory. Since P_4 was metabolised in all models systems after 24 hours (Skosana *et al.*, 2019), we also investigated the time required for P_4 to be metabolised in the three breast cancer cell lines prior to the 24 hour time period.

The following research questions were addressed in the human MDA-MB-231, T47D and MCF-7 BUS breast cancer cell lines:

1. Are R5020, GES, NOMAC and DRSP, differentially metabolised within the same model system?
2. Should progestin metabolism occur, is the metabolism cell-line specific?

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3. Does the half-life ($t\frac{1}{2}$) of P_4 differ between cell lines?

2.2. Materials and Methods

2.2.1. Cell culture

The triple negative human MDA-MB-231 breast cancer cell line was a kind gift from Prof. Adrienne Edkins (Rhodes University, Grahamstown, South Africa), while the T47D human breast cancer cell line was obtained from Prof. Iqbal Parker (University of Cape Town, South Africa). The cell lines were maintained in phenol red Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, South Africa), 100 U/mL penicillin and 100 μg/mL streptomycin (1% penicillin-streptomycin) (Sigma-Aldrich, South Africa), as well as an additional 2 mM L-glutamine (Sigma-Aldrich, South Africa) for the MDA-MB-231 cells. The MCF-7 BUS (originally termed MCF-7 BOS) human breast cancer cell line was received from Prof. Ana Soto (Tufts University, Boston, USA) and was maintained in phenol red DMEM supplemented with 5% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin. All cell lines were maintained in 75 cm^2 culture flasks (Bio-Smart Scientific, South Africa) and incubated at 37ºC in an atmosphere of 90% humidity and 5% CO_2. Cells used for experiments were performed within the first 30 passages since thawed from storage and were regularly tested for mycoplasma infection through Hoechst staining (Freshney, 1987). Only mycoplasma negative cells were used for experiments.

2.2.2. Test compounds

R5020 [17,21-dimethylpregna-4,9(10)-diene-3,20-dione] was purchased from Perkin Elmer Life and Analytical Science, South Africa. The following steroids were purchased from Sigma-

Aldrich, South Africa: P_4 [4-pregnene-3,20-dione], GES [17α-ethinyl-17β-hydroxy-18-methylestra-4,15-dien-3-one], NOMAC [17α-acetoxy-6-methyl-19-norpregna-4,6-dione-3,20-dione], DRSP [6β,7β,15β,16β-dimethylene-3-oxo-17α-pregn-4-ene-21,17 carbolactone] and testosterone (T) [17β-hydroxy-4-androsten-3-one]. All stock solutions were made up in absolute ethanol and stored at -20°C in light-protective glass vials. For experiments, these compounds were diluted in phenol red-free culturing medium to a final ethanol concentration of 0.1% (v/v). Control incubations were thus performed in the presence of 0.1% (v/v) ethanol.

2.2.3. Steroid metabolism

2.2.3.1 Treatment of cells

Cell lines were maintained as described in Section 2.2.1 and seeded into 24-well plates at a density of 5 x 10^4 cells per well in supplemented phenol red DMEM. The next day, the cells were rinsed with 500 µL pre-warmed 1x phosphate buffered saline (PBS) and the medium replaced with phenol red-free DMEM supplemented with 10% (v/v) charcoal-stripped (CS)-FBS and 1% (v/v) penicillin-streptomycin. After 24 hours, cells were treated with 100 nM R5020, P_4, GES, NOMAC or DRSP for 24 hours. In a separate experiment, cells were also treated with 100 nM P_4 for 0.5, 1.5, 3, 6, 12 and 24 hours. Treatments were conducted in either phenol red-free DMEM supplemented with 10% (v/v) CS-FBS and 1% (v/v) penicillin-streptomycin (MDA-MB-231 cells) or unsupplemented phenol red-free DMEM (T47D and MCF-7 BUS cell lines). As shown in Addendum B, Figure B1, the MDA-MB-231 cells were treated using supplemented phenol red-free DMEM as they do not survive 24-hour incubations in serum free DMEM. As a negative (no cell) control, medium containing 100 nM of each compound was added to a 24-well plate and incubated at 37°C. After incubation with the respective ligands, 500 µL of the medium was transferred to clean glass test tubes and stored at -20°C.

2.2.3.2 Methyl tert-butyl ether (MTBE) steroid extraction

Samples were thawed and T (1.5 ng) was added to all samples and the calibration series samples as an internal standard for the extraction. The series of standards ranging between 0 – 100 ng/mL was prepared by serially diluting a standard mastermix of steroids including 1 µg/mL R5020, P_4, GES, NOMAC and DRSP in unsupplemented phenol red-free DMEM, or in the case of the MDA-MB-231 cells phenol red-free DMEM supplemented with 10% (v/v) CS-FBS and 1% (v/v) penicillin-streptomycin. Steroids in the samples and standards were extracted using MTBE (Sigma-Aldrich, South Africa) in a 3:1 ratio (1.5 mL MTBE:0.5mL sample). All samples were vortexed thoroughly, allowing the non-polar steroids to move out of the polar media into the non-polar MTBE solution. These samples were placed at -20˚C overnight to allow the polar (aqueous) phase to freeze. The aqueous phase was discarded and the MTBE layer containing the steroids was transferred to a pyrolyzed glass test tube and evaporated in a fume hood overnight at room temperature. Samples were reconstituted in 150 µL 50% (v/v) methanol and stored at -20˚C until analysis.

2.2.3.3 Ultra-High Performance Supercritical Fluid Chromatography – Tandem Mass Spectrometry (UHPSFC-MS/MS) analysis

The steroids were separated by ultra-high performance supercritical fluid chromatography – tandem mass spectrometry (UHPSFC-MS/MS) (ACQUITY, Waters, Milford, USA) using a Bridged Ethylene Hybrid (BEH) 1.7 µm particle size column with liquid CO_2 (mobile phase A) and 100% methanol (mobile phase B). The injection volume was 2 µL and the steroids separated at a constant flow rate of 1.9 mL/min over a period of 2.5 minutes as described in (Skosana *et al.*, 2019) (Addendum A1). The automated back pressure regulator was set to 1 700 psi and the column temperature to 60°C. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. Using an

electrospray probe in the positive ionisation mode (ESI+), all steroids were analysed in multiple reaction monitoring (MRM) mode (Addendum B, Table B1). The capillary voltage was set to 3.8 kV, the desolvation temperature to 350°C, desolvation gas to 900 L/h, and cone gas to 150 L/h. Data were collected and analysed using the MassLynx software (version 4.1) (Waters Corporation).

2.2.4. Data and statistical analysis

Analysis of results were performed using The GraphPad Prism® v7.00 software package (GraphPad Software, La Jolla, California, USA). Unpaired *t*-tests were used to assess statistical differences between the amount of steroid present in the medium when added to cells versus the no cell control, and multiple *t*-tests were used for comparisons between the progestogens. To determine the rate constant (K) and half-life (t½) values for P_4, non-linear regression, one phase decay analysis was performed with K being used to determine the t½ calculated using the equation, *t½ = ln(2)/K*. Statistically significant differences are represented by *, **, *** indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical differences ($p > 0.05$) is indicated by ns.

2.3. Results and Discussion

2.3.1. While R5020, GES and NOMAC are not metabolised in any of the breast cancer cell lines, the metabolism of DRSP is cell-line specific.

To investigate whether R5020, GES, NOMAC and DRSP are metabolised in the MDA-MB-231, T47D and MCF-7 BUS breast cancer cell lines, each cell line was treated with 100 nM of each of the above-mentioned progestins for 24 hours, prior to steroid extraction and analysis

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by UHPSFC-MS/MS. Medium containing 100 nM of the progestin was also added to wells containing no cells (no cell negative control). As described in Skosana *et al.*, (2019) adsorption of the progestogens to the plastic cell culture plates was corrected for in the experimental design. Unlike P_4 that is metabolised in all three breast cancer cell lines, we show no significant metabolism of R5020, GES and NOMAC in any of these cell lines (Figure 2.1A-C). The result showing no metabolism of R5020 in the T47D cells is consistent with the study by Horwitz and co-authors showing no metabolism of R5020 in T47Dco cells (Horwitz *et al.*, 1986), a variant of the T47D cell line which is insensitive to estradiol-induced PR expression and expresses equimolar levels of the PR (Horwitz *et al.*, 1982). Lack of metabolism of R5020 in any of the cell lines is consistent with the description of R5020 in the literature as a non-metabolizable progestin or PR agonist (O'Connell *et al.*, 2011; Morrill *et al.*, 2012; Ting *et al.*, 2015). The fact that R5020 is not metabolised in the MDA-MB-231, T47D and MCF-7 BUS cell lines suggests that in experiments investigating effects of R5020 in these cell lines, the concentration of R5020 would be constant within a 24 hour treatment period, and that the observed responses would be elicited by R5020 itself and not metabolites of R5020. Moreover, as P_4, but not R5020, is metabolised in all three breast cancer cell lines, our results suggest that R5020 should be the preferred reference ligand to use rather than P_4 when comparing effects of progestins and/or investigating molecular mechanisms of the liganded PR in these breast cancer cell lines. Indeed, it has also been highlighted in literature that R5020 is a more appropriate ligand to use as it is more stable than P_4 (Sathyamoorthy and Lange, 2020). Interestingly, Figure 2.1A and 2.1B show that DRSP is metabolised by 26% in the MDA-MB-231 cells and 34% in the T47D cells, but not in the MCF-7 BUS cells (Figure 2.1C). The fact that DRSP exhibits cell-specific metabolism suggests that the steroidogenic enzymes able to metabolise DRSP are either not present in the MCF-7 BUS cell line, or are expressed at much lower levels.

A MDA-MB-231

	R5020	P4	GES	NOMAC	DRSP
R5020		***	ns	ns	*
P4			***	***	***
GES				ns	**
NOMAC					***
DRSP					

B T47D

	R5020	P4	GES	NOMAC	DRSP
R5020		***	ns	ns	*
P4			***	***	***
GES				ns	*
NOMAC					ns
DRSP					

C MCF-7 BUS

	R5020	P4	GES	NOMAC	DRSP
R5020		***	ns	ns	ns
P4			***	***	***
GES				ns	ns
NOMAC					ns
DRSP					

Figure 2.1. Figure legend on the following page.

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Figure 2.1. R5020, GES and NOMAC are not metabolised in the MDA-MB-231, T47D and MCF-7 BUS breast cancer cell lines, while DRSP is metabolised in the MDA-MB-231 and T47D cells. Medium containing 100 nM R5020, P_4, GES, NOMAC and DRSP was added for 24 hours to a 12-well plate containing no cells (negative control) as well as to the **(A)** MDA-MB-231**, (B)** T47D and **(C)** MCF-7 BUS cell lines. The steroids were extracted and quantified using UHPSFC-MS/MS. The results are plotted as the % steroid remaining, with the amount of the progestogen in the negative control (no cells) for metabolism set as 100% and the amount of the progestogen present in the medium after incubation with the cells, set as a percentage relative to that. The results shown are the averages (± SEM) of at least two independent experiments with each condition performed in quadruplicate. Statistical differences relative to the no cell control are shown on the graph, while differences between progestogens are indicated in the tables below the graph. Significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns.

Although only DRSP is metabolised when considering the panel of progestins and the specific cell lines investigated in this chapter, we have shown that the progestin ETG is metabolised in all three cell lines, while MPA and NET are metabolised in the T47D cells, and NES is metabolised in the MCF-7 BUS cells (Skosana *et al.*, 2019). Although we did not identify any progestin metabolites, our study is the first to investigate the metabolism of GES, NOMAC and DRSP in human breast cancer cell lines *in vitro*. However, it is known that these progestins can be metabolised *in vivo*, as several metabolites of DRSP have been detected in human serum, urine and faeces (Krattenmacher, 2000; Bachmann and Kopacz, 2009; Wiesinger *et al.*, 2015), while metabolites of GES and NOMAC have been detected in human serum (Lello, 2010; Kuhl, 2011) and metabolites of GES have also been detected in urine (Stanczyk and Roy, 1990).

Generally, considering our published results (Skosana *et al.*, 2019) and the data in this chapter, there does not seem to be a correlation between the metabolism of progestins structurally related to P_4 (MPA, NES and NOMAC) or T (NET, LNG and GES). Differences observed

between cell lines could, however, be due to the expression of different steroidogenic enzymes responsible for the metabolism of progestins, or differences in the isoforms or levels of these enzymes. Although the precise mechanism for the differential metabolism of progestins in these breast cancer cell lines has not been elucidated, the results highlight the importance of investigating the metabolism of individual progestins in a chosen model system. These results makes one cognisant of the fact that publications where mechanisms of progestogens are investigated in the MDA-MB-231, T47D and MCF-7 BUS cell lines, may not reflect the effects of the progestin itself but rather the effects of a combination of the progestin and its metabolites, or effects of only the progestin metabolites.

2.3.2. The half-life of P_4 varies within the different cell lines.

As shown in our recent publication (Skosana *et al.*, 2019) and Figure 2.1, P_4 is extensively metabolised across all three cell lines at 24 hours, with the MDA-MB-231 and MCF-7 BUS cell line metabolising P_4 by 81% and 79%, respectively, with essentially complete metabolism in the T47D cell line (98%). Metabolism of P_4 in these breast cancer cell lines was not unexpected as it is known that the cell lines express the steroidogenic enzymes responsible for the metabolism of P_4. These enzymes include 5α-reductase type1/2 (SRD5A1/2), 20α-hydroxysteroid dehydrogenase (20α-HSD; AKR1C1), 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5; AKR1C3) and 3α-hydroxysteroid dehydrogenase type 2 (3α-HSD2; AKR1C2) (Piao *et al.*, 1997; Wiebe and Lewis, 2003; Lewis *et al.*, 2004; Wiebe, 2006; Hevir *et al.*, 2011; Louw-du Toit *et al.*, 2017a), which have been shown to metabolize P_4 to either 4-pregnenes or 5α-pregnanes in the MDA-MB-231, T47D and MCF-7 cell lines (Wiebe and Lewis, 2003). To analyse and compare the metabolism of P_4 over time (0.5 – 24 hours) in the three breast cancer cell lines, the MDA-MB-231, T47D and MCF-7 BUS cells were treated with 100 nM P_4 prior to steroid extraction and UHPSFC-MS/MS.

Figure 2.2. Metabolism of P$_4$ over time is cell line-dependent. Medium containing 100 nM P$_4$ was added to a 12-well plate containing no cells (negative control), as well as to the **(A)** MDA-MB-231, **(B)** T47D and **(C)** MCF-7 BUS cell lines for 0.5, 1.5, 3, 6, 12 and 24 hours. P$_4$ was then extracted from the medium and quantified using UHPSFC-MS/MS. The results are plotted as the % P$_4$ remaining, with the amount of P$_4$ in the negative control (no cells) for metabolism set as 100% and the amount of P$_4$ present in the medium after incubation with the cells set as a percentage relative to that (% indicated in the bars). The results shown are the averages (± SEM) of at least two independent experiments with each condition performed in quadruplicate. **(D)** To compare the metabolism of P$_4$ between the MDA-MB-231, T47D and MCF-7 BUS cells at the specific time points, the data in **A-C** were replotted. Statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns.
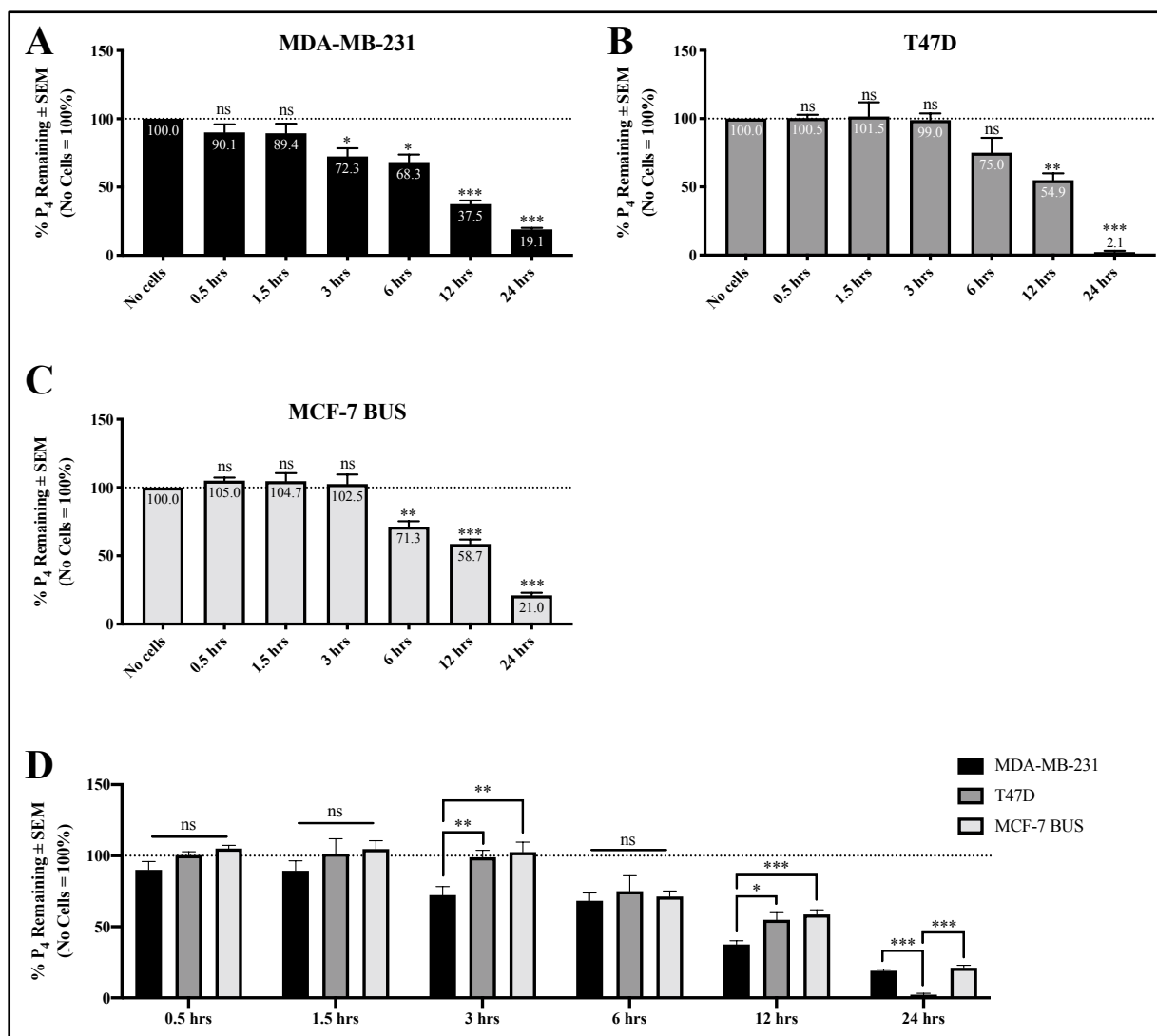
A no cell control for each time point was included as a negative control. Results show that P_4 was already metabolised by 28% at 3 hours in the MDA-MB-231 cells, (Figure 2.2A), while no metabolism was observed at 3 hours in the other two cell lines (Fig 2.2B and C). In the MCF-7 BUS cells, P_4 metabolism was first observed at 6 hours (Figure 2.2C), with the 29% metabolism similar to the percentage P_4 metabolism observed at 6 hours in the MDA-MB-231 cells (32%). Although it appears that metabolism of P_4 also occurs at 6 hours in the T47D cells (25%), this was not statistically significant. While it may be possible that this difference, or other differences, may also be significant but not within the statistical power of the current experiments, only statistically significant differences are discussed below. Significant metabolism of P_4 in the T47D cell was, however, observed at 12 hours (Figure 2.2B), with the percentage P_4 metabolised (45%) being similar to the percentage P_4 metabolised in the MCF-7 BUS cells at 12 hours (41%). However, P_4 metabolism at 12 hours in both the T47D and MCF-7 BUS cells was significantly less than that observed in the MDA-MB-231 cells at the same time point (63% metabolism) (Figure 2.2D). A number of other studies have also previously shown that P_4 can be metabolised in breast cancer cell lines (Horwitz *et al.*, 1983, 1986; Fennessey *et al.*, 1986; reviewed in Wiebe, 2006). For example, one study showed that 100 nM P_4 was completely metabolised after 16 hours in the T47D cell line (Horwitz *et al.*, 1983), while two other studies from the same group showed complete metabolism of 1 μM P_4 at 20 hours in both the T47Dco (Fennessey *et al.*, 1986; Horwitz *et al.*, 1986) and MCF-7 cell lines (Horwitz *et al.*, 1986). Although we did not investigate P_4 metabolism at 16 or 20 hours, our results showing complete metabolism of P_4 at 24 hours together with the results of the above-mentioned studies, suggest that complete metabolism of P_4 may in fact be between 16 and 24 hours in the T47D cells. In terms of P_4 metabolism in the MCF-7 cells, Wiebe and colleagues have also shown metabolism of 21% and 78% in MCF-7 cells at 6 hours and 24 hours, respectively, which is in agreement with the 29% and 79% we observe at these time points in

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the MCF-7 BUS cell line (Figure 2.2C) (Wiebe *et al.*, 2006). To determine the overall difference in P_4 metabolism between these three breast cancer cell lines over time, the half-life (t½), referring to the time needed for a 50% reduction in P_4, was determined. Results show that P_4 has a t½ of ~9 hours in the MDA-MB-231 cells (Figure 2.3A), ~8 hours in the T47D cells (Figure 2.3B) and ~12 hours in the MCF-7 BUS cells (Figure 2.3C). The statistical analysis in Figure 2.3D shows that the half-life for P_4 in the MDA-MB-231 and T47D cells are similar, while the half-life for P_4 in the MCF-7 BUS cells is significantly different to the half-life observed for P_4 metabolism in both the MDA-MB-231 and T47D cells. Two studies investigating the rate of P_4 metabolism in the T47Dco breast cancer cell line more than 30 years ago, report half-life values of ~2 hours (Horwitz *et al.*, 1983) and ~5 hours (Horwitz *et al.*, 1986), which is much faster than the t½ we determined in the T47D cells (~8 hours). The discrepancy in our half-life for the T47D cells compared to those reported by Horwitz and colleagues in the T47Dco cells is quite striking. Although we cannot discount the fact that differences may be due to the T47Dco cells being a variant of the wildtype T47D cells, this is unlikely. Bearing in mind that our t½ values were determined from only two independent experiments and that only two time points, 12 hours apart, showed significant metabolism, it is possible that our t½ values may not be accurate. However, if the t½ value determined for P_4 in the T47D cells is indeed correct, the discrepancy between our half-life and that determined by Horwitz and colleagues could be due to the different methodologies employed. Horwitz *et al.* used the gas chromatography (GC)/MS method for detection, which is known to be less sensitive than the newly developed UHPSFC-MS/MS method (reviewed in Storbeck *et al.*, 2018) used in this study. It is thus possible that lower concentrations of P_4 are detected by UHPSFC-MS/MS, but could not be by GC/MS.

Figure 2.3. The half-life of P₄ is similar between MDA-MB-231 and T47D cells, with both metabolising P₄ faster than MCF-7 BUS cells. Results in Figure 2.2 were reanalysed to determine t½ using non-linear regression, one phase decay analysis and replotted for the **(A)** MDA-MB-231, **(B)** T47D and **(C)** MCF-7 BUS cell lines. The results are plotted as the % P₄ remaining, with the amount of P₄ in the negative control (no cells) set as 100% and the amount of P₄ present in the medium after incubation with the cells set as a percentage relative to that. The t½ values were compared in **(D).** The results shown are the averages ($\pm$ SEM) of at least two independent experiments with each condition performed in quadruplicate. Statistically significant differences are represented by * indicating $p < 0.05$, while no statistical significance ($p > 0.05$) is indicated by ns.

2.4. Conclusion

Considering the clinical link between some progestins and breast cancer, these cell lines are often used to investigate progestin mechanisms, but do not seem to consider progestin metabolism. The results from this chapter and our recent publication (Skosana *et al.*, 2019)

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show for the first time that progestins commonly used in female reproductive medicine are metabolised *in vitro*, in the human MDA-MB-231, T47D and MCF-7 BUS breast cancer cell lines, in a progestin- and cell line-specific manner. As discussed in Skosana *et al.*, these *in vitro* results have both physiological and pharmacological implications if translated *in vivo*. For example, the differential metabolism of progestins suggest that for some progestins used in contraception and/or MHT, active metabolites could be produced which may influence physiological processes involved in the development and progression of breast cancer. Indeed it has been shown that 5α-P$_4$, a metabolite of P$_4$, increases growth and detachment as well as decreases apoptosis of breast cancer cells (reviewed in Wiebe, 2006; Wiebe *et al.*, 2015). Furthermore, progestin metabolites may confound the results of studies where progestins are compared in terms of pharmacological characteristics, such as biocharacter, efficacy and potency.

Although metabolism of P$_4$ occurred in all the cell lines investigated, our preliminary results show that the t½ values of P$_4$ in the MDA-MB-231 and T47D cells are similar to each other, but significantly shorter than that determined in the MCF-7 BUS cells. Differences in the half-life of P$_4$ between model systems has further implications as numerous studies use P$_4$ as a reference ligand when investigating mechanisms of the PR or when comparing progestin responses to P$_4$ (Tegley *et al.*, 1998; Attardi *et al.*, 2002b, 2010; Austin *et al.*, 2002; Richer *et al.*, 2002; Bray *et al.*, 2005; Bain *et al.*, 2015; Finlay-Schultz *et al.*, 2015; Mohammed *et al.*, 2015; González-Orozco *et al.*, 2020). Taken together, our published results, together with the results in this chapter, have been insightful into the metabolism of P$_4$ and a number of clinically used progestins in three breast cancer cell lines *in vitro*. Further investigations are needed to understand the mechanisms behind the observed differential metabolism of these progestins, and to identify active progestin metabolites.

Chapter 3

Evaluating the role of the progesterone receptor isoforms and ratios on the regulation of gene expression by progestogens

3.1. Background and aims

Progestins, or synthetic progestogens, are used by women worldwide for various applications in female reproductive medicine including, but not limited to, contraception, MHT and the treatment of endometriosis (Speroff and Darney, 1996; Hapgood *et al.*, 2004; Gellersen *et al.*, 2009; Africander *et al.*, 2011; Stanczyk *et al.*, 2013). There are many different progestins available for clinical use which have been classed into four consecutive generations (Stanczyk *et al.*, 2013; Schindler, 2014). Despite the therapeutic benefits of progestins, a number of negative side-effects such as an increased risk of developing breast cancer, have been associated with the use of some progestins in MHT (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; Chlebowski *et al.*, 2013; Manson *et al.*, 2013; Collaborative Group on Hormonal Factors in Breast Cancer, 2019) and contraception (Li *et al.*, 2012; Mörch *et al.*, 2017; Graafland *et al.*, 2020). As these compounds were designed to mimic the actions of the natural progestogen, P_4, by binding to the PR [reviewed in (Africander *et al.*, 2011; Stanczyk *et al.*, 2013; Schindler, 2014)], negative effects are often thought to be due to off-target activity via other members of the steroid receptor family, such as the GR, AR and MR. It is thus not surprising that a number of studies, including ours, have investigated the mechanisms of action of different progestins via these receptors (Attardi *et al.*, 2002, 2010; Austin *et al.*, 2002; Koubovec *et al.*, 2005; Sasagawa *et al.*, 2008b; Ronacher *et al.*, 2009; Escande *et al.*, 2009; Africander *et al.*, 2013, 2014; Bain *et al.*, 2014; Louw-du Toit *et al.*, 2017b; Kumar *et al.*, 2017; Louw-du Toit *et al.*, 2020). Similar studies for progestins via the PR, particularly comparative studies and those distinguishing between the two PR isoforms, PR-A and PR-B, are scant. Our results in Chapter 2 and in Skosana *et al.*, (2019) (Addendum A1) showing cell-specific metabolism of progestins, highlight one of the multiple reasons why non-parallel comparisons of progestin activity across different cell lines may not be an accurate reflection of progestin activity. The importance of investigating the role of the individual

isoforms is highlighted by the fact that PR-A and PR-B can regulate similar, but also distinct sets of genes, while both have been implicated in the development and progression of breast cancer (Richer *et al.*, 2002; Jacobsen *et al.*, 2002, 2005; Scarpin *et al.*, 2009; Santos *et al.*, 2010; Jacobsen and Horwitz, 2012; Giulianelli *et al.*, 2012; Singhal *et al.*, 2018; Truong *et al.*, 2019). The role of the PR isoforms in the breast, however, is complex and can be influenced by the levels at which the isoforms are expressed (Graham *et al.*, 2005; McFall *et al.*, 2015; Lamb *et al.*, 2018; Horwitz and Sartorius, 2020; Sathyamoorthy and Lange, 2020). In normal breast tissue, the isoforms are expressed at equivalent levels, while in breast tumours the ratio of the PR isoforms often vary. Although equivalent levels of the PR isoforms have been detected in some tumours, a few tumours overexpress PR-B relative to PR-A, while the majority of tumours overexpress PR-A relative to PR-B (Graham *et al.*, 1995; Graham and Clarke, 2002; Mote *et al.*, 2002, 2015; Hopp *et al.*, 2004; Rojas *et al.*, 2017; Lamb *et al.*, 2018; McFall *et al.*, 2018; Singhal *et al.*, 2018). Where PR-A is overexpressed relative to PR-B, various ratios have been shown which commonly range between 1.2:1 and 5.5:1 (Graham *et al.*, 1995; Hopp *et al.*, 2004; Rojas *et al.*, 2017; McFall *et al.*, 2018), while at least one study has shown excessively high PR-A:PR-B ratios ranging between 35.9:1-115.3:1 (Graham *et al.*, 1995).

In this Chapter, the primary aim was to directly compare the activities of P_4 and selected progestins from all four generations on gene expression via the individual PR isoforms, and to elucidate whether the PR-B-mediated effects are modulated by the co-expression of PR-A, either at equivalent levels or in excess.

The objectives of this chapter were four-fold:

1. To perform a side-by-side pharmacological characterisation of P_4 and selected progestins from different generations in terms of their relative agonist efficacy

(maximal response) and potency (EC_{50}) for transactivation and transrepression via human PR-A and PR-B. These studies were performed using promoter-reporter constructs in the MDA-MB-231 breast adenocarcinoma cell line transiently transfected with the respective PR isoform.

2. To examine the effects of co-expression of PR-B and PR-A, as well as increasing concentrations of PR-A on the agonist activity of the progestogens for transactivation and transrepression via PR-B, the MDA-MB-231 breast cancer cell line was transiently transfected with both PR isoforms at a PR-A:PR-B ratio of 1:1, 2:1 and 5:1.

3. To directly compare the regulation of physiologically relevant genes endogenously expressed in the human T47D breast cancer cell line, which expresses both PR isoforms, by P_4 and the selected progestins, under estrogenic and non-estrogenic conditions.

4. To determine the role of PR-A and PR-B in the progestogen-induced regulation of gene expression observed in objective 3, and examine whether this regulation is influenced by increased expression of PR-A.

3.2. Materials and Methods

3.2.1. Cell culture

The triple negative human MDA-MB-231 and T47D breast cancer cell lines were maintained as described in Section 2.2.1 of Chapter 2. The COS-1 monkey kidney cell line was purchased from the American Type Culture Collection (ATCC) and maintained in phenol red Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, South Africa), 100 U/mL penicillin and 100 μg/mL streptomycin (1% (v/v) penicillin-streptomycin) (Sigma-Aldrich,

South Africa). All cell lines were maintained in 75 cm^2 culture flasks (Bio-Smart Scientific, South Africa) and incubated at 37ºC in an atmosphere of 90% humidity and 5% CO$_2$. Cells used for experiments were performed within the first 30 passages once thawed from storage and were regularly tested for mycoplasma infection through Hoechst staining (Freshney, 1987). Only mycoplasma negative cells were used for experiments.

3.2.2. Test compounds

The following steroids were purchased from Sigma-Aldrich, South Africa: MPA [6α-methyl-17-acetoxy pregn-4-ene-3,20-dione], NET [19-nor-17α-ethynyltestosterone], LNG [3β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one], NES [16-methylene-17α-acetoxy-19-nor-4-pregnen-3,20-dione], 17β-estradiol (E$_2$) [17β-estra-1,3,5(10)-etriene-3,17 diol] and phorbol myristate acetate (PMA) [phorbol-12-myristate-13-acetate]. NET was used in this study as it is the active metabolite following the rapid metabolism of NET-acetate (NET-A) (Stanczyk and Roy, 1990), and previous studies have shown that NET-A and NET elicit similar effects in endocervical and breast cancer cell lines (Govender *et al.*, 2014; Perkins, PhD dissertation, 2018). R5020, P$_4$, GES, NOMAC and DRSP were previously described in Chapter 2, Section 2.2.2. All stock solutions and experimental dilutions were made up as described in Section 2.2.2.

3.2.3. Plasmids

Human PR-A and PR-B encoding cDNA expression vectors (pSG5-hPR-A and pSG5-hPR-B) (Kastner *et al.*, 1990) were a kind gift from Dr. Eric Kalkhoven (University Medical Centre, Utrecht, Netherlands), while the pSG5-empty cDNA expression vector containing no eukaryotic promoter or enhancer sequences was received from Prof. Gunnar Mellgren (University of Bergen, Norway). The cDNA expression vectors encoding the human GR (pRS-

hGRα) (Giguère *et al.*, 1986) was a kind gift from Prof. R. Evans (Howard Hughes Medical Institute, La Jolla, CA, USA), while the expression vector expressing the human AR (pSV-ARo) (Brinkmann *et al.*, 1989) was obtained from Prof. Frank Claessens (University of Leuven, Leuven, Belgium). The plasmid expressing human ERα (pSG5-ERα) was received from Prof. Frank Gannon (European Molecular Biology Laboratory, Heidelberg, Germany) (Flouriot *et al.*, 2000). The pTAT-2xPRE-E1b-luciferase promoter-reporter construct driven by the E1b promoter and containing two copies of the progesterone response element (PRE) from the rat tyrosine amino transferase (TAT) gene (Jenster *et al.*, 1997), was a gift from Prof. Guido Jenster (Erasmus University of Rotterdam, Rotterdam, Netherlands), while the 5xNFκB-luciferase promoter-reporter construct was purchased from Stratagene (Houston, Texas). All plasmid DNA was purified using the NucleoBond® Xtra Maxi Plasmid Preparation Kit (Macherey-Nagel, California, USA) according to the manufacturer's instructions.

3.2.4. Western blot analysis

MDA-MB-231 and T47D breast cancer cell lines were seeded into 12-well tissue culture plates (Bio-Smart Scientific, South Africa) at a density of 1 x 10^5 cells per well and incubated for 48 or 72 (siRNA transfections) hours. For positive controls, COS-1 cells (l x 10^5 cells per well in a 12-well tissue culture plate) were transiently transfected with 250 ng of an expression vector for a specific steroid receptor, while COS-1 cells transfected with the pSG5-empty vector were used as a negative control. Cells were washed with 1x PBS and total protein harvested in 2x Laemmli sodium dodecyl sulfate (SDS)-sample buffer [100 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 5% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 2% (v/v) β-mercaptoethanol] (Sambrook *et al*., 1989). All protein samples were denatured by boiling for 10 minutes at 97ºC prior to separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 15 minutes, followed by 200 V for 1 hour in 1x SDS-PAGE electrophoresis buffer (35 mM SDS,

250 mM Tris, 1.92 M glycine). The separated proteins were subsequently electroblotted to nitrocellulose membranes (AEC Amersham, South Africa) in ice-cold 1x transfer buffer [25 mM Tris-HCl, 192 mM glycine, 1% (w/v) SDS, 10% (v/v) methanol] at 180 mA for 2 hours. Membranes were blocked for 90 minutes at room temperature with agitation using 10% (w/v) fat-free milk powder prepared in 1x Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBST). The membranes were subsequently probed with primary antibodies specific for PR-A/B (Leica Biosystems, UK), ERα, AR, GR, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, USA) (Table 3.1) at 4ºC for 16 hours with agitation. GAPDH was used as the loading control. The membranes were washed with 1x TBST once for 15 minutes and 3x for 5 minutes prior to incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 3.1) (Santa Cruz Biotechnology, USA) for 90 minutes at room temperature with agitation. Membranes were washed with 1x TBST once for 15 minutes and twice for 5 minutes, followed by a 5-minute wash in 1x TBS. Proteins were visualised by enhanced chemiluminescence using the Bio-Rad Clarity™ Western ECL substrate (Bio-Rad Laboratories, Inc.) and MyECL Imager (Pierce Thermo Scientific Inc., USA). Images were quantified using ImageJ™ Software (v.1.8).

Table 3.1. Details of antibodies used for western blot analyses.

Protein	Size (kDa)	Primary Antibody (Dilution)	Secondary Goat Antibody (Dilution)
PR-A/B	94 (PR-A) 114 (PR-B)	Mouse anti-human PGR-312 L-CE (1:1 000)	anti-mouse (1:2 000)
ER-α	66	Rabbit anti-human ER-α HC-20 (1:1 000)	anti-rabbit (1:2 000)
AR	110	Mouse anti-human AR 441 (1:1 000)	anti-mouse (1:5 000)
GR	90	Rabbit anti-human GR H-300 (1:3 000)	anti-rabbit (1:4 000)
GAPDH	37	Mouse anti-human GAPDH 0411 (1:2 000)	anti-mouse (1:3 000)

3.2.5. Promoter-reporter assays

The human MDA-MB-231 breast cancer cells were seeded into 10 cm tissue culture dishes (Bio-Smart Scientific, South Africa) at a density of 2 x 10^6 cells per dish, in supplemented phenol red DMEM. After 24 hours, the cells were rinsed with 10 mL pre-warmed 1x PBS (Quantum Biotechnologies, South Africa) and the medium replaced with phenol red-free DMEM supplemented with 10% (v/v) charcoal stripped (CS)-FBS and 1% (v/v) penicillin-streptomycin. For transactivation assays, the cells were transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase construct and 900 ng of pSG5-PR-A or pSG5-PR-B plasmids, or 900 ng pSG5-PR-B plasmid and 900 ng (1:1), 1 800 ng (2:1) or 4 500 ng (5:1) pSG5-PR-A plasmid. The pSG5-empty vector was used to ensure a constant total DNA concentration of 14.4 μg in all experiments. For transrepression assays, the cells were transiently transfected with 3 000 ng of the 5xNFκB-luciferase reporter plasmid and 1 500 ng pSG5-PR-A or pSG5-PR-B plasmids, or 1 500 ng pSG5-PR-B plasmid and 1 500 ng (1:1), 3 000 ng (2:1) or 7 500 ng (5:1) pSG5-PR-A plasmid. The pSG5-empty vector was used to ensure a constant DNA concentration of 12 μg in all experiments. X-tremeGENE-HP transfection reagent (Roche Molecular Biochemicals) was used for all transfections following the manufacturer's instructions. After 24 hours, all transfected cells were seeded into 96-well plates at a density of 1 x 10^4 cells per well and allowed to settle. For transactivation assays, cells were treated with either 0.1% (v/v) ethanol or increasing concentrations of R5020, P_4, MPA, NET, LNG, GES, NES, NOMAC or DRSP in phenol red-free DMEM supplemented with 10% CS-FBS and 1% penicillin-streptomycin for 24 hours. For transrepression, cells were treated for 24 hours with 10 ng/mL PMA in the absence (0.2% (v/v) ethanol) or presence of increasing concentrations of the selected progestogens. All cells were subsequently washed with 1x PBS, lysed with 25 μL passive lysis buffer [0.2% (v/v) Triton-X-100, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-ethylenediaminetetra-acetic acid (EDTA) and 1.44 mM

EDTA] and stored at -20°C. Cell lysates were thawed and the luciferase activity, in relative light units (RLUs), was determined using the Promega Luciferase Assay System (Promega, USA) and a Veritas™ microplate luminometer (Turner Biosystems, USA). The RLU values were normalized to the protein concentration (mg/mL) of each lysate determined by the Bradford protein assay (Bradford, 1976). Normalising to protein concentrations is an accepted method for bulk transfections where cells are subsequently reseeded into a number of wells (Visser *et al.*, 2013).

3.2.6. Total RNA isolation and cDNA synthesis

The human T47D breast cancer cells were seeded into 10 cm dishes at a density of 2×10^6 cells per dish. The following day, cells were transiently transfected with 4 500 ng of either the pSG5-PR-A expression vector or the pSG5-empty vector (to ensure that all cells were transfected with the same total amount of DNA). Twenty-four hours later, the transfected cells were seeded into 12-well plates at 1×10^5 cells per well in supplemented phenol red-free DMEM. The next day, the cells were treated with either 0.2% (v/v) ethanol or 100 nM R5020, P_4, MPA, NET, LNG, GES or DRSP in the absence or presence of 100 nM E_2 in serum-free phenol red-free DMEM. The treatment time for each gene was optimised and is specified in the relevant figure legends. Total RNA was isolated using TRI Reagent® (Sigma-Aldrich, South Africa) according to the manufacturer's instructions. Briefly, cells were washed with 1x PBS and lysed with 400 µL TRI Reagent®. The lysates were transferred into 1.5 mL microcentrifuge tubes and incubated at room temperature for 5 minutes. Following the addition of 80 µL chloroform (Merck, South Africa), the solution was vortexed for 15 seconds before incubation at room temperature for 3 minutes, and centrifugation at 18 407 x g at 4°C for 15 minutes. The aqueous phase was subsequently transferred to a new microcentrifuge tube and an equal volume of ice-cold isopropanol was added. Samples were vortexed for 15 seconds and incubated at room

temperature for 15 minutes. RNA was pelleted by centrifugation at 18 407 x g for 15 minutes at 4°C. The supernatant was discarded, and the RNA pellet washed with 500 μL 75% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water. The samples were vortexed for approximately 1 minute before centrifugation at 6 010 x g for 10 minutes at 4°C. The supernatant was removed and the pellet air dried for 5 – 10 minutes on ice. RNA was dissolved in DEPC-treated water and the concentration and purity determined spectrophotometrically at 260/280 nm using a NanoDrop (ND-100 Spectrophotometer) (Thermo Fisher Scientific, USA). The samples were electrophoresed in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 nM EDTA) at 60 V for 1 hour on a 1% (w/v) denaturing formaldehyde agarose gel and visualised using the MyECL Imager. The integrity of the isolated RNA was confirmed by the presence of intact 28S and 18S ribosomal RNA bands on a denaturing agarose gel (data not shown). All RNA samples were stored at – 80ºC. Total RNA extracted from the T47D cell line was reverse transcribed using the ImProm-II^{TM} Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. Briefly, cDNA was synthesized from 0.5 μg total RNA based on oligo(dT) priming. To denature RNA secondary structures, the template-primer mixture was incubated at 70ºC for 5 minutes, followed by a 5-minute incubation on ice. The following components were subsequently added per reverse transcription reaction: ImProm-II 1X reaction buffer, $MgCl_2$ (final concentration of 1 mM), dNTPs (final concentration of 0.5 mM of each nucleotide), recombinant RNasin ribonuclease inhibitor (final concentration of 10 U) and ImProm-II reverse transcriptase (final concentration of 80 U). The samples were incubated at 25ºC for 5 minutes to allow for annealing of primers, 60 minutes at 45ºC for extension, and 70ºC for 15 minutes to inactivate the reverse transcriptase. All cDNA samples were stored at -20°C.

3.2.7. Realtime quantitative polymerase chain reaction (qPCR)

Realtime qPCR was conducted using the LightCycler® 96 (Roche applied Science, South Africa), as per the manufacturer's guidelines. The PCR reaction was prepared using SYBR Green (KAPPA SYBR® Fast ABI Prism®, Roche, South Africa), forward and reverse primers (final concentration of 0.5 µM each) and sterile PCR-grade water to a final volume of 9 µL. Then, 1 µL of either sterile PCR grade water (no template) or cDNA was added. The forward and reverse primers used in this thesis have all been previously described and the details are shown in Table 3.2.

Table 3.2. Details of primer sets used for quantitative analyses of gene expression.

Gene	Primers (5' - 3')	Amplicon Length (bp)	Primer efficiency	Reference
GATA3	F: CAGACCACCACAACCACACTCT R: GGATGCCTTCCTTCTTCATAGTCA	124	1.99	(Izzo *et al.*, 2014)
SOX4	F: CTTGACATGATTAGCTGGCATGATT R: CCTGTGCAATATGCCGTGTAGA	104	2.06	(Wang *et al.*, 2015)
TGFβ1	F: CAATTCCTGGCGATACCTCAG R: AACCACTGCCGCACAACT	96	2.08	(Ekhteraei-Tousi *et al.*, 2015)
BAX	F: GAGAGGTCTTTTTCCGAGTG R: GGTGAGGAGGCTTGAGGAGT	234	2.03	(Ding *et al.*, 2014)
FOXO1	F: ACGAGTGGATGGTCAAGAGC R: GCACACGAATGAACTTGCTG	120	2.02	(Diep *et al.*, 2013)
Ki67	F: CGGACTTTGGGTGCGACTT R: GTCGACCCCGCTCCTTTT	203	2.11	(Chottanapund *et al.*, 2013)
GAPDH	F: TGAACGGGAAGCTCACTGG R: TCCACCACCCTGTTGCTGTA	307	2.00	(Ishibashi *et al.*, 2003)

Abbreviations: GATA3, GATA-binding protein 3; SOX4, Sex-determining region Y-related high mobility group box 4; TGFβ1, transforming growth factor beta 1; FOXO1, Forkhead box protein O1; Ki67, antigen Ki-67; BAX, BCL-2-like protein 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Melting curve analysis confirmed the presence of a single amplicon. Standard curves generated from a cDNA dilution series were used to determine the amplification efficiency of each primer set, and these primer efficiencies are shown in Table 3.2. The relative transcript levels of the target genes were normalised to the relative GAPDH transcript levels using the Pfaffl mathematical equation (Pfaffl, 2001).

3.2.8. Small interference RNA (siRNA) transfection

The human T47D breast cancer cells were seeded into 10 cm dishes at a density of 2×10^6 cells per dish. The following day, the cells were transiently transfected with either 10 nM validated non-silencing scrambled sequence control (NSC) siRNA (Qiagen, USA), validated siRNA directed against both PR isoforms (a combination of four target-specific siRNAs – in this thesis referred to as PR-A/B siRNA (Qiagen, USA)) or directed against PR-B only (a combination of two target-specific siRNAs targeting only PR-B (Hardy *et al.*, 2008)). DharmaFECT transfection reagent (Dharmacon, USA) diluted in OptiMEM reduced serum media (Gibco, Thermo Fisher Scientific, South Africa) was used for all siRNA transfections according to the manufacturer's instructions. Cells were incubated with the siRNA transfection mix for 48 hours before seeding the transfected cells into 12-well plates at a density of 1×10^5 cells per well using supplemented phenol red-free DMEM. The next day, the cells were treated with the test compounds in serum free DMEM for various times, as indicated in the figure legends, prior to harvesting RNA for realtime qPCR. PR protein knockdown levels were confirmed using western blot analysis as described in Section 3.2.4.

3.2.9. Data and statistical analysis

The GraphPad Prism® v7.00 software package (GraphPad Software, USA) was used for analysis of results, graphical representation and statistical analysis. One-way analysis of

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variance (ANOVA) with the Bonferroni (compares all pairs of columns) post-test, or two-way ANOVA with the Bonferroni post-test, was used for statistical analyses. Unpaired *t*-tests were used for statistical analysis where experiments were not conducted in parallel. Unless otherwise stated, the error bars indicate the standard error of the mean (SEM) of three or more independent experiments.

3.3. Results and Discussion

3.3.1. Progestogens display differential agonist efficacies and potencies via both PR-A and PR-B.

Although a number of studies have reported efficacies (maximal response) and potencies (EC_{50} values) of some progestins for transactivation via the human PR (Markiewicz and Gurpide, 1994; Tegley *et al.*, 1998; Lim *et al.*, 1999; Attardi *et al.*, 2002, 2010; Austin *et al.*, 2002; Madauss *et al.*, 2004; Zhang *et al.*, 2005; Bray *et al.*, 2005; Abdel-Hafiz *et al.*, 2009; Escande *et al.*, 2009; Bain *et al.*, 2015), most studies did not specify the isoform involved or compare these parameters for different progestins, via the individual PR isoforms in the same model system. We thus directly compared the agonist efficacies and potencies of selected progestins from different generations to each other, P_4 and R5020 for transactivation via either human PR-A or PR-B.

Human MDA-MB-231 breast cancer cells transiently transfected with a PRE-containing luciferase reporter construct and an expression vector for either PR-A or PR-B, were treated with increasing concentrations of the selected progestogens for 24 hours. Although these cells endogenously express the GR (Horwitz *et al.*, 1978; Leo *et al.*, 2004) (Addendum C, Figure C1) to which some progestins can bind (Koubovec *et al.*, 2005; Ronacher *et al.*, 2009), the

transcriptional activity of the progestogens in the absence of transfected PR-A and PR-B is negligible (Addendum C, Figure C2). In addition, as the MDA-MB-231 cells do not endogenously express PR-A, PR-B, AR, or ERα (Addendum C, Figure C1), this cell system thus allows for the direct comparison of the activities of the progestogens via the specific transiently transfected PR isoform. The efficacies and potencies of the progestogens for PR-A and PR-B are summarised in Table 3.3. Given that natural P_4, but not R5020, is metabolised in the MDA-MB-231 cells (Chapter 2), together with the evidence in the literature that R5020 is more stable than P_4 (Sathyamoorthy and Lange, 2020), R5020 was used as the reference ligand in this chapter. Here, only statistically significant results will be discussed, even though there may be other differences that seem significant but not within the statistical power of these experiments. In fact, we have recently reported that establishing statistically significant differences for efficacy and potency values determined by dose-response analysis, when multiple ligands are investigated in parallel, is challenging (Enfield *et al.*, 2020) (Addendum A2).

Results in Figure 3.1A and 3.1B show that the 2nd generation progestin LNG displays a similar agonist efficacy to R5020 via PR-A, while all other progestins and P_4 display a lower efficacy. Although this suggests that LNG is a full agonist via PR-A, while P_4 and the other progestins are partial agonists, LNG, NET and GES have indistinguishable efficacies. No significant difference in efficacy is observed between P_4, the 1st generation MPA and the 4th generation progestins NES, NOMAC and DRSP, while the 1st generation NET, LNG and 3rd generation GES were significantly more efficacious than P_4 via PR-A (Figure 3.1B). Notably, NET, LNG and GES are all structurally related to testosterone, while all other progestins, except DRSP, are structurally related to P_4. Interestingly, the results in Figures 3.1D and 3.1E show that the efficacy of R5020, P_4, LNG, GES and NES via PR-B are all indistinguishable, suggesting that they are all full PR-B agonists, while MPA, NET, NOMAC and DRSP display partial agonist

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Figure 3.1. continues on the following page.

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D

E

F

	P$_4$	MPA	NET	LNG	GES	NES	NOMAC	DRSP
P$_4$		***	ns	ns	ns	ns	***	***
MPA			**	**	***	**	ns	ns
NET				ns	ns	ns	ns	**
LNG					ns	ns	*	**
GES						ns	***	***
NES							*	***
NOMAC								ns
DRSP								

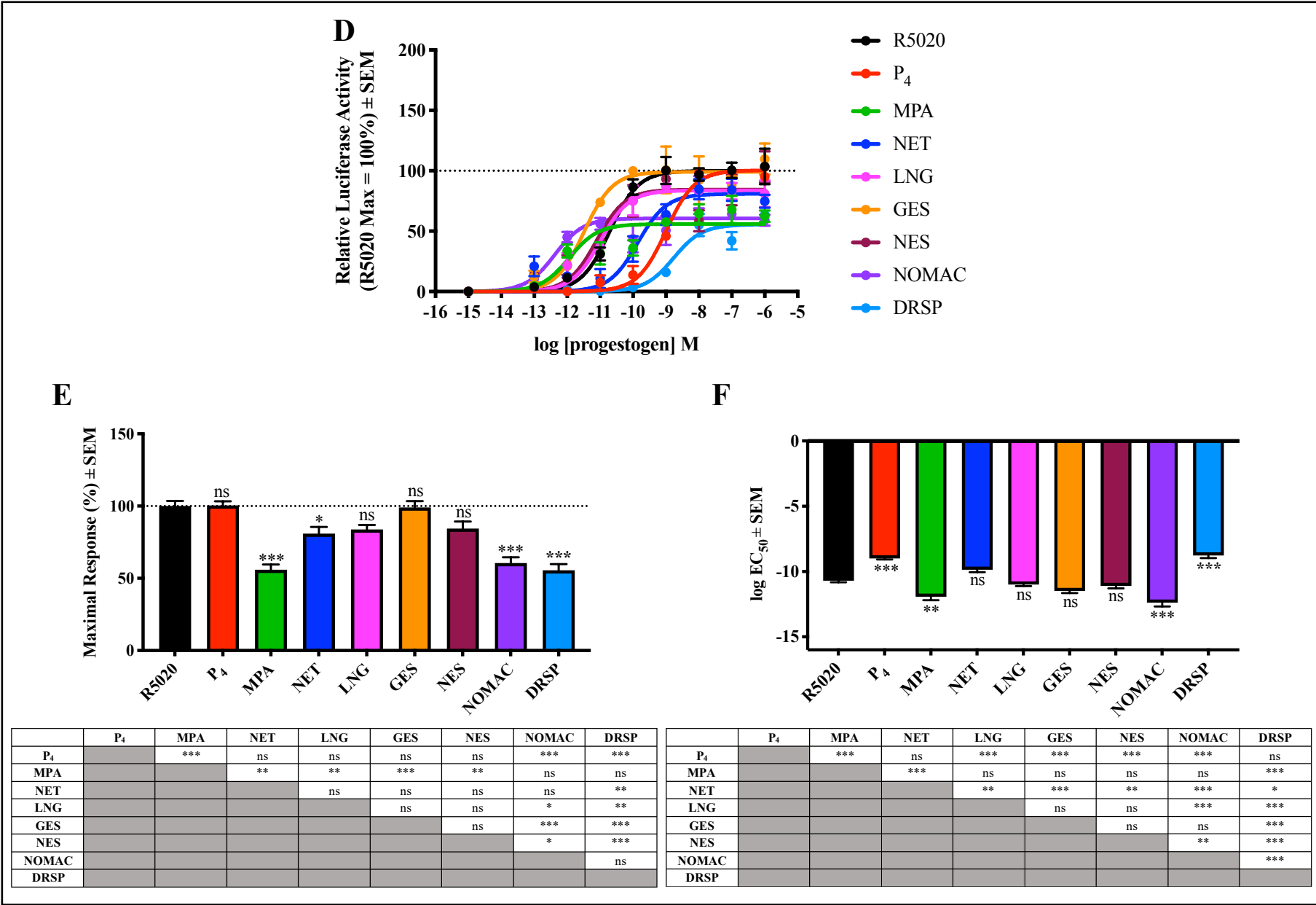|	P$_4$	MPA	NET	LNG	GES	NES	NOMAC	DRSP
P$_4$		***	ns	***	***	***	***	ns
MPA			***	ns	ns	ns	ns	***
NET				**	***	**	***	*
LNG					ns	ns	***	***
GES						ns	ns	***
NES							**	***
NOMAC								***
DRSP								

Figure 3.1. Figure legend on the following page.

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Figure 3.1. While all progestogens, except LNG, are partial agonists via PR-A, only MPA, NET, NOMAC and DRSP are partial agonists via PR-B. The MDA-MB-231 cells were transiently transfected with 9 000 ng pTAT-2xPRE-E1b-luciferase reporter construct and 900 ng of either **(A)** pSG5-PR-A or **(D)** pSG5-PR-B. Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 24 hours. Luciferase activity was normalised to protein concentration. Results are shown as relative luciferase activity, where the maximal response with R5020 was set as 100%, and all other responses were calculated relative to this. Plots are shown for the maximal responses and $logEC_{50}$ values of the progestogens via **(B and C)** PR-A and **(E and F)** PR-B. The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns. Statistically significant differences relative to R5020 (reference agonist) are shown on the graph, while significant differences between progestogens are indicated in the tables below the graph.

activity as they display lower efficacies. Again, it is noteworthy that the efficacies of LNG, GES, NES and NET are indistinguishable from one another. At least one other study has reported that MPA is less efficacious than P_4 via the PR transiently transfected into the CV-1 cell line (Tegley *et al.*, 1998), albeit that the authors did not specify the isoform investigated. Given that our results show that the efficacies of P_4 and MPA are indistinguishable via PR-A, while MPA is less efficacious than P_4 via PR-B, it is likely that PR-B was used in that study. To our knowledge, only one other study has directly compared the efficacies for R5020, P_4, MPA and NET. Unlike our results showing that MPA displays a lower efficacy than R5020, P_4, and NET via PR-B in the MDA-MB-231 cells, these authors showed that MPA was more efficacious than the other ligands (Markiewicz and Gurpide, 1994). The reason for the discrepancy between our studies may be due to the fact that only PR-B, and negligible GR, were expressed in our system, while their study used T47D cells which not only express both PR isoforms, but also other competing steroid receptors. Collectively, when comparing the

efficacies of the progestogens via PR-A or PR-B to each other, there is no clear pattern between the generations of progestins or within a generation. This was also seen for the potencies of the progestins. Although the 4th generation progestins were designed to be more PR-specific compared to the earlier generation progestins (Sitruk-Ware, 2004), the progestins within this generation are not always the most potent via either PR-A or PR-B. For example, the 3rd generation GES is more potent than the 4th generation NOMAC via PR-A (Figure 3.1A and C). In fact, NOMAC displays a similar potency to the 1st generation progestins MPA and NET, as well as 2nd generation LNG. On the other hand, the other 4th generation progestins NES and DRSP, are more potent than both 1st generation progestins, but similar to the 2nd and 3rd generation LNG and GES, respectively. NES is the only 4th generation progestin that is more potent than P_4 via PR-A, while NOMAC and DRSP are as potent as P_4. Conversely, NES and NOMAC are more potent than P_4 via PR-B (Figure 3.1D and F), while DRSP displays a similar potency to P_4. Interestingly, DRSP, the only spironolactone-derived progestin, has a lower potency than all of the selected progestins via PR-B. While NES and NOMAC are more potent than NET, these 4th generation progestins display a similar potency to MPA and GES. In addition, NES is similar to LNG, while NOMAC is more potent compared to both NES and LNG.

Although several studies have investigated the potencies of R5020, P_4, MPA, NET, LNG, GES, NES and DRSP via the PR (Table 3.3), these were mostly non-parallel studies conducted in model systems expressing other steroid receptors to which progestins can bind, which may confound these results. Moreover, most of these studies did not distinguish between the isoforms. This study is thus the first to directly compare the agonist efficacies and potencies of P_4 and the selected progestins for transactivation in the same model system via either human PR-A or PR-B (Table 3.3), and the first study to report the efficacy and potency for NOMAC via the human PR-A and PR-B. Discrepancies in potency values between our study and those

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reported in the literature for the PR (Table 3.3) could be attributed to differences in the systems used, such as the cell lines used, co-expression of PR-A and PR-B, density of steroid receptors, presence of specific steroidogenic enzymes, and/or the specific response element in the reporter construct. For example, the study by Bain and colleagues reported higher potency values for P4, NET, LNG, GES and DRSP, in U2OS cells stably expressing a reporter construct containing multiple copies of the PRE, but did not specify which PR isoform was investigated or the concentration of PR transfected into the cells (Bain *et al.*, 2015). Knowledge of all of the aforementioned parameters is important, as we have shown that the efficacies and potencies of some progestins are influenced by the concentration of the PR-B expression plasmid transiently transfected into the MDA-MB-231, COS-1 and U2OS cells (Enfield *et al.*, 2020). The context of the promoter is also important, as it has been shown that P4 has a higher transcriptional activity via PR-B in CV-1 cells transiently transfected with a PRE2tk- luciferase versus an MMTV-luciferase reporter construct (Vegeto *et al.*, 1993). In addition, P4, LNG, GES and DRSP can bind to the AR (Louw-du Toit *et al.*, 2017b), which is endogenously expressed in the U2OS cells (Gnanapragasam *et al.*, 2000), while it is also known that NET is metabolised in U2OS cells but not MDA-MB-231 cells (Skosana *et al.*, 2019). Indeed, it is likely that differential metabolism of progestogens across model systems may contribute to the large range in potency values reported for the PR in literature (Table 3.3). EC50 values reported for R5020 (Attardi *et al.*, 2002), P4 (Attardi *et al.*, 2002, 2010; Bray *et al.*, 2005), MPA (Attardi *et al.*, 2002; Bray *et al.*, 2005), NET- A, LNG (Bray *et al.*, 2005; Attardi *et al.*, 2010), NES (Attardi *et al.*, 2010) and DRSP (Bray *et al.*, 2005) were from studies performed in T47D cells endogenously co-expressing both PR-A and PR-B. The majority of these values were higher than those reported in our study. The potencies for P4 and NET reported by Bray and colleagues, however, are similar to that reported in our study via PR-B, but higher compared to PR-A (Table 3.3). For those studies that do specify the PR isoform, albeit limited, some

report similar potency values to those determined in our study, while others report different values. For example, the potency of 60.5 pM reported by Lim and colleagues via PR-A (Lim *et al.*, 1999) is similar to that (81 pM) determined by us. Similarly, the values. reported via PR-B, for R5020 in HeLa cells (Abdel-Hafiz *et al.*, 2009), as well as LNG and NES in HEK293 cells (Kumar *et al.*, 2017), are consistent with the values reported in our study (Table 3.3).

Table 3.3. Relative agonist efficacies (maximal response) and potencies (EC_{50}) of the progestogens for transactivation via either PR-A or PR-B on a synthetic PRE-containing reporter construct.

| Ligand | Maximal Response (%) ± SEM | | EC_{50} (pM) ± SEM | | EC_{50} (pM) |
	PR-A	PR-B	PR-A	PR-B[a]	Reported in literature for the PR[b]
R5020	100.0 ± 5.2	100.0 ± 3.5	81.3 ± 28.4	18.03 ± 6.19	2.23[§], 60.5[$], 120[§], 160[*], 290[*], 390[*], 5000[##]
P4	47.1 ± 3.3	100.4 ± 2.9	2.0 ± 1.3	791.0 ± 265.0	98[§], 400[§], 580[*], 800[§], 1000[*], 2330[*], 2900[#], 3090[*], 3500[*], 5810[#], 25 000[*]
MPA	43.4 ± 2.5	55.9 ± 3.6	4.9 ± 2.9	0.71 ± 0.57	50[*], 100[§], 120[*], 150*[#], 680[*]
NET	74.1 ± 6.2	80.9 ± 4.6	8.5 ± 7.1	339.7 ± 180.4	53[§], 200[§], 380[*], 400[*], 580[*], 909[*], 1550[#]
LNG	81.8 ± 5.5	83.8 ± 3.1	0.7 ± 0.4	8.22 ± 3.82	5.8[§], 169[*], 190[*], 200[*], 342[#], 570[*]
GES	74.1 ± 3.7	99.1 ± 4.3	0.08 ± 0.05	3.3 ± 1.3	126[#]
NES	57.0 ± 3.7	84.5 ± 4.8	0.04 ± 0.03	7.9 ± 3.5	8.2[§], 29.7[*]
NOMAC	43.0 ± 2.7	60.6 ± 3.9	2.3 ± 1.6	0.4 ± 0.2	-
DRSP	64.0 ± 4.2	55.6 ± 4.3	0.1 ± 0.09	1707 ± 775	3380[*], 21 800[#],

[a]The EC_{50} values of R5020, P4, MPA, NET and LNG, via PR-B, have been published in (Enfield *et al.*, 2020) Addendum A2).
[b]References for R5020, P4, MPA, NET and LNG [(Markiewicz and Gurpide, 1994) and references within (Enfield *et al.*, 2020)]. References for GES, NES and DRSP (Bray *et al.*, 2005; Attardi *et al.*, 2010; Bain *et al.*, 2015; Kumar *et al.*, 2017).
[*]T47D cells expressing both PR isoforms.
[#]Isoform not specified in U2OS or CV-1 cells, or [##]Hela cells containing only the PR LBD, structurally common to both PR-A and PR-B.
[$]Potencies via PR-A.
[§]Potencies via PR-B.

However, the potency of P_4 and NET reported by Kumar *et al.*, (2017) is ~10 and ~3 times lower than what was reported in our study (Table 3.3), while the values reported for MPA and NET by Sasagawa and colleagues were ~83 times and ~1.4 times higher, respectively (Sasagawa *et al.*, 2008b). Similarly, a ~6-fold difference in potency for R5020 via PR-B was observed between the potency reported in our study and that of Lim and co-workers (Lim *et al.*, 1999).

When comparing the efficacies and potencies per progestogen via PR-A and PR-B, statistical analysis shows that like P_4, the 3rd (GES) and 4th generation progestins (NES and NOMAC) (Figure 3.2A) are more efficacious via PR-B than PR-A. We find that the maximal response of R5020 is higher via PR-B compared to PR-A (Addendum C, Figure C3), which is in line with a previous study showing an efficacy of ~13-fold via PR-A and 40-fold via PR-B for R5020 (Lim *et al.*, 1999). Although higher folds were obtained in our study for PR-A (25-fold) and PR-B (85-fold), both our study and the study by Lim and colleagues showed a ~3-fold difference for R5020 between the PR isoforms. These differences were not due to different expression levels of PR-A and PR-B (Figure 3.2C and D). In contrast, the earlier generations (MPA, NET and LNG) and DRSP, the 4th generation progestin with the unique structure, display similar efficacies via both PR isoforms. Surprisingly, P_4 and most progestins, except R5020, MPA and NOMAC, are more potent via PR-A (Figure 3.2B). It is known that the conformation of a steroid receptor is dependent on the ligand binding to the receptor, and that the ligand-induced changes in the conformation of the LBD will also influence the structure of the DBD and ultimately its interaction with DNA (Raynaud *et al.*, 1980; Spilman *et al.*, 1986; Rayasam *et al.*, 2005; Connaghan-Jones *et al.*, 2007; Bain *et al.*, 2014). For the PR, it has been shown that PR agonists, such as R5020, P_4, and MPA, as well as antagonists, such as RU486 and ZK98299, induce different conformations in the LBD domain of the PR (Daux *et al.*, 1978; Allan *et al.*, 1992a; Allan *et al.*, 1992b), resulting in the modulation of the DBD structure

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Figure 3.2. Figure legend on the following page.

Figure 3.2. While P₄, GES, NES and NOMAC are more efficacious via PR-B, P₄ and most progestins are more potent via PR-A. The **(A)** maximal response and **(B)** $logEC_{50}$ values of the progestogens for PR-A and PR-B (from Figure 3.1B, C, E and F) were replotted as grouped data and two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used to determine differences for PR-A versus PR-B. **(C)** Total protein of the untreated cells was harvested to perform western blot analysis using antibodies specific for the PR (PR-A and PR-B), as well as GAPDH (loading control). A representative western blot is shown. **(D)** PR expression levels relative to GAPDH expression was quantified using ImageJ Analysis Software and one-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis. Statistically significant differences are represented by * and ***, indicating $p < 0.05$ and $p < 0.001$, respectively, or by a and b, where significant differences are indicated by different letters. No statistical significance ($p > 0.05$) is indicated by ns.

and thus dictating to which DNA sequences the PR can bind (Daux *et al.*, 1978). Furthermore, the interaction of the AF2 with co-activators and co-repressors is influenced by the conformational changes of Helix 12 in the LBD (Brzozowski *et al.*, 1997; Williams and Sigler, 1998; Huang *et al.*, 2013). Moreover, as it is known that post-translational modifications also influence activity [reviewed in (Abdel-Hafiz and Horwitz, 2014; Knutson *et al.*, 2017)], the conformation of the full-length PR isoform will determine whether there are differential hormone-dependent, isoform-specific post-translational modifications. Given that progestins all differ in structure, it is thus probable that progestin-specific differences in conformation of the LBD will influence activity. Although PR-A and PR-B are transcribed from the same gene and have identical LBDs, PR isoform-specific conformational changes in response to R5020 and RU486 have previously been reported (Tung *et al.*, 2006; Goswami *et al.*, 2014). While it is likely that the differences in activity between the progestins is linked to the various structures of the progestins, studies directly comparing the differences or similarities in conformational changes of the PR isoforms upon binding to multiple progestins have not been conducted.

3.3.2. The density of PR-A, alone or co-expressed with PR-B, influences both the agonist efficacy and potency of most progestogens.

It is known that receptor density can modulate the efficacy, potency and biocharacter (agonist versus antagonist) of a ligand. For example, the potency of the GR agonist, dexamethasone, and efficacy of MPA for the GR has been reported to increase with increased expression levels of the GR (Robertson *et al.*, 2013), while GR levels can determine whether MPA acts as a GR agonist or antagonist (Zhao *et al.*, 2003). For PR-B specifically, the potency of R5020 via PR-B increased when PR-B levels increased (Abdel-Hafiz *et al.*, 2009). Knowing that receptor levels can influence the biocharacter of a ligand and that different concentrations of PR-A are found in breast cancer tumours, we next investigated whether the density of PR-A influences the efficacy and potency of the progestogens. MDA-MB-231 cells transiently transfected with the PRE-containing luciferase reporter construct as well as increasing concentrations of PR-A (2x and 5x more), were treated with increasing concentrations of the progestogens for 24 hours. Results show that the efficacy and potency of most, but not all, progestins are modulated by increasing the expression levels of PR-A (Figure 3.3). While the density of PR-A does not influence the efficacy of the natural ligand P_4, the efficacies of the progestins are differentially influenced. Although the efficacies for MPA, NET, LNG and NES decrease at both concentrations of PR-A, only the efficacy for NET decreases in a concentration-dependent manner (Figure 3.3B). In contrast, the efficacies of R5020, GES and DRSP decrease only at the highest concentration of PR-A (5x), while the efficacy of NOMAC decreases only when the expression of PR-A is doubled (2x). Notably, in the absence of ligand the increase in PR-A expression at both 2x and 5x decreases the basal luciferase activity (Addendum C, Figure C4). Indeed, unliganded effects of PR-A have previously been reported (Jacobsen *et al.*, 2002; Richer *et al.*, 2002; Khan *et al.*, 2012; Daniel *et al.*, 2015; Truong *et al.*, 2019). Thus, this decrease in basal activity may partly explain the observed decrease in efficacy for MPA, NET,

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Figure 3.3. The density of PR-A influences the potency of P₄ and most progestins, while the efficacy of all progestins, but not P₄, are also modulated. The MDA-MB-231 cells were transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase reporter construct and either **(A)** 900 ng (1x), 1 800 ng (2x) or 4 500 ng (5x) pSG5-PR-A, and treated with either the 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 24 hours. Luciferase activity was normalised to protein concentration. Results are shown as relative luciferase activity for which the maximal response for R5020 via 1x PR-A was set to 100% and all other responses calculated relative to this. The results shown are the averages (± SEM) of at least two independent experiments with each condition performed in triplicate. The **(A)** maximal response and **(B)** $logEC_{50}$ values determined at the different levels of PR-A were directly compared and two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis. Statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Absence of stars indicate no significant differences ($p > 0.05$).

LNG and NES, at both 2x and 5x more PR-A. However, it is not that straightforward as the efficacy of all progestogens does not decrease at both concentrations. For example, the efficacy of NOMAC decreases only at 2x PR-A, while the efficacies of R5020, GES and DRSP decrease only at 5x PR-A. Although the potencies of NES and DRSP are not influenced by the density of PR-A, the potencies of the other progestins are influenced in a density-specific manner (Figure 3.3C). For example, the potency of R5020 and GES decrease to a similar extent at both 2x and 5x PR-A, while the potency of NET increases. Furthermore, the potency of both MPA and NOMAC increase at 2x PR-A, while their potencies at 1x and 5x PR-A are similar. In contrast, the potency of LNG only decreases at 5x PR-A. Even though the efficacy of P₄ did not change with increased PR-A expression, its potency increases at 2x PR-A, and decreases at 5x PR-A. Collectively, the data show that the density of PR-A influences the efficacy and potency in a progestin-specific manner, which is independent of structural derivation or generation.

It is well-known that PR-A and PR-B are generally expressed at equal levels in the healthy

breast, while the levels of PR-A are often higher than PR-B in the majority of breast cancer tumours (Graham *et al.*, 1995, 2005; Hopp *et al.*, 2004; Mote *et al.*, 2015; Rojas *et al.*, 2017). Furthermore, PR-A is known to repress the activity of PR-B and other steroid receptors (Tung *et al.*, 1993; Vegeto *et al.*, 1993; Chalbos and Galtier, 1994; McDonnell *et al.*, 1994; Giangrande *et al.*, 2000; Patel *et al.*, 2018). Thus, we next assessed whether the agonist efficacies and potencies of the progestogens for transactivation via PR-B would be influenced when co-expressed with PR-A at equimolar, or excess concentrations. MDA-MB-231 cells transiently transfected with the PRE-containing luciferase reporter construct as well as PR-A and PR-B in ratios of 1:1, 2:1 or 5:1, were treated with increasing concentrations of the progestogens for 24 hours and the resulting dose-response curves, as well as graphs comparing the efficacies and potencies at each ratio are shown in Addendum C, Figure C5 and C6. The efficacies and potencies of the progestins at all densities of PR-A and all PR-A:PR-B ratios are summarised in Table C1 and C2 of Addendum C. Western blot analysis confirmed the expression of PR-A relative to PR-B in the correct ratios of 1:1, 2:1 and 5:1 (Figure 3.4A and B). Interestingly, a comparison of the basal level of transcription when PR-A and PR-B are co-expressed versus the expression of PR-B only, shows a decrease in activity in a PR-A concentration-dependent manner (Addendum C, Figure C7). This decrease is not due to the increased PR-A decreasing PR-B levels (Figure 3.4A and B), suggesting that the unliganded PR-A may be inhibiting the basal activity of PR-B. The results in Figure 3.4 show that compared to PR-B only there is a significant decrease in efficacy for all progestogens when PR-A and PR-B are co-expressed, irrespective of whether at equal or excess concentrations of PR-A relative to PR-B (Figure 3.4C and Figure C5). While for R5020 and DRSP this decrease is similar at all ratios, this is not the case for all ligands. For example, the efficacies for P_4 and GES at the 2:1 and 5:1 ratio were lower than the efficacy at the 1:1 ratio,
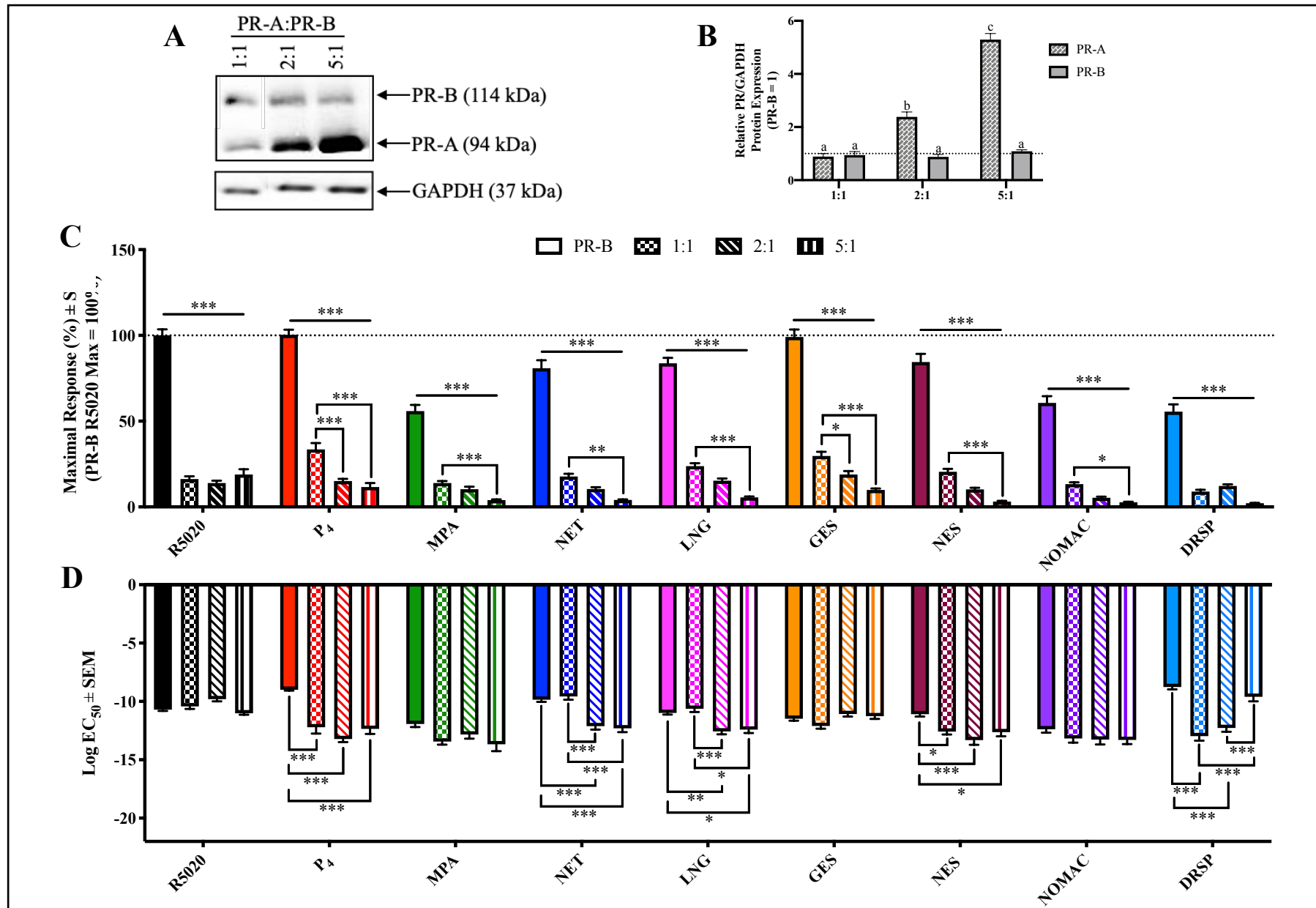
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Figure 3.4. While the efficacies of all progestogens decrease, the potencies of P₄, NET, LNG, NES and DRSP increase with the increased expression of PR-A relative to PR-B. MDA-MB-231 cells transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase reporter construct and either 900 ng pSG5-PR-B only or in combination 900 ng (1:1), 1 800 ng (2:1) or 4 500 ng (5:1) pSG5-PR-A, were treated with either the 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 24 hours. Luciferase activity was normalised to protein concentration. Responses of the progestogens in the presence of different PR-A:PR-B ratios were set relative to the maximal response of R5020 via PR-B only set as 100%. Results shown are representative of at least three biological repeats with each condition performed in triplicate (±SEM). **(A)** Total protein of the untreated cells was harvested to perform western blot analysis using antibodies specific for the PR (PR-A and PR-B), as well as GAPDH (loading control). A representative western blot is shown. **(B)** PR expression levels relative to GAPDH expression was quantified using ImageJ Analysis Software and one-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis. **(C)** Maximal responses and **(D)** logEC$_{50}$ values of the progestogens via PR-B only, and those at the various PR-A:PR-B ratios (data taken from Addendum C, Figure C5 and C6) were plotted as grouped data and two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used to determine differences between PR-B vs. PR-A:PR-B at 1:1, 2:1 and 5:1. Statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, or by the letters a, b and c, where significant differences are indicated by different letters. Absence of stars indicate no significant differences ($p > 0.05$).

whereas there was no difference in efficacies between the 2:1 and 5:1 ratio. In contrast, there is only a difference in efficacies between the 1:1 and 5:1 ratio for MPA, NET, LNG, NES and NOMAC. Contrary to the efficacies of all progestogens decreasing when PR-A and PR-B are co-expressed, the potencies were influenced in a progestogen-specific manner (Figure 3.4D). It is important to note that when setting the dose-response curves of the progestogens in the presence of PR-A and PR-B co-expression, relative to the maximal response of R5020 via PR-B (Addendum C, Figure C6), a typical sigmoidal curve is not always observed due to responses via PR-B being so much higher. In our opinion, a more accurate analysis is obtained when responses of the progestogens are set relative to the maximal response of R5020 for PR-B only, and relative to the maximal response of R5020 for each respective PR-A and PR-B co-

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expression ratio (Addendum C, Figure C5). Thus, potencies discussed hereafter refers to the potencies reported in Addendum C, Table C2. Unlike the potencies of R5020, MPA, GES and NOMAC that are not influenced by the co-expression of PR-B and PR-A at equal concentrations or the excess expression of PR-A at any ratio, the potency of P_4 and NES increase whenever PR-A is co-expressed with PR-B (Figure 3.4D). In contrast, the potency of DRSP increases when PR-A:PR-B are expressed at the 1:1 and 2:1 ratio compared to when only PR-B is expressed. However, when PR-A:PR-B are expressed in a 5:1 ratio the potency is similar to that when only PR-B is expressed. Although the potencies of both NET and LNG are similar when PR-B is expressed alone, compared to PR-B co-expressed with PR-A at similar levels, it is more potent when PR-A is expressed at 2x and 5x that of PR-B. The decrease in the maximal responses of all progestogens or increase in the potencies of P_4, NET, LNG, NES and DRSP are not due to PR-A modulating the expression levels of PR-B (Figure 3.4B). Given that PR-A has previously been shown to repress the activity of PR- B (Tung *et al.*, 1993; Vegeto *et al.*, 1993; Chalbos and Galtier, 1994; McDonnell *et al.*, 1994; Giangrande *et al.*, 2000; Patel *et al.*, 2018), and that the equivalent and excess co-expression of PR-A with PR-B resulted in a decrease of the basal transcriptional activity of PR-B (Addendum C, Figure C7), the decrease in efficacy observed for all progestins in the presence of PR-A:PR-B co-expression, is perhaps not surprising.

To the best of our knowledge, this is the first study to determine agonist efficacies and potencies of the selected progestogens in the same system where both PR-A and PR-B are expressed at varying ratios. Previous studies have however reported potencies for R5020, P_4, MPA, NET, LNG, NES and DRSP in the T47Dco breast cancer cell line (Attardi *et al.*, 2002, 2010; Bray *et al.*, 2005) which is reported to constitutively express equal levels of PR-A and PR-B (Horwitz *et al.*, 1982; Richer *et al.*, 2002; Jacobsen *et al.*, 2002). However, the potencies for these progestogens in the aforementioned studies were lower than the potencies for the 1:1 ratio

in this study, and could be attributed to the different cell lines used or differences in expression levels of the PR. Considering that the density of PR-A only influences the efficacies and potencies of the progestogens, and that when PR-A and PR-B are co-expressed the efficacies of all progestogens at all ratios decrease, it may be argued that PR-B is modulating the activity of PR-A. To better understand the role of PR-A and PR-B, we replotted the data in Figures 3.3 and 3.4 to directly compare the efficacies and potencies of the progestogens when PR-A is expressed in excess, in the absence and presence of PR-B, to determine whether PR-B may in fact be influencing the activity of PR-A (Addendum C, Figure C8A and 8B). The results show that the efficacies of all progestogens decreased in the presence of PR-A and PR-B, despite the concentration of PR-A (Addendum C, Figure C8C and 8D), while the potencies were minimally influenced (Addendum C, Figure C8E and 8F). While it may be that PR-A is modulating the activity of PR-B, or *vice versa,* it is also possible that the progestogens may preferentially bind to a specific isoform. However, studies comparing the binding affinities of the progestogens between PR-A and PR-B, as well as whether the binding affinities are influenced at various PR isoform densities, are lacking. We could not determine the binding affinities of the selected progestins for PR-A and PR-B, since radiolabelled R5020 (reference agonist in this study) is currently not commercially available in South Africa. Although radiolabelled P_4 is available, the use of this radiolabelled ligand is not ideal given that it is rapidly metabolised in the MDA-MB-231 cell line, as well as eight other cell lines commonly used to characterise progestogen binding and transcriptional activity (Chapter 2 and (Skosana *et al.*, 2019) (Addendum A1)). Alternatively, since the efficacy of all progestogens decrease compared to PR-B only, as well as compared to excess PR-A only, it could be that the isoforms are not influencing each other's activity but that this is simply the activity observed when the isoforms are co-expressed. With the exception of the potency for GES, which falls within the reported serum concentration range, the potencies of the selected progestins for PR-A at all

densities, and for PR-B whether alone or co-expressed with different levels of PR-A, are well below the concentrations found in serum of women using these progestins in MHT or contraception (Perkins *et al.*, 2018; Bick *et al.*, 2021). Therefore, it is likely that similar progestin-specific responses may be observed *in vivo*.

Our results support the importance of characterising breast cancer tumours in terms of the presence and amount of the respective PR isoforms, and not only total PR. Although this concept has been suggested for many years (Hopp *et al.*, 2004; Hagan and Lange, 2014; Diep *et al.*, 2015), identifying specific isoform expression in breast cancer is not yet common practice, most likely due to the lack of an antibody that is specific to only PR-A (Lamb *et al.*, 2018). Furthermore, the implications of these data do not only extend to breast cancer, but also to other tissues where the PR isoforms are expressed either equally or with the dominant expression of one of the isoforms. For example, in normal female tissues such as the endometrium, adrenal gland and skin, PR-B is predominantly expressed relative to PR-A, while PR-B is expressed alone in the lung and liver (Asavasupreechar *et al.*, 2020). Furthermore, in the pancreas and ovary PR-A is predominantly expressed (40:1), whereas PR-A is expressed alone in endometrial stromal cells (Asavasupreechar *et al.*, 2020). While PR-A is predominantly expressed in the normal ovary, in ovarian cancer PR-B is either the dominant isoform or even expressed alone (Akahira *et al.*, 2000, 2002; Diep, *et al.*, 2015). A similar observation has been shown in the brain, where in human chordomas (Camacho-Arroyo *et al.*, 2000) and astrocytomas (González-Agüero *et al.*, 2001) PR-B is the predominant isoform.

3.3.3. Progestogens display differential agonist activity for transrepression via a synthetic NFκB promoter via both PR-A and PR-B.

In addition to transactivation, the PR can also repress the expression of target genes by tethering to other transcription factors such as NFκB and AP-1 (Kalkhoven *et al.*, 1996; Kobayashi *et*

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al., 2010; Proietti *et al.*, 2018). With this background, we thus investigated the agonist activity of P_4 and the selected progestins for transrepression on a synthetic luciferase reporter construct, linked downstream of five copies of a NFκB site, co-transfected into the MDA-MB-231 cells with the human PR-A or PR-B expression vector. As attempts to perform dose-response experiments for the transrepression assay were thwarted by large variability between biological repeats, transfected cells were treated with 10 ng/mL PMA in the absence or presence of a single concentration (100 nM) of the progestogens for 24 hours. PMA was used to activate the NFκB promoter prior to progestogen treatment (Lee et al., 2002).

Results in the inserts of Figure 3.5A and 3.5B show that the PMA-mediated induction on the NFκB promoter is repressed by R5020 via PR-A (26%) and PR-B (42%), respectively. The results also show that P_4 and the selected progestins from all four generations, except NES, are agonists for transrepression via both PR-A (Figure 3.5A) and PR-B (Figure 3.5B), albeit to a different extent. NES is the only progestin to show repression in the absence of transfected receptor (Addendum C, Figure C9A), which is not statistically different to the repression observed by NES in cells transfected with either PR-A or PR-B (Addendum C, Figure C9B). These results suggest that neither PR-A nor PR-B is mediating the repression by NES. Given that the MDA-MB-231 cells express endogenous GR (Horwitz *et al.*, 1978) and that NES can bind to the GR, albeit only previously shown for calf (Kumar *et al.*, 2000), it is likely that the GR is mediating the repression by NES. Thus, NES was excluded from the analyses in Figure 3.5 and 3.6.

All progestins, except NET, has higher agonist activity for transrepression than both R5020 and P_4 via PR-A (Figure 3.5A). As for transactivation, the repression of the progestogens via PR-A is independent of generation or structure. For example, 1st generation MPA, structurally related to P_4, and 4th generation DRSP which is derived from spironolactone, are similarly

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A — PR-A

	P4	MPA	NET	LNG	GES	NOMAC	DRSP
P4		***	ns	***	***	***	***
MPA			***	***	***	*	ns
NET				*	***	***	***
LNG					ns	ns	***
GES						ns	*
NOMAC							ns
DRSP							

B — PR-B

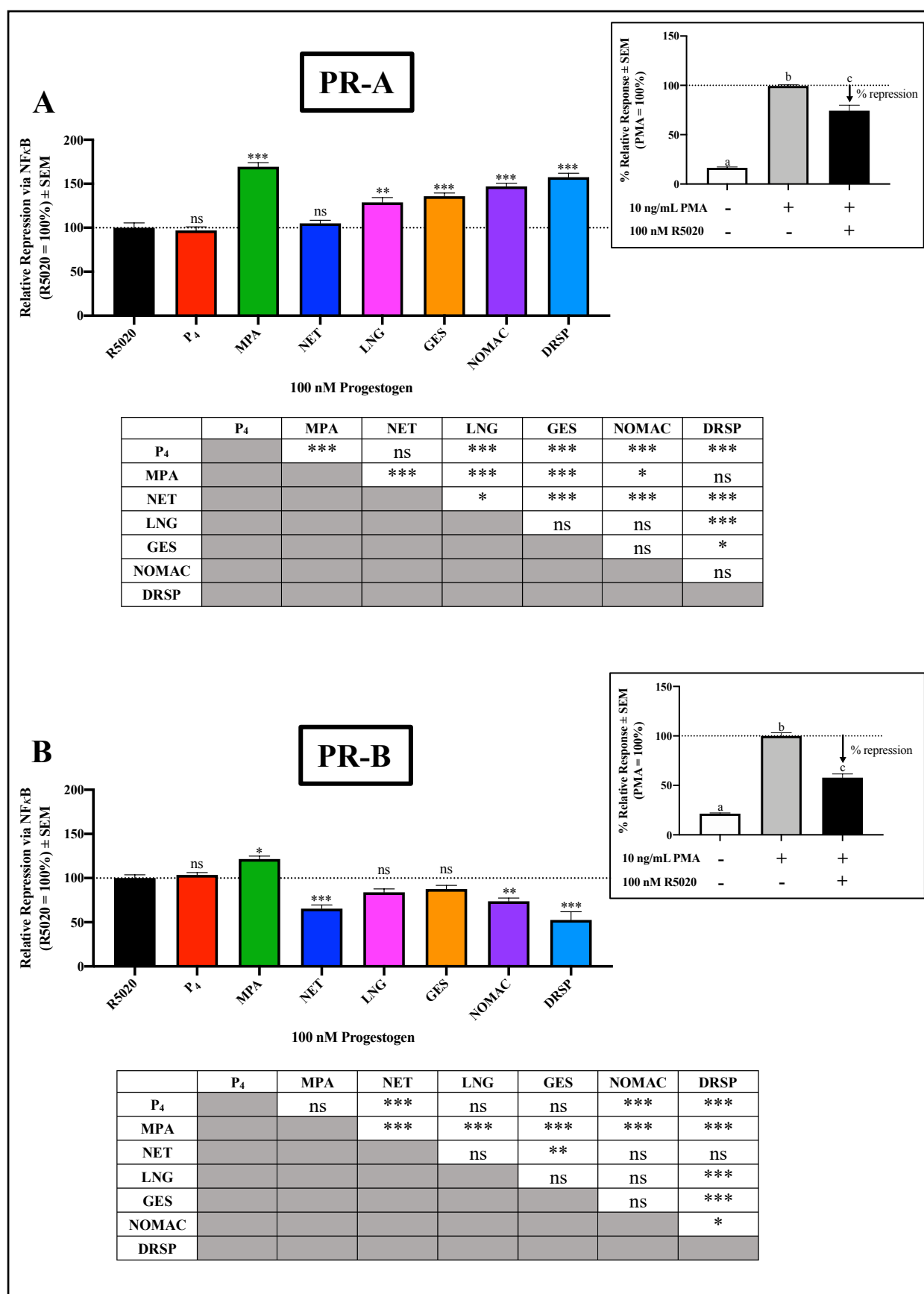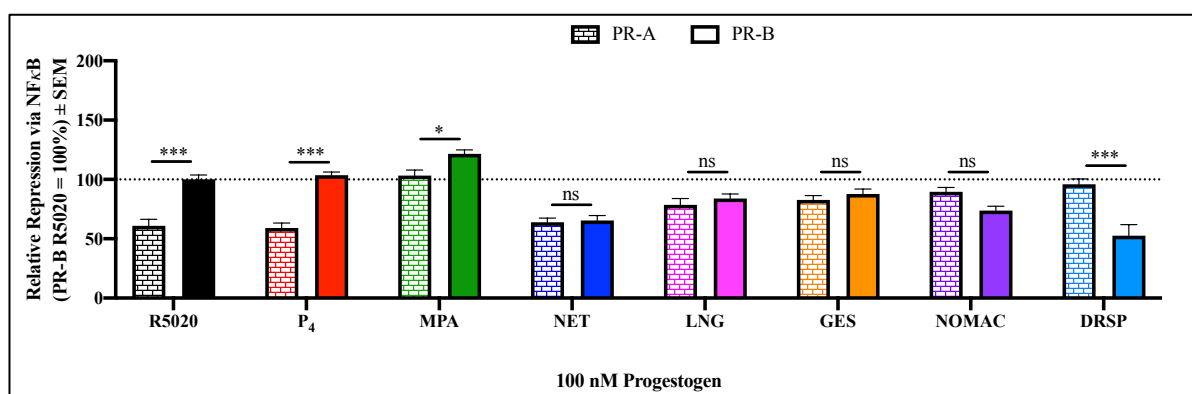|	P4	MPA	NET	LNG	GES	NOMAC	DRSP
P4		ns	***	ns	ns	***	***
MPA			***	***	***	***	***
NET				ns	**	ns	ns
LNG					ns	ns	***
GES						ns	***
NOMAC							*
DRSP							

Figure 3.5. Figure legend on the following page.

Figure 3.5. All progestogens are agonists for transrepression via both PR isoforms, albeit to different extents. MDA-MB-231 cells transiently transfected with 3 000 ng of the 5xNFκB-luciferase reporter plasmid and 1 500 ng of **(A)** pSG5-PR-A or **(B)** pSG5-PR-B were treated with 0.2% (v/v) ethanol (vehicle control) or 10 ng/mL PMA, in the absence or presence of 100 nM progestogens, for 24 hours. Luciferase activity was measured and normalised to protein concentration. **(A and B insert)** The PMA response was set as 100% and the response of R5020 was calculated as a percentage of this. The percentage repression by 100 nM R5020 via PR-A **(A insert)** or PR-B **(B insert)** (indicated by the arrow) was set as 100% in **(A)** and **(B).** The percentage repression by the progestogens was expressed as a percentage of the R5020 response (100% repression). The results shown are the averages (±SEM) of at least two biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, or by letters a, b and c, where the values that differ significantly from other values are assigned a different letter. No statistical significance ($p > 0.05$) is indicated by ns. Statistically significant differences relative to R5020 (reference agonist) are shown on the graph, while significant differences between progestogens are indicated in the tables below the graph.

efficient at mediating repression via PR-A, and the 2nd and 3rd generation testosterone derived LNG and GES, have similar transrepressive activity to the 4th generation NOMAC, derived from P$_4$. These observations are also true for progestogen agonist activity via PR-B (Figure 3.5B). R5020, P$_4$, LNG and GES display similar agonist activities for transrepression via PR-B, while NET, NOMAC and DRSP show lower activity, and MPA higher activity, compared to R5020.

When comparing the transrepression of the progestogens via PR-A versus PR-B, results show that most progestins exhibit similar activities via the PR isoforms. However, R5020, P$_4$ and MPA display more repression via PR-B, while DRSP displays more repression via PR-A (Figure 3.6). Interestingly, for transactivation, we also showed that R5020, the potent PR agonist, and P$_4$, the natural PR ligand, are more efficacious via PR-B. Although not statistically significant for transactivation, the trend that MPA has a higher efficacy via PR-B and DRSP a

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Figure 3.6. Like P$_4$, R5020 and MPA exhibit higher repression via PR-B, while DRSP has a higher activity via PR-A. The responses in Figure 3.5 **(A)** and **(B)** were replotted as grouped data and two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used to determine differences for PR-A versus PR-B. The results shown are averages (±SEM) of at least two biological repeats with each condition performed in triplicate. Statistically significant differences are represented by *, ***, indicating $p < 0.05$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns.

higher efficacy via PR-A was also observed. Surprisingly, while NES is an agonist for transactivation via both PR isoforms, it is not an agonist for transrepression on the NFκB promoter via either PR-A or PR-B. Interestingly, it has been shown that NES, as well as NET-A, LNG and GES can dissociate between transactivation and transrepression via the MR (Africander *et al.*, 2013; Louw-du Toit *et al.*, 2020), and that MPA can do the same via the GR (Bamberger *et al.*, 1999), while a non-steroidal anti-inflammatory ligand, known as Compound A, does the same via PR-B (Robertson *et al.*, 2013). However, these ligands all favour transrepression and not transactivation via the respective steroid receptors, unlike NES that favours transactivation via PR-A and PR-B. Ligands dissociating between transrepression and transactivation and favouring transrepression, is best described for the GR [reviewed in (Adcock, 2000; De Bosscher and Haegeman, 2009), and are proposed to be ideal anti-inflammatory compounds. This is due to the negative side-effects of glucocorticoids being

associated with transactivation, while the beneficial anti-inflammatory activity is linked to transrepression [reviewed in (De Bosscher and Haegeman, 2009)].

Although our results suggest that NES favours transactivation rather than transrepression, we cannot exclude the possibility that the transrepressive effects of NES via the PR isoforms may be promoter specific. Indeed, our group has recently shown that MPA and NOMAC are MR agonists for transrepression via the NFκB-containing promoter used in this study, but not via an AP-1-containing promoter (Louw-du Toit *et al.*, 2020). Further research is thus required to elucidate whether NES can cause transrepression via the PR on alternate promoters to confirm whether NES does in fact have dissociative properties, whereby transactivation over transrepression is preferentially induced. These data highlight the importance of investigating the activities of the individual progestins for both transactivation and transrepression to fully understand the mechanism of progestogens via the PR, and to distinguish between the activities of PR-A and PR-B.

3.3.4. Progestogens display greater agonist activity for transrepression when PR-A and PR-B are co-expressed at equivalent levels, compared to an excess of PR-A relative to PR-B.

As for transactivation, we determined whether the density of PR-A would influence repression of the progestogens, and whether the repression of the ligands via PR-B is modulated when PR-A and PR-B are co-expressed either at equivalent levels, or 5x excess of PR-A. MDA-MB-231 cells transiently transfected with the NFκB-containing luciferase reporter construct and 5x PR-A only, or PR-A and PR-B (1:1 and 5:1), were thus treated as described in Section 3.3.3. The results in Figure 3.7 show that when the concentration of PR-A increases, so does the repression by all progestogens.

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Figure 3.7. Increasing the expression levels of PR-A enhances the repression by all progestogens. MDA-MB-231 cells transiently transfected with 3 000 ng 5xNFκB-luciferase reporter plasmid and 1 500 ng (1x used in Figure 3.5A) or 7 500 (5x) pSG5-PR-A, were treated with 0.2% (v/v) ethanol (vehicle control) or 10 ng/mL PMA in the absence or presence of 100 nM progestogens for 24 hours. Luciferase activity was measured and normalised to protein concentration. The percentage repression by R5020 via 1x PR-A was set as 100% and the percentage repression for the other progestogens via 1x PR-A and 5x PR-A was set relative to this. The results shown are the averages (±SEM) of at least two biological repeats with each condition performed in triplicate. An unpaired *t*-test was used for statistical analysis to assess differences between 1x PR-A and 5x PR-A, and statistically significant differences are represented by ***, indicating p < 0.001.

Results show that when PR-A is co-expressed with PR-B at equivalent levels, the agonist activity for transrepression by all progestogens increase (Figure 3.8). This is in contrast to the decrease in agonist activity for transactivation observed at 100 nM progestogen when the PR-A:PR-B ratio is increased (Figure 3.4). Even though we showed that NES is not an agonist for transrepression via PR-A or PR-B alone, when PR-A levels are 5x higher and when PR-A is co-expressed with PR-B at either ratio, the repressive activity by NES increases (Addendum C, Figure C10). The repression by NES in the absence of PR-A and PR-B was subtracted to correct for any low-level responses via endogenous steroid receptors. Thus, here the repression by NES is consistent with a PR-dependent mechanism in a density-dependent manner. When a 5x excess PR-A is present relative to PR-B, however, the activity is generally lower than

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when expressed at equal ratios (Figure 3.8). Furthermore, the repression by P_4, NET and GES are similar when PR-A is co-expressed with PR-B at a ratio of 5:1 compared to when PR-B is expressed alone. In contrast, the repression by R5020, LNG, NOMAC and DRSP is increased, while that by MPA is decreased, when PR-A is co-expressed with PR-B at a ratio of 5:1 compared to the expression of PR-B only.

Figure 3.8. Progestogens display greater effects on transrepression when PR-A and PR-B are present at equivalent expression levels compared to when PR-B is expressed alone or in the presence of 5x more PR-A. The data shown in Figure 3.5 and Addendum C, Figure C11 were reanalysed to present the responses of the progestogens in the presence of different PR-A:PR-B ratios relative to the repression by R5020 via PR-B only, set as 100%. The results shown are averages (±SEM) of at least two biological repeats with each condition performed in triplicate. Two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Absence of stars indicate no significant differences ($p > 0.05$).

As for transactivation, the data were replotted in attempt to better understand the role of PR-A and PR-B in the progestogen-induced transrepression. Responses when PR-A and PR-B are co-expressed were thus set relative to the PR-A 1x or PR-A 5x responses. Results show that when PR-A and PR-B are co-expressed at equivalent levels, the repression by all progestogens increases compared to 1x PR-A only (Addendum C, Figure C11A). However, when PR-A

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expression levels are 5x more than that of PR-B, progestogen-specific effects are observed. Repression by MPA, NET and LNG decrease, while there is no difference in repressive activity for R5020, P_4, GES, NOMAC and DRSP (Addendum C, Figure C11B). Further experiments are required to determine the underlying mechanism behind the differential responses of the progestins at the various densities of PR-A relative to PR-B.

This is the first study to compare, in parallel, the agonist activity for transrepression of these selected progestogens from all four generations to each other, R5020 and P_4 via either PR-A or PR-B in the same model system, and evaluate whether this activity is influenced by the levels of PR-A or the ratio of PR-A:PR-B. Understanding the physiological implications of the aforementioned transrepression results warrants an understanding of inflammation in breast cancer development and progression (DeNardo and Coussens, 2007). Indeed, due to the role that inflammation and the immune system play in aiding neoplastic progression, it is now considered a hallmark of cancer [reviewed in (Hanahan and Weinberg, 2011). The fact that all progestogens, except NES, are shown to be PR-A and PR-B agonists for transrepression, may suggest that inflammation-induced breast cancer development and progression may be inhibited by these progestogens via the respective PR isoform. This may also be true for NES in tissues where the PR isoforms are equally expressed and when there is dominant expression of PR-A. However, the concept that the PR-mediated effects of the progestogens are involved in the inhibition of inflammation-induced breast cancer is complex, as one has to consider that these progestogens are also agonists for transactivation, and that the balance of transactivation and transrepression inevitably leads to the final physiological response. Further studies are needed to assess the precise association between the PR isoforms, inflammation and breast cancer. Taken together, the aforementioned results for transactivation and transrepression via the PR isoforms highlight the importance of understanding progestin- and PR isoform-specific effects, as well as evaluating both the transactivation and transrepression mechanisms of PR-

A and PR-B, particularly in tissues where there is discordance between the expression of the two isoforms.

3.3.5. Progestogens differentially regulate endogenous genes in T47D cells in a ligand- and gene-specific manner.

Having shown that the progestins and P_4 display differential effects via PR-A or PR-B exogenously expressed in the MDA-MB-231 cell line on synthetic PRE- and NFκB-containing promoters, we next investigated their regulation of a number of endogenous genes in the human T47D breast cancer cell line known to endogenously express both PR-A and PR-B (Horwitz *et al.*, 1978). All of the selected genes are known to play a role in breast cancer development and/or progression, with some genes such as GATA3, FOXO1, SOX4, TGFβ1 and Ki67 previously shown to be regulated by some progestins (Elizalde *et al.*, 1990; Jeng and Jordan, 1991; Graham *et al.*, 1999; Mrusek *et al.*, 2005; Labied *et al.*, 2006; Kyo *et al.*, 2011; Diep *et al.*, 2013; Nakamura *et al.*, 2013; Izzo *et al.*, 2014; Mohammed *et al.*, 2015; Clare *et al.*, 2016; Diep *et al.*, 2016b; Truong *et al.*, 2019). In fact, effects of R5020, P_4 or MPA on GATA3 and FOXO1 expression were previously shown to be mediated via both PR isoforms (Diep *et al.*, 2013; Nakamura *et al.*, 2013; Izzo *et al.*, 2014; Diep *et al.*, 2016b; Truong *et al.*, 2019). However, studies investigating the effects of multiple progestins in parallel on the expression of these genes, while also investigating the role of the PR isoforms, are lacking. Effects of the progestogens were also examined in the presence of E_2, given that progestins are often used in combination with an estrogen in both contraception and MHT [reviewed in (Perkins *et al.*, 2018)], and that concerns have previously been raised about the relevance of investigating PR activity in the absence of estrogen (Carroll *et al.*, 2017; Sathyamoorthy and Lange, 2020).

T47D breast cancer cells were thus treated with 100 nM of the selected progestogens in the absence and presence of 100 nM E_2 for time points previously optimised and indicated in the

relevant figure legends. Realtime qPCR results in Figure 3.9 show both progestogen- and gene-specific effects. All progestins increase the mRNA expression of Ki67 (Figure 3.9E) and FOXO1 (Figure 3.9D) to various extents, while GATA3 expression (Figure 3.9A) is decreased by all progestogens and to the same extent. However, differential regulation of SOX4 (Figure 3.9B) and TGFβ1 (Figure 3.9C) mRNA expression is observed. While most of the selected progestogens increase SOX4 expression, LNG and GES have no effect. TGFβ1 mRNA expression on the other hand, is significantly decreased by R5020, P_4 and MPA, while NET, GES and DRSP have no effect. The effect of LNG is less clear as the statistical analysis indicates that it is similar to the vehicle, suggesting no effect on TGFβ1 expression, but also similar to R5020, P_4 and MPA. In addition, only R5020 and P_4 increase BAX mRNA expression, while all other ligands had no effect (Figure 3.9F).

Results in the presence of equimolar E_2 show that, in most cases, E_2 does not influence the effect of the progestogens on the selected genes. The exceptions are the responses of R5020, NET and GES. While SOX4 expression is increased with the co-treatment of GES and E_2 (Figure 3.9B), TGFβ1 expression is downregulated (Figure 3.9C). Similarly, the R5020-mediated increase in Ki67 expression is decreased in the presence of E_2 (Figure 3.9E), with a similar result for the NET-mediated upregulation of Ki67 (Figure 3.9E), SOX4 (Figurer 3.9B) and FOXO1 (Figurer 3.9D) expression. In contrast, E_2 lifts the R5020-mediated downregulation of TGFβ1 (Figure 3.9C). While E_2 only increases the expression of TGFβ1, Ki67 and BAX, and decreases the expression of SOX4, E_2 does not regulate GATA3 and FOXO1 mRNA expression. As there is no difference in BAX mRNA expression in the presence of E_2 only versus E_2 plus all progestogens, except GES, the observed increase with E_2 and progestogens are probably the effects of E_2 only, likely mediated by the endogenously
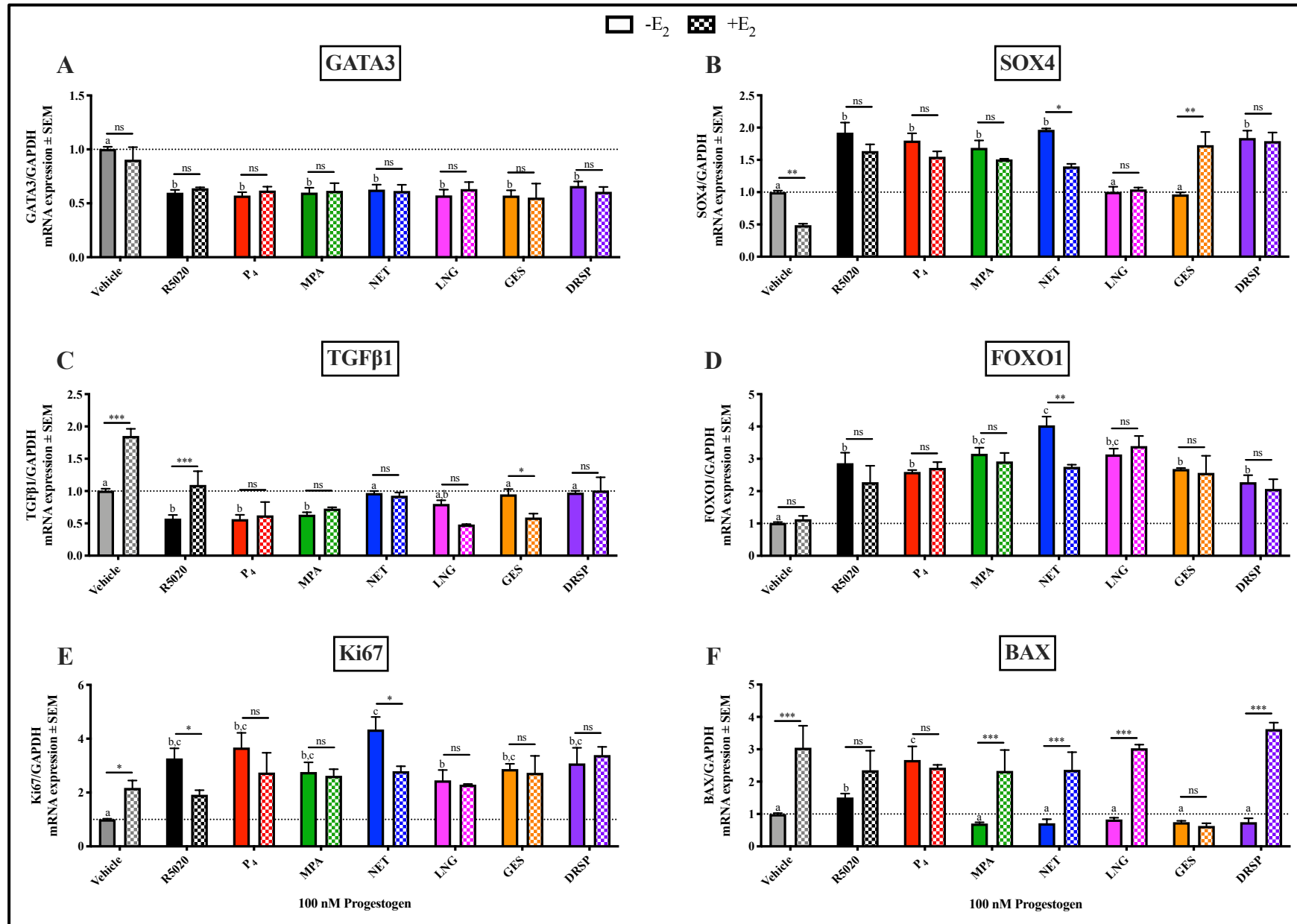
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Figure 3.9. Figure legend on the following page

Figure 3.9. P_4 and the progestins regulate the mRNA expression of genes involved in breast cancer development and/or progression in a progestogen- and gene-specific manner, with E_2 having minimal effect on progestogen regulation. T47D cells were treated with either the 0.2% (v/v) ethanol (vehicle control) or 100 nM progestogens, in the absence or presence of 100 nM E_2, for 2 hours (GATA3 and BAX), 12 hours (SOX4, TGFβ1 and FOXO1) or 24 hours (Ki67). Total RNA was isolated, cDNA synthesized, and realtime qPCR performed to determine the mRNA expression levels of **(A)** GATA3, **(B)** SOX4, **(C)** TGFβ1, **(D)** FOXO1, **(E)** Ki67 and **(F)** BAX. GAPDH was used as the reference gene. Relative GATA3, SOX4, TGFβ1, FOXO1, Ki67 and BAX mRNA expression of treated cells was calculated relative to the vehicle control, set as 1. The results shown are averages (±SEM) of at least three biological repeats. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used to compare the progestogen effects to the vehicle and each other (first bar of every group), and statistical differences are indicated with letters a, b and c. Two-way ANOVA with Bonferroni's post-test was used to compare the responses in the absence and presence of E_2 per treatment group, and statistically significant differences are represented by *, **, ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No significant differences ($p > 0.05$) are indicated using ns. One-way ANOVA with Dunnett's (compares all columns to the control) post-test was also used to compare the effects of E_2 only versus E_2 in the presence of the progestogens and the statistical analysis indicate the following: **GATA3:** E_2 versus E_2 + R5020, P_4, MPA, NET, LNG and DRSP ($p > 0.05$); E_2 versus E_2 + GES ($p < 0.05$). **SOX4:** E_2 versus E_2 + R5020, P_4, MPA, NET, GES and DRSP ($p < 0.001$); E_2 versus E_2 +LNG ($p < 0.01$); **TGFβ1:** E_2 versus E_2 + R5020 and DRSP ($p < 0.05$); E_2 versus E_2 + P_4, MPA, NET ($p < 0.01$); E_2 versus E_2 + LNG and GES ($p < 0.001$); **FOXO1:** E_2 versus E_2 + R5020 and DRSP ($p > 0.05$); E_2 versus E_2 + P_4, NET and GES ($p < 0.05$); E_2 versus E_2 + MPA and LNG ($p < 0.01$); **Ki67:** E_2 versus E_2 + all progestogens ($p > 0.05$); **BAX:** E_2 versus E_2 + R5020, P_4, MPA, NET, LNG and DRSP ($p > 0.05$), GES ($p < 0.05$).

expressed ERα. GES does not influence the expression of BAX and surprisingly, the upregulation of BAX by E_2 is not observed in the presence of GES. Given that GES is an agonist for both PR isoforms (Figure 3.1 and 3.5), it may be that GES induces a conformation in the PR that results in the inhibition of E_2-induced upregulation of BAX mRNA expression via ERα. Indeed, it is known that the PR and ERα associate (Migliaccio *et al.*, 1998; Giulianelli *et al.*, 2012; Daniel *et al.*, 2015; McFall *et al.*, 2015, 2018; Mohammed *et al.*, 2015; Singhal *et al.*, 2016), and that the PR can inhibit ERα activity (McFall *et al.*, 2015, 2018; Mohammed *et*

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al., 2015; Singhal *et al.*, 2018). Although Mohammed and co-workers did not specify the PR isoform involved, a role for PR-A has been indicated in recent studies (McFall *et al.*, 2015, 2018; Singhal *et al.*, 2018). Surprisingly, although GES does not regulate SOX4 mRNA expression, and E_2 downregulates its expression, SOX4 is upregulated in the presence of both GES and E_2. The mechanism underlying this response is unclear and additional studies will be required to understand the role of the PR isoforms, as well as that of the ER in the responses of GES under estrogenic conditions. Interestingly, the suppression of SOX4 by E_2 is lifted by all progestogens, while the E_2-mediated upregulation of TGFβ1 is inhibited by all the progestogens.

The lack of GATA3 regulation by E_2 in our study, is in line with a previous study in MCF7 cells (Hoch *et al.*, 1999), while the observed downregulation of GATA3 expression by MPA is in agreement with a previous study showing that 10 nM MPA decreases GATA3 mRNA in T47D cells (Izzo *et al.*, 2014). The authors showed that repression by MPA occurred through the PR binding to a putative PRE within the promoter region (Izzo *et al.*, 2014). While we did not investigate the mechanism of GATA3 regulation, our study is the first to show that the selected panel of progestins from the different generations also decrease GATA3 mRNA expression (Figure 3.9A). GATA3 is a known tumour suppressor (Dydensborg *et al.*, 2009; Yan *et al.*, 2010; Takaku *et al.*, 2018), that is constitutively downregulated in most breast cancers (Dydensborg *et al.*, 2009; Yan *et al.*, 2010; Yoon *et al.*, 2010). These results thus suggest that the progestins may be further contributing to the downregulation of GATA3 expression in breast cancer, supporting a role for these progestins in breast cancer development and progression.

Although we have not found evidence of the selected progestogens regulating SOX4 expression in breast cancer cells, a study more than 20 years ago showed that 10 nM of a

progestin structurally derived from P_4, Org2058, upregulates SOX4 mRNA expression in T47D cells, while the expression is downregulated by E_2 (Graham *et al.*, 1999). Considering that an increase in SOX4 expression in breast cancer has been implicated in more invasive breast cancer tumours and therefore a worse prognosis (Song et al., 2015), our results suggest that the selected progestogens which increase SOX4 expression, may enhance the migration and invasion of T47D cells, while those with no effect, such as the 2nd generation LNG and 3rd generation GES, may not.

While our study is the first to directly compare the effects of progestins from different generations to each other, P_4 and R5020 on TGFβ1 mRNA expression in the same model system, effects of MPA, NET and R5020 have previously been investigated in non-parallel studies. Consistent with our results, one study showed that MPA downregulates TGFβ1 mRNA expression in a mouse-mammary tumour model (Elizalde *et al.*, 1990). In contrast, MPA and R5020 had no effect on TGFβ1 mRNA expression in the MCF-7 breast cancer cell line (Jeng and Jordan, 1991). However, Jeng and co-workers used a 10-fold lower concentration of R5020 and MPA, suggesting that the effects of these progestins on TGFβ1 expression may be either cell line- or dose-dependent. Cell line-specific effects are, however, not observed for NET, as 100 nM NET had no effect on TGFβ1 mRNA expression in MCF-7 cells, in agreement with our results for 100 nM NET in the T47D cells (Jeng and Jordan, 1991). Our results showing that E_2 does not modulate the effects of MPA on TGFβ1 mRNA expression in T47D cells are also consistent with a previous study in MCF-7 cells (Jeng and Jordan, 1991). However, Jeng and co-workers showed that E_2 and R5020 alone, or in combination, has no effect on TGFβ1 mRNA expression, which contradicts our results showing abrogation of R5020-mediated downregulation of TGFβ1 mRNA expression by E_2. Understanding the physiological implications of these aforementioned effects on TGFβ1 is not easy. In the early stages of breast cancer, TGFβ1 acts as a tumour suppressor, while during the late stages it has been shown to

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enhance breast cancer progression (Seoane and Gomis, 2017). Since we show that R5020 and P$_4$, like, MPA, downregulate TGFβ1 expression, and that it has been hypothesized that the proliferative effects of MPA are facilitated by the downregulation of growth inhibitors such as TGFβ1 (Elizalde *et al.*, 1990), it is likely that R5020 and P$_4$ may also enhance proliferation through a similar mechanism. On the other hand, an increase in TGFβ1 expression in breast cancer is also associated with facilitating the loss of cell-cell adhesion and gain of metastatic characteristics (Moustakas and Heldin, 2014), suggesting that the downregulation of TGFβ1 expression by R5020, P$_4$ and MPA could in fact lead to the inhibition of processes involved in metastatic behaviour.

Considering that FOXO1 is a well-known PR-regulated gene, it is not surprising that all of the selected progestogens upregulate FOXO1 expression, albeit to different extents. The upregulation of FOXO1 mRNA expression by R5020 has previously been shown in T47D cells endogenously expressing both PR-A and PR-B, and in T47D cells stably expressing either PR-A or PR-B (Truong *et al.*, 2019). A similar result was found in the ES-2 ovarian clear cell adenocarcinoma cell line stably expressing either PR-A or PR-B (Diep *et al.*, 2013; Diep *et al.*, 2016b). Similarly, P$_4$ (Kyo *et al.*, 2011) and MPA (Labied *et al.*, 2006; Kyo *et al.*, 2011; Nakamura *et al.*, 2013) have been shown to upregulate FOXO1 mRNA and protein expression in both human endometrial stromal primary cells and the endometrial epithelial EM-E6/E7/TERT cell line. FOXO1 is a transcription factor belonging to the Forkhead Box O subfamily of transcription factors, involved in the regulation of cellular processes including metabolism, cellular differentiation, apoptosis and cell cycle progression (Brosens and Lam, 2013). Despite reports of FOXO1 being a tumour suppressor (Zhao *et al.*, 2010), both FOXO1 and the PR have been associated with enhancing cancer stem cell (CSC) behaviours (Truong *et al.*, 2019). The fact that the progestogens all increase FOXO1 expression in breast cancer

cells, may thus suggest that the selected progestins may play a role in enhancing CSC behaviour.

In the cancerous breast, the balance between proliferation and apoptosis is often disrupted, aiding in the survival of cancerous cells (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Ki67 is a well-known marker of proliferation, while BAX is pro-apoptotic (Hanahan and Weinberg, 2011; Stevanovic *et al.*, 2019). The results showing that all progestogens increase expression of the proliferation marker Ki67, whereas only R5020 and P_4 increase the pro-apoptotic BAX, suggesting that, all the selected progestins from the four generations may in fact play a role in breast cancer development by swaying the balance between proliferation and apoptosis to the side of proliferation. The finding that R5020 and P_4 have similar effects on BAX expression, while the rest of the progestins do not, is quite significant as most mechanistic studies use R5020 and P_4 to investigate the mechanisms underlying PR activity. This finding highlights the importance of not making assumptions about progestin activity based on results obtained using only R5020 or P_4.

Similar to our study, it has previously been shown that 10 nM R5020 upregulates Ki67 mRNA expression in T47D breast cancer cells (Clare *et al.*, 2016), and that E_2 has no effect on the response of MPA on Ki67 mRNA expression (Mrusek *et al.*, 2005). However, findings from our laboratory showing that both 1 nM (Louw-du Toit et al., in preparation for Biochemical Journal) and 100 nM R5020 (Figure 3.9) has no effect on the E_2-induced upregulation of Ki67 mRNA in T47D cells, is contrary to evidence that the E_2-induced increase in Ki67 protein expression is inhibited by 10 nM R5020 in primary breast cancer explant tissue (Mohammed *et al.*, 2015). While evidence of progestin effects on BAX expression is scarce, at least one study has shown that 10 nM MPA (Ory *et al.*, 2001) has no effect on BAX mRNA expression in the T47D cells, while another study showed that BAX mRNA expression increased in human

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endometrial tissue of women using LNG-containing intra-uterine devices (Orbo *et al.*, 2010). The results from this study with 100 nM MPA and those from our laboratory with 1 nM MPA (Louw-du Toit et al., in preparation for Biochemical Journal) are in line with those of Ory and colleagues.

Taken together, the regulation of a specific gene by the progestins and P_4 is often, but not always, similar, with progestin-specific effects being observed on SOX4, TGFβ1 and BAX. Furthermore, the presence of E_2 generally has minimal effects on the response of the progestogens and *vice versa*. Differences observed between some progestins may be explained by the involvement of different or more than one steroid receptor, as multiple steroid receptors are expressed in the T47D cell line. Considering the array of *cis*-regulatory elements within the promoter regions of the selected genes to which steroid receptors are known to bind [(Tseng *et al.*, 2003); reviewed in (Payne and Freishtat, 2012; Proietti *et al.*, 2018), an alternative explanation could be proposed. It could be that the progestins induce differential conformational changes in the LBD of a specific steroid receptor, leading to a change in the DBD structure and thus influencing where the receptor binds to DNA, and possibly resulting in progestin-specific cofactor recruitment.

3.3.6. The progestogen-induced regulation of the selected genes is not solely mediated by the PR isoforms.

Having shown differential activity of the progestins via PR-A and PR-B in reporter assays, and that excess expression of PR-A relative to PR-B influences the activities of P_4 and the selected progestins, we next evaluated the contribution of the PR isoforms in the progestogen-induced regulation of five of the selected endogenous genes, and whether excess levels of PR-A would influence these responses. To evaluate the role of the PR isoforms, T47D cells were transiently transfected with either 10 nM NSC siRNA or siRNA directed against PR-B only (PR-B siRNA)

or against both PR-A and PR-B (PR-A/B siRNA), while the cells were transfected with 4 500 ng pSG5-hPR-A for the investigation into the influence of excess PR-A. Western blot analysis confirmed that PR-A/B siRNA reduced both PR-A (97%) and PR-B (98%) protein levels, while the PR-B siRNA only reduced PR-B levels (87%) (Figure 3.10A and C). Confirmation of PR-

A expression levels approximately 5 times more than that of PR-B was also shown following the transient transfection of PR-A into the T47D cells (Figure 3.10C and D). Notably, in our laboratory the ratio of endogenous PR-A:PR-B expression levels in the T47D cells are ~2:1 and not 1:1 as previously reported (Sartorius *et al.*, 1994; Khan *et al.*, 2012; McFall *et al.*, 2015; Yu *et al.*, 2017).

The realtime qPCR results in Figure 3.11 show that the role of the PR and PR isoform ratio in the progestogen-induced regulation of the selected genes is complex. When only PR-B is silenced, the upregulation of FOXO1 by all progestogens is abrogated (Figure 3.11C), suggesting that the progestogens regulate FOXO1 expression via PR-B. In contrast, silencing only PR-B or both PR-B and PR-A did not abrogate the progestin-induced increase of Ki67 mRNA expression (Figure 3.11D), suggesting that none of the progestin effects are mediated by either PR isoform. However, the P_4-induced upregulation of Ki67 is partially inhibited when the levels of PR-B are reduced, and this reduction is similar when both isoforms are silenced, indicating that PR-B may partially be involved in Ki67 upregulation. Interestingly, when both PR-A and PR-B are silenced, we observe an increase in Ki67 expression in a ligand-independent manner. The expression of GATA3 (Figure 3.11A) and TGFβ1 (Figure 3.11B), however, were differentially affected by the silencing of PR-B or total PR. For example, when only PR-B is silenced, the P_4-, MPA-, GES- and DRSP-mediated repression of GATA3 (Figure 3.11A), as well as the R5020-mediated repression of TGFβ1 (Figure 3.11B) is lifted, while the responses of the other progestins remain unchanged.

Figure 3.10. Western blot analyses confirming PR expression levels in T47D cells. (A) Decrease in PR expression levels by siRNA specifically targeting PR-B or both PR-A and PR-B. T47D cells were either untransfected (UT) or transiently transfected with 10 nM non-silencing scrambled sequence control (NSC) siRNA or siRNA directed against only PR-B or both PR isoforms. **(C) Increased PR-A expression following transient transfection of PR-A in the T47D cells.** T47D cells were transiently transfected with 4 500 ng of either pSG5-empty vector or pSG5-PR-A. For both **(A and C)**, total protein was harvested to perform western blot analysis using antibodies specific for the PR (PR-A and PR-B), as well as GAPDH (loading control), and representative western blots are shown. **(B and D)** PR-A and PR-B expression levels relative to GAPDH expression was quantified using ImageJ Analysis Software, with relative protein expression levels of PR-B in the UT T47D cells set to **(B)** 100% or **(D)** 1. Percentage knockdown is shown by the arrows. The results shown are averages (±SEM) of at least three biological repeats. Two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented with letters a, b and c, where the values that differ significantly from other values are assigned a different letter.

Thus it is likely that the effects of these progestins on GATA3 and TGFβ1 is mediated by PR-B. The result for MPA is in line with a previous study showing that the downregulation of GATA3 by MPA is PR-B-mediated (Izzo *et al.*, 2014). Unlike Izzo and colleagues (Izzo *et al.*, 2014), however, we do not show a role for PR-A in the regulation of GATA3 by MPA.
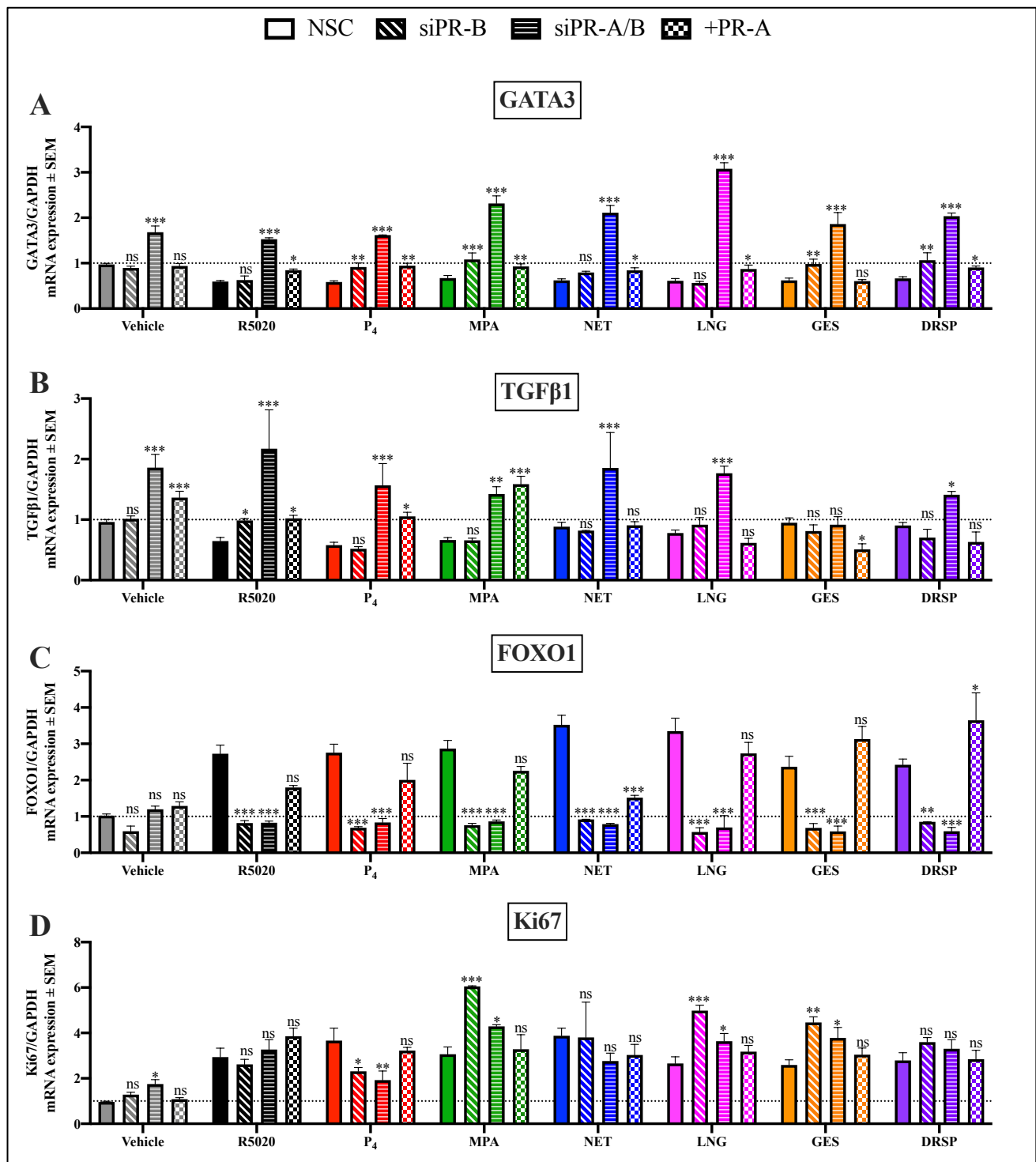
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Figure 3.11. The role of the PR-isoforms and dysregulated isoform ratio in progestogen-mediated regulation is progestogen- and gene-dependent. The T47D cells were transiently transfected as in Figure 3.10. Cells were treated with either the 0.1% (v/v) ethanol (vehicle control) or 100 nM progestogens for 2 hours (GATA3), 12 hours (TGFβ1 and FOXO1) or 24 hours (Ki67). Total RNA was isolated, cDNA synthesized and realtime qPCR performed to determine the relative mRNA expression levels of **(A)** GATA3, **(B)** TGFβ1, **(C)** FOXO1 and **(D)** Ki67. GAPDH was used as the reference gene. The responses of all progestogens are set relative to the vehicle control of the T47D cells transfected with the NSC siRNA which is set as 1. The results shown are averages (±SEM) of at least three biological repeats. Two-way ANOVA with Bonferroni's post-test was used for statistical analysis to compare the responses within all treatment groups after PR-B or PR-A/B siRNA knockdown, or excess PR-A expression, to the NSC siRNA (first bar of every group). Statistically significant differences are represented with either *, ** or ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical differences ($p > 0.05$) are indicated by ns. One-way ANOVA with Dunnett's (compares all columns to the control) post-test was also used to compare the effects of the progestogens to the ligand independent effects at a specific transfection condition and statistical analysis indicated the following: **GATA3:** siPR-A/B vehicle versus R5020, P_4, MPA, NET, GES and DRSP ($p > 0.05$); vehicle versus LNG ($p < 0.001$); **TGFβ1:** siPR-A/B vehicle versus all progestogens ($p > 0.05$); **TGFβ1:** +PR-A vehicle versus R5020, P_4 and MPA ($p > 0.05$); vehicle versus NET ($p < 0.05$); vehicle versus LNG, GES and DRSP ($p < 0.001$); **Ki67:** siPR-A/B vehicle versus P_4 and NET ($p > 0.05$); vehicle versus R5020 and DRSP ($p < 0.05$); vehicle versus LNG and GES ($p < 0.01$); vehicle versus MPA ($p < 0.001$).

Indeed, identifying the role of PR-A in the progestogen-induced downregulation of GATA3 (Figure 3.11A) and TGFβ1 (Figure 3.11B) is complex, given that the silencing of both PR-A and PR-B results in an increase in GATA3 and TGFβ1 expression in a ligand-independent manner. Considering that the LNG-induced upregulation of GATA3 expression is significantly different to that of the unliganded effect, it is probable that PR-A is suppressing the regulation of GATA3 by LNG, mediated by a steroid receptor other than the PR. Indeed it has been shown that PR-A has the ability to repress the activity of the GR, AR, MR and ERα, all of which LNG can bind (Vegeto *et al.*, 1993; Abdel-Hafiz *et al.*, 2002). Finally, we also evaluated the role of the PR isoforms in the upregulation of the pro-apoptotic gene BAX by R5020 and P_4. Silencing of PR-B only did not inhibit these responses, while the silencing of both PR-A and PR-B caused

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a complete loss of BAX upregulation by R5020 and P$_4$ (Addendum C, Figure C12), suggesting a PR-A-mediated mechanism for both R5020 and P$_4$.

Effects of excess PR-A on gene regulation in the T47D cells also appears to be dependent on the specific gene and progestogen (Figure 3.11). When increasing the expression of PR-A relative to PR-B in the T47D cells, there was an increase in the mRNA expression of TGFβ1 in the absence of ligand (Figure 3.11B), suggesting a ligand-independent regulation of TGFβ1. In the presence of ligand and 5-fold more PR-A than PR-B, the suppression of TGFβ1 by R5020, P$_4$ and MPA observed in T47D cells endogenously expressing a 2:1 PR-A:PR-B ratio, is lifted. Interestingly, GES causes a decrease in TGFβ1 expression when PR-A is in excess to PR-B. For GATA3, the suppression by all progestogens is lifted, except for GES, in T47D cells expressing PR-A in excess. Although other studies have not investigated the regulation of GATA3 by the selected progestins used in our study, one study 16 years ago showed that the progestin Org2058 decreased GATA3 expression in cells expressing a ~5:1 ratio of PR-A:PR-B (Graham *et al.*, 2005). This downregulation is in agreement with our findings for GES, but not the other progestogens, suggesting progestin-specific regulation of GATA3. Although we show that the downregulation of GATA3 by some progestins is mediated via PR-B, the role for PR-A in progestin-induced effects is unclear. However, these results indicate that the response of the progestogens will be affected by the ratio of PR-A:PR-B. Interestingly, while GES does not regulate TGFβ1 in the untransfected T47D cells, in the presence of excess PR-A, the ligand-independent upregulation of TGFβ1 expression is reduced. This suggests that GES, possibly acting via another steroid receptor, is most likely inhibiting this basal increase in TGFβ1 expression. Indeed, GES can bind to the AR (Louw-du Toit *et al.*, 2017b), MR (Louw-du Toit *et al.*, 2020), as well as ERα (Louw-du Toit *et al.*, 2017b).

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Our results showing that upregulation of FOXO1 expression is mediated by PR-B, is not surprising as previous studies in ES-2 ovarian cancer and T47D breast cancer cells have shown that FOXO1 is regulated in a PR-isoform specific manner (Diep *et al.*, 2016b; Truong *et al.*, 2019). Similar to what was observed in our study, in an ovarian cancer cell line, R5020 via PR-B upregulates FOXO1 expression (Diep *et al.*, 2016b), while the study by Truong and colleagues showed that in the presence of R5020, both PR isoforms can upregulate FOXO1 mRNA expression with PR-A causing a stronger regulation in the T47D cells (Truong *et al.*, 2019). The discrepancy between the results in our study versus the study by Truong and colleagues, could be due to the fact that our study made use of siRNA, while the latter study used T47D cells engineered to express only one isoform. However, this is the first study to show that the selected progestins used in contraception and/or MHT, like P_4 and R5020, upregulate FOXO1 mRNA expression via a PR-B-dependent mechanism. Interestingly, with the expression of excess PR-A, the upregulation of FOXO1 by the uniquely structured DRSP is enhanced. While the mechanism behind this response is unclear, there is the possibility that the DRSP bound PR is interacting with STAT3 (Liu and Ogle, 2002; Proietti *et al.*, 2011). This further increase with DRSP has not been shown for any of the other progestins in our panel, but has been observed for another progestin, Org2058, in the T47D cells expressing a similar ratio to our study (Graham *et al.*, 2005). Surprisingly, the P_4-induced upregulation of Ki67 mRNA expression appears to be only partially mediated by PR-B, whereas upregulation by the progestins is not PR-mediated. We thus hypothesize that these progestins may be acting via other steroid receptors to which they can bind. For instance, MPA has previously been shown to be an agonist via the GR and AR (Ronacher *et al.*, 2009; Africander *et al.*, 2014), with NET, LNG and GES being reported to be agonists for ERα and the AR (Louw-du Toit *et al.*, 2017b).

In summary, the results show that the effect of the progestins are not only PR isoform-specific, but gene-dependent as well. Furthermore, the differences observed between the progestogens

is most likely due to the differences in structure eliciting varied conformational changes in the PR isoforms, which in turn will influence homo- or heterodimer formation, co-factor recruitment and thus influencing activity (reviewed in Grimm et al., 2016) (Hapgood et al., 2018). Alternatively, the possibility of PR-A forming complexes with other steroid receptors and how this is influenced in the presence of PR-B and when the ratio is dysregulated, can also not be excluded.

3.4. Conclusion

Although progestins were designed to be PR ligands, it is surprising that published studies examining the relative efficacies and potencies of progestins via the PR are scarce. Moreover, studies seldom investigate different progestins in parallel in the same model system, or assess progestin activity specifically via PR-A and PR-B, respectively. This is the first study to determine the relative efficacies and potencies of a number of progestins, from different generations, and that of P_4 and the PR-specific agonist R5020, in parallel, in the same model system, via either PR-A or PR-B for transactivation. We also show for the first time that the efficacies and potencies of most of these progestins via PR-A are in fact influenced by the density of PR-A. This is also the first study to directly compare the agonist activity of these progestins for transrepression via PR-A and PR-B, respectively, a mechanism often overlooked when investigating PR action. Collectively, we show that despite all progestins being designed to mimic the activity of P_4, their activities are not always similar to each other or P_4, via the PR isoforms. We also show that the progestins and P_4 exhibit differential effects when the PR isoforms are co-expressed. Given that PR-A is often expressed in excess relative to PR-B in breast cancer tumours, we also assessed the activities of the progestins when PR-A is in excess of PR-B, and show that agonist activities for transactivation and transrepression are influenced in a progestogen- and ratio-specific manner. Interestingly, the EC_{50} values of all progestins for

the PR isoforms, expressed individually or co-expressed at different ratios, are either within or below the reported serum concentration ranges of the progestins in women (Bick et al., 2021), suggesting that these activities may likely be mimicked *in vivo*. Like others, we have shown that PR-A in excess may be inhibiting the activity of the progestogens via PR-B on a minimal PRE-containing promoter. However, in the context of more complex endogenous promoters containing various *cis*-regulatory elements, we do not observe this inhibition, but rather that the effects are gene- and progestogen-specific. While further studies will be needed to map the specific mechanism underlying the differential activities of each progestogen via the respective PR isoform, this study is the first to conduct a parallel comparison of multiple progestins via PR-A and/or PR-B in the same *in vitro* model system. The findings of this study contribute an important starting point to future studies investigating progestin mechanism via the PR, especially in progestin target tissues where the PR isoforms may be differentially expressed. Lastly, this study highlights the importance of investigating the actions of individual progestins and not extrapolating the mechanism/activity of one progestin to all progestogenic compounds, and the importance of distinguishing between the actions of the two PR isoforms.

Chapter 4

A direct comparsion of progesterone and progestin effects on hallmarks of breast cancer and the influence of the progesterone receptor isoform ratios

4.1. Background and Aims

Breast cancer is the leading oncology-related cause of death amongst women in developed countries (Jemal *et al.*, 2011; Torre *et al.*, 2015). In a developing country like South Africa, women have a 1 in 27 chance of developing breast cancer in their lifetime (Cancer in South Africa Full Report, National Cancer Registry 2014). While clinical studies have suggested that some progestins increase the risk of developing breast cancer (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; Marjoribanks *et al.*, 2017; Collaborative Group on Hormonal Factors in Breast Cancer, 2019), not all progestins have been evaluated and the mechanism by which progestins supposedly increase this risk is unknown. Bearing in mind that progestins were designed to mimic P_4 by binding to the PR, and that the role of the PR in breast cancer has recently gained traction (Daniel *et al.*, 2015; McFall *et al.*, 2015; Mohammed *et al.*, 2015; Singhal *et al.*, 2018; Truong *et al.*, 2019), research into the role of the PR in mediating the effects of progestins on breast cancer development and progression are warranted. Like most cancers, breast cancer has various hallmarks or biological processes during the multistep transformation of a normal cell into a cancer cell. These hallmarks include, but are not limited to, uncontrolled cell proliferation, evading programmed cell death (apoptosis), as well as activation of invasion and metastasis (Hanahan and Weinberg, 2000, 2011; Sledge and Miller, 2003). Migration is the ability of the transformed cancer cell to move within tissues or between organs, while invasion is when a transformed cell gains the ability to penetrate a tissue barrier [Reviewed in (Kramer *et al.*, 2013)]. Although a few studies have investigated the effects of some progestins, or the role of the PR, on some hallmarks, these studies mainly focus on cell proliferation, whereas side-by-side comparisons of the progestins or evaluation of PR isoform-specific effects on other hallmarks of cancer are scarce. Performing a direct comparison of the effects of a wider variety of progestins on these hallmarks is essential, as not all progestins have been evaluated in terms of breast cancer risk, and our results in Chapter 2

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and 3 highlight the importance of direct comparisons. Moreover, the significance of evaluating PR-A- and PR-B-specific effects is highlighted by our results in Chapter 3, as well as evidence in the literature showing divergent roles of these isoforms in breast cancer development and progression (Richer *et al.*, 2002; Truong *et al.*, 2019).

The aim of this Chapter was thus to directly compare the effects of P_4 and a selected panel of progestins from different generations on the hallmarks of tumour cells, and to establish the role of PR-A and PR-B. As PR-A and PR-B are co-expressed in some breast tumours, and the levels of PR-A are enhanced relative to PR-B in most breast cancers (Graham and Clarke, 2002; Mote *et al.*, 2015; Rojas *et al.*, 2017; McFall *et al.*, 2018), responses were also evaluated in the presence of equivalent PR-A and PR-B expression levels and when PR-A is expressed in excess in ratios of 2:1 and 5:1.

The objectives of this chapter were three-fold:

1. To compare the effects of the selected progestins from different generations to each other and P_4 in parallel, on cell growth and survival in the MDA-MB-231 breast cancer cell line transiently transfected with PR-A or PR-B.

2. To examine the effects of increasing concentrations of PR-A on progestogen-induced effects on cell growth and survival in the MDA-MB-231 breast cancer cell line exogenously co-expressing PR-A and PR-B at various ratios, as well as the T47D breast cancer cell line endogenously expressing both PR-A and PR-B.

3. To directly compare the effects of the selected progestins from different generations to each other and P_4 on migration and invasion of the MDA-MB-231 breast cancer cell line, and evaluate the influence of increasing concentrations of PR-A.

4.2. Materials and Methods

4.2.1. Cell culture

The human MDA-MB-231 and T47D breast cancer cell lines were maintained as described in Section 2.2.1 of Chapter 2.

4.2.2. Test compounds

R5020, P_4, MPA, NET, LNG, GES, NES, NOMAC and DRSP are described in Chapter 3, Section 3.2.2. Tamoxifen was purchased from Sigma-Aldrich, South Africa.

4.2.3. Plasmids

All steroid receptor cDNA expression vectors used in this chapter are described in Chapter 3, Section 3.2.3.

4.2.4. Western blot analysis

Western blot analysis was performed as described in Chapter 3, Section 3.2.4.

4.2.5. Cell viability assay

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell viability assay was used to measure effects on cell proliferation as previously described (Verhoog *et al.*, 2007). MDA-MB-231 and T47D cells were seeded into 10 cm dishes at a density of 2 x 10^6 cells per dish. The following day, the MDA-MB-231 cells were transiently transfected with 900 ng of either pSG5-PR-A or pSG5-PR-B only or 900 ng (1:1), 1 800 ng (2:1) or 4 500 ng (5:1) pSG5-PR-A in the presence of 900 ng pSG5-PR-B, while the T47D cells were transfected with 4 500 ng of either pSG5-PR-A or the pSG5-empty vector. The X-tremeGENE-HP transfection

reagent was used for transfections following the manufacturer's instructions. After 24 hours, the transfected MDA-MB-231 and T47D cells were seeded into 96-well plates at a density of 1×10^3 and 1×10^4 cells per well, respectively. The next day the cells were treated with 0.1% (v/v) ethanol, increasing concentrations (MDA-MB-231) or 100 nM (T47D) of R5020, P_4, MPA, NET, LNG, GES, NES, NOMAC or DRSP in phenol red-free DMEM supplemented with 10% (v/v) charcoal stripped (CS)-FBS and 1% (v/v) penicillin-streptomycin. After 48 hours, the cells were retreated with the test compounds and incubated for an additional 48 hours (MDA-MB-231) or 24 hours (T47D). Four hours before the end of the incubation period, 50 µL of the colorimetric MTT solution (5 mg/ml) (Sigma-Aldrich, South Africa) and 150 µL serum-free phenol red-free DMEM was added to each well. During the four-hour incubation the yellow MTT thiazole solution was reduced by metabolically active cells to form insoluble purple formazan crystals. The MTT solution is discarded and the crystals solubilised in 200 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, South Africa). The absorbance was measured at 550 nm using the Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific, South Africa).

4.2.6. Caspase-Glo® 3/7 apoptosis assay

The Caspase-Glo® 3/7 apoptosis assay kit (Promega, Anatech, South Africa) detects the activity of caspase-3 and caspase-7, which cleaves the caspase-3/7 prosubstrate as it recognises the DEVD tetrapeptide sequence found within the prosubstrate. After substrate cleavage, aminoluciferin is released and reacts with luciferase to produce light. The luminescence measured is thus proportional to the caspase activity. The kit was used to measure apoptosis according to the manufacturer's instructions. Briefly, MDA-MB-231 and T47D cells were seeded and transfected as described in Section 4.2.5. After 24 hours, the transfected cells were seeded into High Binding Isoplate–96 HB white framed, clear bottomed 96-well plates

(PerkinElmer, Massachusetts, USA) at 1 x 10^3 (MDA-MB-231) or 1 x 10^4 (T47D) cells per well using phenol red-free DMEM containing 10% (v/v) CS-FBS and 1% (v/v) penicillin-streptomycin. The following day, the cells were treated for 24 hours with 0.1% (v/v) ethanol or 100 nM of the selected progestogens in either phenol red-free DMEM supplemented with 10% (v/v) CS-FBS and 1% (v/v) penicillin-streptomycin (MDA-MB-231), or serum-free DMEM (T47D). The following experimental controls were included: cell culture medium without cells (blank – to be subtracted from sample absorbances) and cells treated with 25 µM tamoxifen (Sigma-Aldrich, South Africa) (positive control). Following treatment with the test compounds and controls, the Caspase-Glo® 3/7 substrate was added to the wells and the plates incubated at room temperature for 2 hours. The luminescence (in RLUs) of each sample was measured using a Veritas microplate luminometer (Turner Biosystems, USA).

4.2.7. Anchorage-independent growth assay

Anchorage-independent growth or soft agar assays were performed as described with a few modifications (Perkins *et al.*, 2017). MDA-MB-231 cells were seeded and transfected as previously described in Section 4.2.5. After 24 hours, 600 µL of phenol red-free DMEM containing 10% (v/v) CS-FBS, 1% (v/v) penicillin-streptomycin and 0.5% (w/v) agar was added to each well of a 12-well plate, and allowed to solidify at room temperature for 1 hour. A second layer of medium containing 0.3% (w/v) agar and 1 x 10^4 cells was then added. The cells were treated with either 0.1% (v/v) ethanol or 100 nM test compound in phenol red-free DMEM supplemented with 10% (v/v) CS-FBS and 1% (v/v) penicillin-streptomycin, for 21 days in total, with fresh compound being added at day 7 and day 14. The colonies formed were fixed by adding 600 µL of 37% (v/v) formaldehyde and stained with 0.005% (w/v) crystal violet. Colonies were counted using ImageJ software (v.1.8) (Schneider *et al.*, 2012; Guzmán *et al.*, 2014).

4.2.8. Migration (Scratch wound-healing) assay

The human MDA-MB-231 cells were seeded into 10 cm dishes at a density of 2×10^6 cells per dish in supplemented phenol red DMEM and transiently transfected as described in Section 4.2.5. Twenty-four hours later the cells were seeded into 24-well plates at a density of 2.5×10^5 cells per well in phenol red-free DMEM supplemented with 10% (v/v) CS-FBS and 1% (v/v) penicillin-streptomycin. Cells were subsequently treated with 5 µg/mL Mitomycin C (Sigma-Aldrich, South Africa), an inhibitor of proliferation, for 2 hours (Tomasz, 1995). The medium was removed and using a pipette tip, a "scratch/wound" was made in the cell monolayer. Cells were washed twice with 1x PBS to remove the cell debris, and subsequently treated for 48 hours with 0.1% (v/v) ethanol or 100 nM of the selected progestogens. Images were captured at T_0 (immediately after the scratch was made) and every 12 hours over a period of 48 hours using the Olympus IX81 Inverted Microscope (Olympus Biosystems GmbH, Germany) (4x objective) and Cell-R Live Imaging software. ImageJ software (v1.49) was used to assess the distance migrated by calculating the difference in distance between the leading edge of the initial wound (T_0) versus that at 12, 24, 36 and 48 hours (T_{12}, T_{24}, T_{36} and T_{48}).

4.2.9. Transwell invasion assay

Polycarbonate transwell inserts containing 8.0 µm pore filter membranes (Corning, Inc., Corning, NY) were used. Each filter membrane was coated with 100 µg/mL matrigel (BD Biosciences, San Jose, CA, USA), and allowed to dry for 60 minutes in an incubator at 37ºC, 5% CO_2 and 90% humidity. MDA-MB-231 cells were seeded and transfected as in Section 4.2.5. Using serum-free DMEM, the transfected cells were then gently seeded on top of the matrigel at 2.5×10^4 cells per insert. After 30 minutes, either 0.1% (v/v) ethanol or 100 nM of the selected progestogens were added to the cell suspension. The inserts were placed into the wells of a 24 well-tissue culture plate containing 600 µL of phenol red-free DMEM

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supplemented with 10% (v/v) CS-FBS (chemo-attractant). After a 24 hour incubation period the transwell inserts were removed, the medium aspirated and excess cells that did not migrate were carefully removed from the upper side of the filter membrane using a cotton swab. Inserts were washed twice with 1x PBS and the cells fixed by placing the inserts into 1 mL of 70% (v/v) ethanol for 10 minutes. Thereafter, the inserts were rinsed twice with 1x PBS and allowed to air dry. Cells were subsequently stained with 0.2% (w/v) crystal violet (Sigma-Aldrich, South Africa) for 20 minutes at room temperature in the dark. Excess crystal violet was removed by carefully washing the inserts with distilled water and then allowing the inserts to air dry. Images of the stained cells were captured using the Carl Zeiss LSM 780 confocal microscope with a 4x objective using ZEN® 2 software. The cells from ten non-overlapping areas (from the centre of the insert as well as the surrounding edges) were counted using a script in Wolfram Mathematica (www.wolfram.com) version 12.0.0. The pixel values above a certain threshold (typically 0.35) were binarized and colour negated. To analyse overlapping cells a distance transform (distance of pixel to nearest background level pixel) was combined with a gradient filter and watershed transform. Subsequently, components with pixel counts larger than 500 (and smaller than 10 000) were selected, and the centroid and disk radius of the components were determined and plotted on the original image for visual inspection of the analysis. Finally, pixel counts were binned and plotted in bar graphs.

4.2.10. Small interference RNA (siRNA) transfection

siRNA transfection of the T47D cells was performed as previously described in Chapter 3, Section 3.2.8. Cells were subsequently seeded for qPCR assays (described in Chapter 3), while for MTT assays, cells were seeded into 96-well plates at a density of 1×10^4 cells per well using supplemented phenol red-free DMEM. The next day, the cells were treated with 100 nM test compounds in serum-free DMEM for 72 hours prior to harvesting for cell viability assays

(Section 4.2.5). PR protein knock down levels were confirmed using western blot analysis as described in Section 3.2.4 of Chapter 3.

4.2.11. Data and statistical analysis

The GraphPad Prism® v7.00 software package (GraphPad Software, USA) was used for analysis of results, graphical representation and statistical analysis. One-way analysis of variance (ANOVA) with Dunnett's (compares all columns to the control) or Bonferroni (compares all pairs of columns) post-test, two-way ANOVA with the Bonferroni post-test or unpaired *t*-tests, was used for statistical analyses. Unless otherwise stated, the error bars indicate the standard error of the mean (SEM) of three or more independent experiments. When comparing to a control, statistically significant differences are represented by *, ** and *** indicating $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. No statistical differences ($p > 0.05$) is indicated by ns. When comparing all means, the letters a, b, c etc. are used, where the values that differ significantly from others are assigned a different letter.

4.3. Results and discussion

4.3.1. All of the selected progestogens are full agonists for proliferation in the MDA-MB-231 cells transiently transfected with PR-A.

The collective effects of both transactivation and transrepression contribute to biological responses, including, but not limited to, cell proliferation, apoptosis, migration and invasion (Nicholson *et al.*, 1999; Hanahan and Weinberg, 2000, 2011). Given that we have shown differential responses of the progestogens on transactivation and transrepression in the presence of both PR isoforms, and when the isoforms are expressed alone (Chapter 3), we next

determined the effects of the selected progestogens on the above-mentioned biological processes in breast cancer cell lines expressing the individual PR isoforms alone or both PR-A and PR-B. First, the agonist efficacies and potencies of the selected panel of progestins from different generations were compared for proliferation in human MDA-MB-231 breast cancer cells transiently transfected with expression vectors for either PR-A or PR-B. Results of the MTT cell viability assay show that all progestogens increase proliferation of the MDA-MB-231 cells transfected with PR-A (Figure 4.1A) and PR-B (Figure 4.1D). Although the maximal responses displayed by some progestins in the dose-response curves appear to be lower than that of R5020, these responses are not statistically different, suggesting that all progestogens have similar efficacies for proliferation and are full agonists via both PR-A (Figure 4.1B) and PR-B (Figure 4.1E). In terms of potency, all progestogens display indistinguishable potencies via PR-A, with the exception of DRSP, which is the least potent progestin (Figure 4.1C). On the other hand, DRSP is equipotent to all the other progestogens via PR-B, with NOMAC being the least potent (Figure 4.1F). No proliferation is observed in the absence of transfected PR (Addendum D, Figure D1), suggesting that the progestogen-induced increase in proliferation is mediated by PR-A or PR-B. When directly comparing the efficacies of the progestogens via PR-A and PR-B, results show that LNG is more efficacious via PR-B, with no differences observed for the other progestogens (Figure 4.1G). In terms of potency, only DRSP is more potent via PR-B and NOMAC is more potent via PR-A (Figure 4.1H). The potencies and efficacies of the progestogens for PR-A and PR-B are summarised in Table 4.1.

Next it was assessed whether the progestin-induced proliferation is modulated when PR-A and PR-B are co-expressed at equivalent levels or with increasing expression levels of PR-A relative to PR-B. The MDA-MB-231 cells were thus transiently transfected with PR-A and PR-B in ratios of 1:1, 2:1 and 5:1, and the resulting dose-response curves, as well as the efficacy and potency plots for the progestogens at the different ratios, are shown in Addendum D, Figure
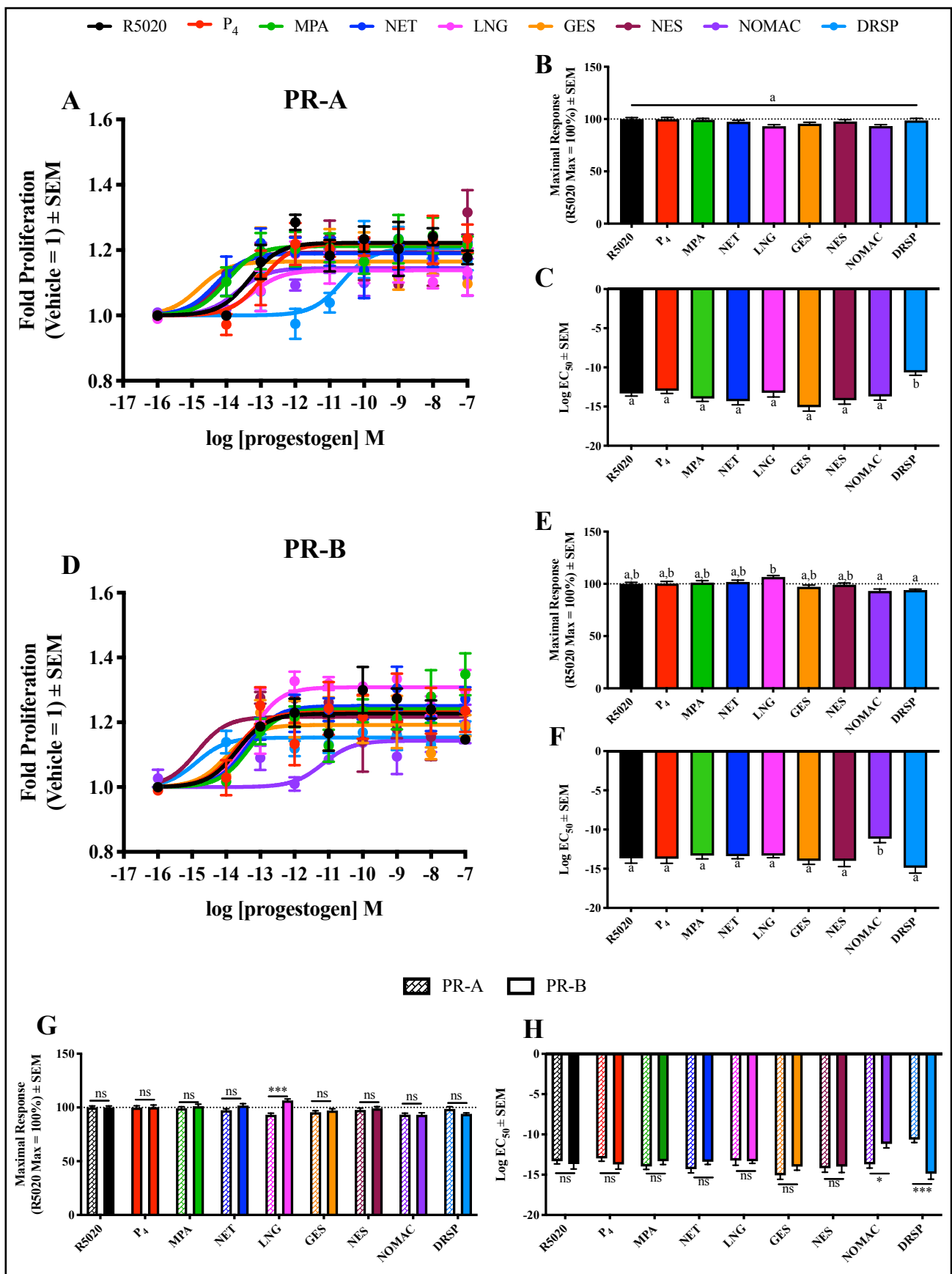
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Figure 4.1. All progestogens are full agonists for breast cancer cell proliferation via PR-A and PR-B. The MDA-MB-231 cells were transiently transfected with 900 ng of either **(A)** pSG5-PR-A or **(D)** pSG5-PR-B. Cells were treated with 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 48 hours, then retreated for a further 48 hours. Proliferation was quantified using the colorimetric MTT cell viability assay. Results are shown as fold proliferation where the vehicle is set as 1, while all other responses were calculated relative to this. Plots are shown for the maximal responses and $logEC_{50}$ values of the progestogens via **(B and C)** PR-A and **(E and F)** PR-B. For a direct comparison of the proliferative response by the progestogens via the two PR isoforms, the **(G)** maximal response and **(H)** $logEC_{50}$ values for PR-A and PR-B were replotted as grouped data. The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-or two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by letters a and b, where the values that differ significantly from other values are assigned a different letter or by * or ***, indicating $p < 0.05$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns.

D2. Notably, regardless of the transfection condition, no proliferation of the MDA-MB-231 cell line was observed in the absence of ligand (Addendum D, Figure D3). To directly compare the responses of the progestogens in cells co-expressing various ratios of PR-A and PR-B to responses in cells expressing only PR-B (Figure 4.2A), the maximal responses of the progestogens (Addendum D, Figure D2) were all set relative to the maximal response of R5020 via PR-B, shown in Figure 4.1. In terms of efficacy, the results show that the co-expression of PR-A and PR-B at all ratios has no significant effect on the efficacy of R5020, P_4, LNG, GES and DRSP compared to when only PR-B is expressed (Figure 4.2A and B). In contrast, the efficacy of MPA and NES increase when the isoforms are expressed equally, while the efficacy of NET decreases when PR-A is double that of PR-B (Figure 4.2B). When PR-A is expressed 5x in excess of PR-B, the efficacy of NOMAC increases compared to PR-B only (Figure 4.2B). The potency of the progestogens is less influenced by the presence and density of PR-A co-expressed with PR-B.

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Table 4.1. Relative agonist efficacies (maximal responses) and potencies (EC$_{50}$) of the progestogens for proliferation via either PR-A or PR-B expressed in MDA-MB-231 cells.

	Maximal Response (%) ± SEM								
Ligand	R5020	P$_4$	MPA	NET	LNG	GES	NES	NOMAC	DRSP
PR-A	100.0 ± 1.4	99.9 ± 1.7	99.3 ± 1.5	97.5 ± 1.4	93.2 ± 1.5	95.6 ± 1.4	97.7 ± 1.8	93.4 ± 1.3	93.9 ± 1.8
PR-B	100.0 ± 1.5	100.3 ± 2.0	101.1 ± 2.0	101.9 ± 1.7	106.6 ± 1.3	97.1 ± 1.7	99.2 ± 1.6	93.2 ± 1.9	94.0 ± 0.89
	EC$_{50}$ (M) ± SEM								
PR-A	4.5 ± 3.0 x 10^{-14}	1.1 ± 0.8 x 10^{-13}	1.0 ± 0.8 x 10^{-14}	4.9 ± 2.2 x 10^{-15}	5.6 ± 3.6x 10^{-14}	1.6 ± 0.7 x 10^{-15}	6.3 ± 1.2 x 10^{-15}	2.0 ± 0.9 x 10^{-14}	2.3 ± 1.6 x 10^{-11}
PR-B	2.1 ± 1.3 x 10^{-14}	1.9 ± 1.2 x 10^{-14}	4.8 ± 3.7 x 10^{-14}	4.1 ± 3.2 x 10^{-14}	4.7 ± 2.8 x 10^{-14}	1.0 ± 0.4 x 10^{-14}	1.3 ± 0.9 x 10^{-15}	6.7 ± 4.6 x 10^{-12}	1.3 ± 0.6 x 10^{-15}

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For instance, MPA is less potent, and NOMAC more potent, when PR-A is double that of PR-B, compared to PR-B and the other ratios, while DRSP is less potent when the isoforms are equally expressed (Figure 4.2C).

Next, we directly compared the progestogen-induced responses in cells expressing the various isoform ratios to responses in cells expressing PR-A only (Figure 4.3). The maximal responses of the progestogens obtained at the different PR-A:PR-B ratios (Addendum D, Figure D2) were set relative to the maximal response of R5020 via PR-A (Figure 4.1). Besides a few exceptions, the results of this comparison (Figure 4.3) is mostly similar to the earlier comparison to PR-B only (Figure 4.2). For example, while the efficacy of NET at the 2:1 ratio is similar to its efficacy via PR-A (Figure 4.3A and B), it is significantly lower than the efficacy via PR-B (Figure 4.2B). In addition, the potency of NOMAC at the 1:1 and 5:1 ratio is similar to that via PR-B (Figure 4.2C), yet less potent via PR-A (Figure 4.3C).

Proliferation was also evaluated in T47D cells which express endogenous PR-A and PR-B at a ~2:1 ratio in our laboratory. Similar to the results observed in the MDA-MB-231 cells when the expression of PR-A:PR-B is at 2:1 (Addendum D, Figure D2B), the results in Figure 4.4 show that R5020, P_4 and all of the selected progestins significantly increase proliferation of the T47D cells, to similar extents. These results also correlate to the observed upregulation of the pro-proliferative gene, Ki67, by all progestogens, mostly to a similar extent, in the T47D cells (Chapter 3, Figure 3.9E). Although we did not perform dose-response analyses in this study, relative efficacies from a previous study in the Africander laboratory (van der Meer, MSc dissertation 2017), reported similar responses as we observe at 100 nM progestogen in the T47D cells. In addition, the relative efficacies of R5020, P_4, MPA, NET-A, LNG, GES and DRSP for proliferation of the T47D cells are similar to those we determined in the MDA-MB-231 cells transfected with both PR-A and PR-B (2:1).
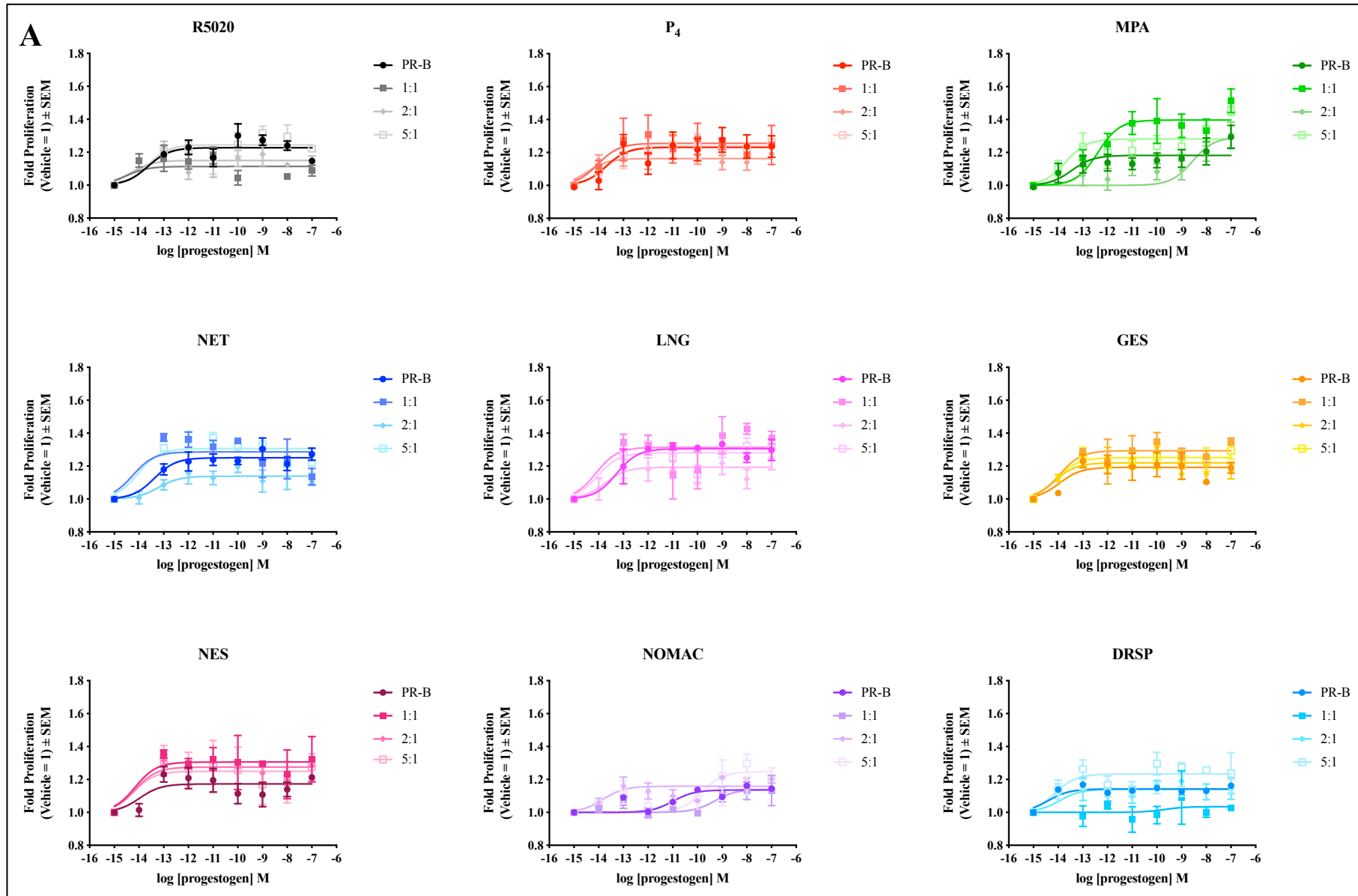
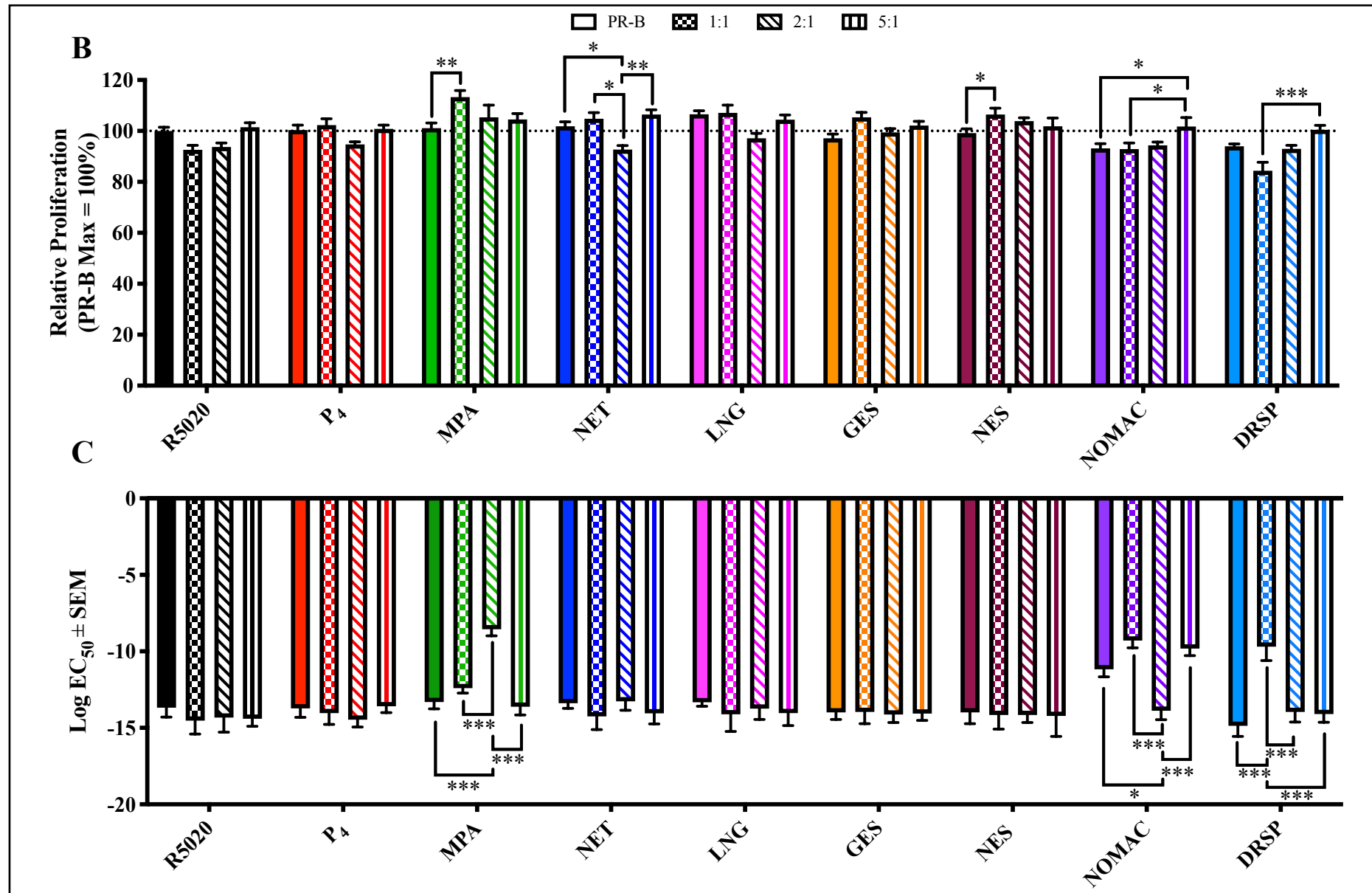**Figure 4.2. continues on the following page.**

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Figure 4.2. Co-expression of PR-B with various levels of PR-A differentially modulates progestogen-induced cell proliferation compared to PR-B only. (A) Direct comparison of the dose response curves for PR-B (from Figure 4.1) and the subsequent ratios (from Addendum D, Figure D2). The **(B)** maximal response and **(C)** EC$_{50}$ values determined at the different PR isoform ratios were set relative to the values obtained via PR-B only, with the maximal response of R5020 via PR-B set to 100%. The results shown are the averages ($\pm$ SEM) of at least three independent experiments. Two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$. Absence of stars indicate no significant differences ($p > 0.05$).

The evidence in the literature on effects of progestins on breast cancer cell proliferation is contradictory, with some studies showing proliferative effects, while others show anti-proliferative effects. In agreement with our study, other studies conducted in various *in vitro* breast cancer cell lines expressing both PR isoforms (T47D, MCF7, BT-474 or HCC1500 cells), have shown that R5020 (Hissom and Moore, 1987), P$_4$ (Carvajal *et al.*, 2005; Liang *et al.*, 2007, 2010), MPA, NET-A, NET (Schoonen *et al.*, 1995b, 1995a; Franke and Vermes, 2003; Seeger *et al.*, 2003a; Krämer *et al.*, 2006; Giulianelli *et al.*, 2012; Ruan *et al.*, 2012; Wargon *et al.*, 2015), LNG and GES (Catherino *et al.*, 1993; Kalkhoven *et al.*, 1994; Schoonen *et al.*, 1995b, 1995a; Seeger *et al.*, 2003a; Krämer *et al.*, 2006; Ruan *et al.*, 2012), as well as DRSP (Ruan *et al.*, 2012), increase cell proliferation. In contrast to our results, R5020, P$_4$, (Horwitz and Freidenberg, 1985; Musgrove *et al.*, 1991; Botella *et al.*, 1994; Formby and Wiley, 1999; Seeger *et al.*, 2003), MPA (Musgrove *et al.*, 1991; Botella *et al.*, 1994; Seeger *et al.*, 2003b), NET (Seeger *et al.*, 2003b) and NOMAC (Botella *et al.*, 1994) have been reported to have anti-proliferative effects in MCF-7, T47D and/or T47Dco breast cancer cell lines. Importantly, none of the above-mentioned studies report efficacies and potencies of the progestogens for proliferation. This information is pertinent as it allows comparisons to serum concentrations in women using these ligands in reproductive medicine.
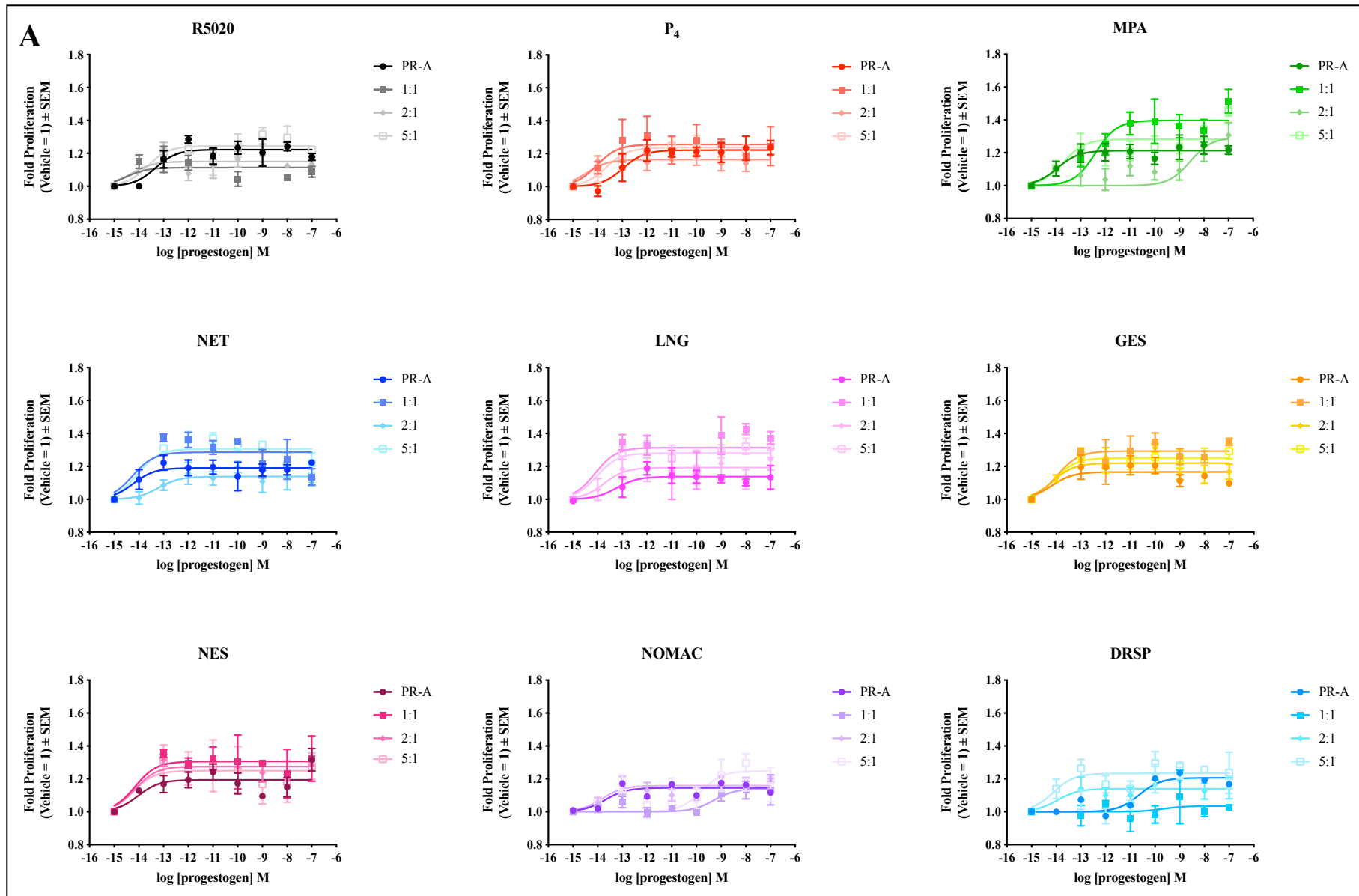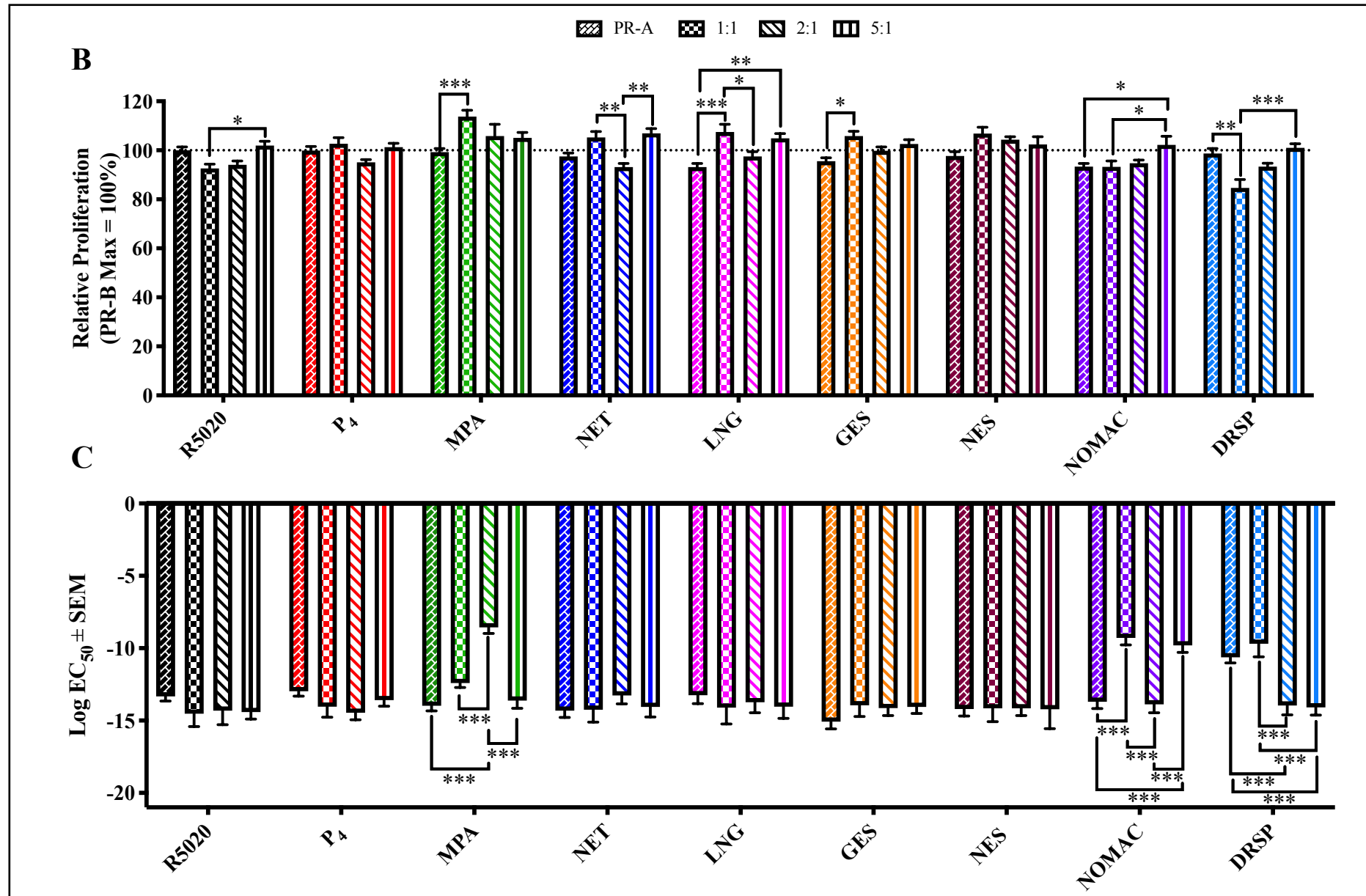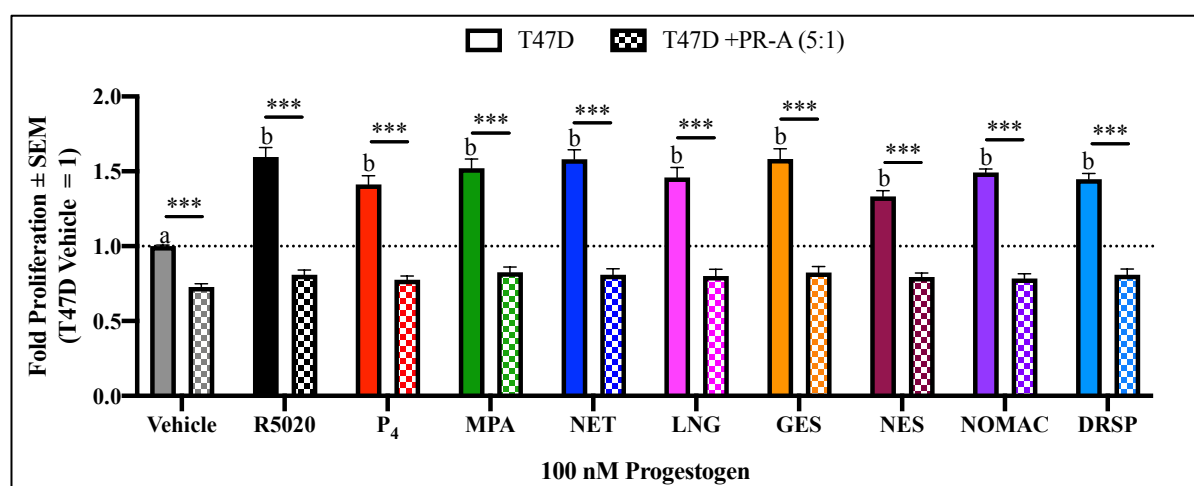
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Figure 4.3. Co-expression of PR-B with various levels of PR-A differentially modulates progestogen-induced cell proliferation compared to PR-A only. (A) Direct comparison of the dose response curves for PR-A (from Figure 4.1) and the subsequent ratios (from Addendum D, Figure D2). The **(B)** maximal response and **(C)** EC_{50} values determined at the different PR isoform ratios were set relative to the values obtained via PR-A only, with the maximal response of R5020 via PR-A set to 100%. The results shown are the averages (±SEM) of at least three independent experiments. Two-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$. Absence of stars indicate no significant differences ($p > 0.05$).

Indeed, the concentration of the progestins found in serum of women are mostly within the nM range after progestin treatment [reviewed in (Perkins *et al.*, 2018; Bick *et al.*, 2021)], and since the potencies of the progestins for proliferation shown in this section are all in the pM range, irrespective of whether one or both PR isoforms are present, it is likely that progestin-induced breast cancer cell proliferation may occur *in vivo*.

For the investigation into the influence of excess PR-A, the T47D cells were transiently transfected with 4 500 ng pSG5-PR-A, resulting in a 5:1 ratio of PR-A to PR-B. The results in Figure 4.4 show that proliferation decreases both in the absence and presence of ligand to the same extent, suggesting that the decrease observed is not due to the progestogens, but due to a ligand-independent decrease in proliferation in the presence of 5x PR-A. Given that PR-A is known to repress the activity of PR-B and other steroid receptors (Tung *et al.*, 1993; Vegeto *et al.*, 1993; McDonnell *et al.*, 1994; Giangrande *et al.*, 2000; Abdel-Hafiz *et al.*, 2002; Patel *et al.*, 2018), it is likely that the unliganded PR-A, when expressed in excess, inhibits the proliferative activity of PR-B, or other steroid receptors such as the AR or ER. Our findings suggest that higher PR-A expression levels relative to PR-B may lead to a positive prognosis in terms of breast cancer, as the excess expression of PR-A significantly decreases proliferation. Interestingly, a recent study has shown that a high PR-A:PR-B ratio is associated
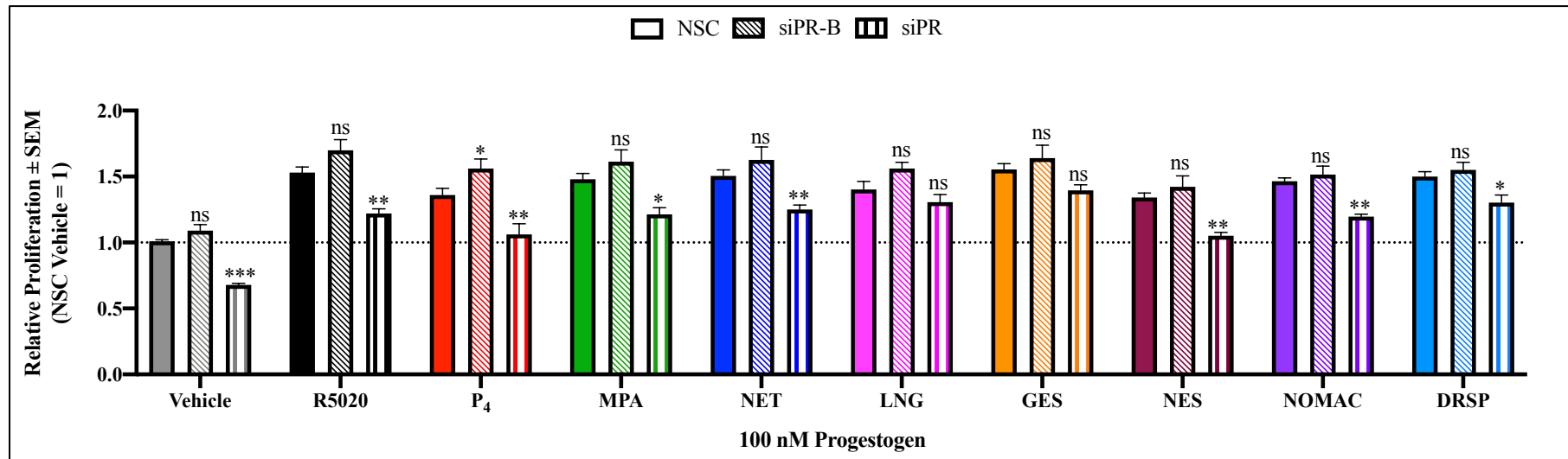
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with a positive prognosis, as the authors observed a decrease in the expression of the proliferation marker Ki67 after treatment with the anti-progestin mifepristone in primary breast cancer tissue with a high PR-A:PR-B ratio (Rojas *et al.*, 2017).

Figure 4.4. All progestogens induce proliferation of the T47D cells, while the excess expression of PR-A inhibits proliferation both in the absence and presence of ligand. The T47D were transiently transfected with 4 500 ng of either pSG5-empty ( ) or pSG5-PR-A ( ). The cells were treated with 0.1% (v/v) ethanol (vehicle control) or 100 nM progestogen for 48 hours, then retreated for a further 24 hours. Proliferation was quantified using the colorimetric MTT cell viability assay. Results are shown as fold proliferation relative to the vehicle control of the pSG5-empty vector transfected T47D cells ( ). The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used to compare the progestogen effects to the vehicle and each other (first bar of every group), and statistical differences are indicated with letters (a and b), with different letters indicating a statistical difference. Two-way ANOVA with Bonferroni's post-test was used to compare the responses of the pSG5 transfected T47D cells with the T47D cells expressing excess PR-A for each treatment group, and statistically significant differences are represented by ***, indicating p < 0.001. One-way ANOVA with Dunnett's (compares all columns to control) post-test was also used to compare the effects of T47D+PR-A (5:1) vehicle versus T47D+PR-A (5:1) progestogens (second bar of every group) and the statistical analysis indicate the following: T47D+PR-A (5:1): vehicle versus all progestogens (p > 0.05).

However, our results, together with the evidence in the literature, suggest that the role of the PR isoforms, as well as dysregulation of the isoform ratio, may be context dependent. Lastly,

in the context of the T47D breast cancer cells endogenously co-expressing both PR isoforms, as well as the GR, AR, MR and ER subtypes, we wanted to determine the role of PR-A and PR-B in the progestogen-induced upregulation of cell proliferation. Thus, proliferation was measured in T47D cells in the absence and presence of transfected 10 nM NSC siRNA or siRNA directed against PR-B only (PR-B siRNA) or against both PR isoforms (PR siRNA) (Figure 4.5). As the same cells were used for both qPCR (Chapter 3) and the cell viability assays, refer to Figure 3.10 for the western blot analysis confirming reduction of both PR-A and PR-B, as well as PR-B only after transient transfection of the respective siRNA. Results indicate that progestogen-induced proliferation of the T47D cells is not mediated via PR-B, as silencing the expression of PR-B did not modulate the effect (Figure 4.5). These results correlate with our findings for the progestins on gene expression, where PR-B is not required for the progestin-induced upregulation of Ki67 mRNA expression (Chapter 3, Figure 3.11D). However, when both PR isoforms are silenced, we show that the P_4- and NES-induced increase in proliferation is completely abolished, suggesting that the response by these progestogens are mediated by PR-A. When both PR isoforms are silenced, the proliferation in the absence and presence of most progestins, except LNG and GES, decreases. However, the proliferative effects of R5020, MPA, NET, NOMAC and DRSP were only partially abolished, indicating the involvement of other steroid receptors in addition to PR-A. In contrast, the results suggest that neither PR-A nor PR-B are required for the proliferative responses of LNG and GES in the T47D breast cancer cell line, as their responses were not modulated when both PR isoforms were silenced. Considering that both LNG and GES are androgenic, and the fact that they are as efficacious as E_2 for transactivation via ERα (Louw-du Toit *et al.*, 2017b), it is likely that either the AR or ERα may be mediating the proliferative effects of these progestins. Further studies are needed to elucidate the possible involvement of these, or other, steroid receptors.

Figure 4.5. While PR-A mediates the proliferative effects of P$_4$ and NES, it only partly mediates the effects of R5020, MPA, NET, NOMAC and DRSP. The T47D cells were transiently transfected with either 10 nM non-silencing scrambled sequence control (NSC) siRNA, or siRNA directed against both PR-A and PR-B or only PR-B. Cells were treated with either the 0.1% (v/v) ethanol (vehicle control) or 100 nM progestogens for 48 hours, then retreated for a further 24 hours. Proliferation was quantified using the colorimetric MTT cell viability assay. The responses of all progestogens are set relative to the vehicle control of the T47D cells transfected with the NSC siRNA, which is set as 1. The results shown are averages (±SEM) of at least 3 biological repeats. Two-way ANOVA with Bonferroni's post-test was used for statistical analysis to compare the response of all treatment groups after PR-B or PR-A/B siRNA knockdown to the NSC siRNA (first bar of every group). Statistically significant differences are represented with either *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. No statistical significance ($p > 0.05$) is indicated by ns.

4.3.2. Progestogens do not influence caspase 3/7 activity in both the MDA-MB-231 cells expressing PR-A and/or PR-B and in the T47D cells, however, the co-expression of PR-B and excess PR-A modulates apoptosis only in the T47D cells.

Tumorigenesis and tumour progression occur because of an imbalance between proliferation and apoptosis (Mommers *et al.*, 1999). Having shown that the selected progestogens increase proliferation of MDA-MB-231 cells transfected with PR-A and PR-B, as well as that of T47D cells endogenously expressing both isoforms, and that increasing the expression of PR-A relative to PR-B only effects the proliferative responses of a few progestogens, we next determined the effects of the progestogens on cell apoptosis. MDA-MB-231 and T47D cells, transiently transfected as for the proliferation assays, were treated with 100 nM progestogens for 24 hours before apoptosis was measured using the Caspase-Glo$^®$ 3/7 apoptosis assay kit. The results show that neither P_4, nor any of the progestins, induce apoptosis in MDA-MB-231 cells transfected with PR-A (Figure 4.6A), or PR-B (Figure 4.6B) or both PR-A and PR-B at equivalent levels (Figure 4.6C), at a PR-A:PR-B ratio of 2:1 (Figure 4.6D) or at a PR-A:PR-B ratio of 5:1 (Figure 4.6E). Tamoxifen, known to induce apoptosis (Zhang *et al.*, 1999), significantly induces caspase 3/7 activity in all cells (Figure 4.6), indicating that the lack of response by the test compounds is not due to the assay not working. These results suggest that in the MDA-MB-231 cells, the progestins have no influence on the caspase 3/7 apoptotic pathway.

Next, we investigated whether the progestogens would induce apoptosis of the T47D cells endogenously expressing multiple steroid receptors, including both PR-A and PR-B. Furthermore, we wanted to assess whether apoptosis would be influenced when PR-A is expressed in excess relative to PR-B. Similar to the results in the transfected MDA-MB-231 cells, none of the progestogens induced apoptosis of the T47D cells (Figure 4.7).

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Figure 4.6. None of the progestins induce apoptosis in MDA-MB-231 cells transfected with either PR-A or PR-B, or MDA-MB-231 cells co-expressing PR-A and PR-B. The MDA-MB-231 cells were transiently transfected with 900 ng of **(A)** pSG5-PR-A, **(B)** pSG5-PR-B, as well as PR-A and PR-B at a **(C)** 1:1, **(D)** 2:1 and **(E)** 5:1 ratio. The cells were treated with either the 0.1% (v/v) ethanol (vehicle control), 25 µM tamoxifen (positive control) or 100 nM progestogen for 24 hours. The Caspase-Glo® 3/7 apoptosis assay kit was used to assess apoptotic activity. Graphs show fold Caspase 3/7 activity with the responses set relative to the vehicle control set as 1. The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are shown by letters a and b, where the values that differ significantly from other values are assigned a different letter.

This is in contrast to the gene expression results (Chapter 3) where we show that R5020 and P_4 increase the expression of the pro-apoptotic gene BAX in the T47D cells. However, we cannot exclude the possibility that the progestogens can in fact influence apoptosis. Apoptosis is characterized by many different traits such as chromosome condensation, cell shrinking, nuclear fragmentation, phosphatidyl serine translocation [reviewed in (He *et al.*, 2009)]. Therefore, apoptosis can be assayed using a number of different approaches besides the measurement of caspase 3 and 7 activity or investigating expression of the pro-apoptotic marker, BAX. Indeed, earlier studies have shown that R5020, MPA, NET, LNG and GES inhibit apoptosis in T47D, MCF7 and HCC1500 breast cancer cells (Ory *et al.*, 2001; Krämer *et al.*, 2006; Moore *et al.*, 2006). These studies used various approaches to investigate apoptosis. For example, the response of MPA was determined by evaluating the mRNA expression of multiple members of the B-cell lymphoma 2 (BCL-2) family of proteins (excluding BAX), with the MPA-mediated suppression of apoptosis further confirmed using Hoechst staining to identify chromatin condensation (Ory et al., 2001). Furthermore, R5020 was shown to decrease apoptosis by examining caspase 3 activation through western blot analysis (Moore *et al.*, 2006) and the decrease in apoptosis in response to NET, LNG and GES was determined using an enzyme-linked immunosorbent assay (ELISA) kit which detected histone-associated DNA fragments in the cytoplasm as a measure of apoptosis (Krämer *et al.*, 2006).

In contrast to our results indicating no apoptosis of the MDA-MB-231 cells exogenously expressing PR-B and excess PR-A, apoptosis of T47D cells expressing endogenous PR-A and PR-B, as well as exogenous excess PR-A, was observed in the absence and presence of all progestogens (Figure 4.7). Although it appears that apoptosis is enhanced in the presence of the progestogens, these differences were not statistically significant. However, one cannot exclude that these responses may in fact be significant, but not within the statistical power of

the current experiments. Notably, the increase in apoptosis of T47D cells expressing excess PR-A in the absence of ligand, correlates with the observed decrease in proliferation of the same cells (Figure 4.4). Taken together, the results for proliferation and apoptosis of T47D cells expressing 5x more PR-A than PR-B, suggest that excess PR-A may be playing a tumour suppressive role in these cells in a ligand-independent manner.

Figure 4.7. Excess PR-A, relative to PR-B, increases apoptosis of T47D breast cancer cells. T47D cells transiently transfected with 4 500 ng of either pSG5-empty (☐) or pSG5-PR-A (▨) were treated with either the 0.1% (v/v) ethanol (vehicle control), 25 µM tamoxifen (positive control) or 100 nM progestogen for 24 hours. The Caspase-Glo® 3/7 apoptosis assay kit was used to assess apoptotic activity. Graphs show fold Caspase 3/7 activity with all responses set relative to the vehicle of the pSG5 transfected T47D cells (☐), set as 1. The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used to compare the progestogen effects to the vehicle and each other (first bar of every group), and statistical differences are indicated with letters (a and b), with different letters indicating a statistical difference. Two-way ANOVA with Bonferroni's post-test was used to compare the responses in the absence and presence of excess PR-A, and statistically significant differences are represented by *, ** or ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. One-way ANOVA with Dunnett's (compares all columns to the control) post-test was also used to compare the effects of T47D+PR-A (5:1) vehicle versus T47D+PR-A (5:1) progestogens and the statistical analysis indicate the following: T47D+PR-A (5:1): vehicle versus all progestogens ($p > 0.05$).

4.3.3. R5020, MPA, NET, LNG, GES and NES exhibit isoform-specific effects on the anchorage-independent growth of the MDA-MB-231 breast cancer cells.

Given that the ability of tumour cells to survive and grow anchorage independently is essential for metastasis, we next used a soft agar colony formation assay to evaluate the effects of the selected progestogens on anchorage-independent growth of the MDA-MB-231 cells exogenously expressing PR-A or PR-B. Cells were treated with 100 nM progestogen for 21 days, and the results show that only P4 and MPA significantly increase colony formation in cells expressing PR-A (Figure 4.8A), while R5020 and MPA increase colony formation in cells expressing PR-B (Figure 4.8B). Similarly, it has been shown that R5020 increases colony formation in T47D cells engineered to only express PR-B (T47D-YB cells) (Hagan *et al.*, 2011; Truong *et al.*, 2019). Although not significantly different compared to the vehicle, this statistical analysis shows that P4, NET, LNG, GES and NES are similar to R5020 and MPA, thus it is probable that these progestogens may also increase colony formation in cells expressing PR-B. Notably, no colonies were formed in MDA-MB-231 cells lacking the PR isoforms when treated with the vehicle or progestogens, suggesting that the progestogen-induced colony formation observed in Figure 4.8 is mediated by the exogenously expressed PR-A or PR-B. The effects on anchorage-independent growth of MDA-MB-231 cells co-transfected with varying ratios of PR-A:PR-B are progestogen- and ratio-specific (Figure 4.9). For example, when the isoforms are present in equimolar concentrations, only R5020 increases colony formation (Figure 4.9A). In contrast, the study by Truong and colleagues showed that R5020 has no effect on colony formation in T47Dco cells (Truong *et al.*, figshare, 2018), presumably expressing equal amounts of the PR isoforms.
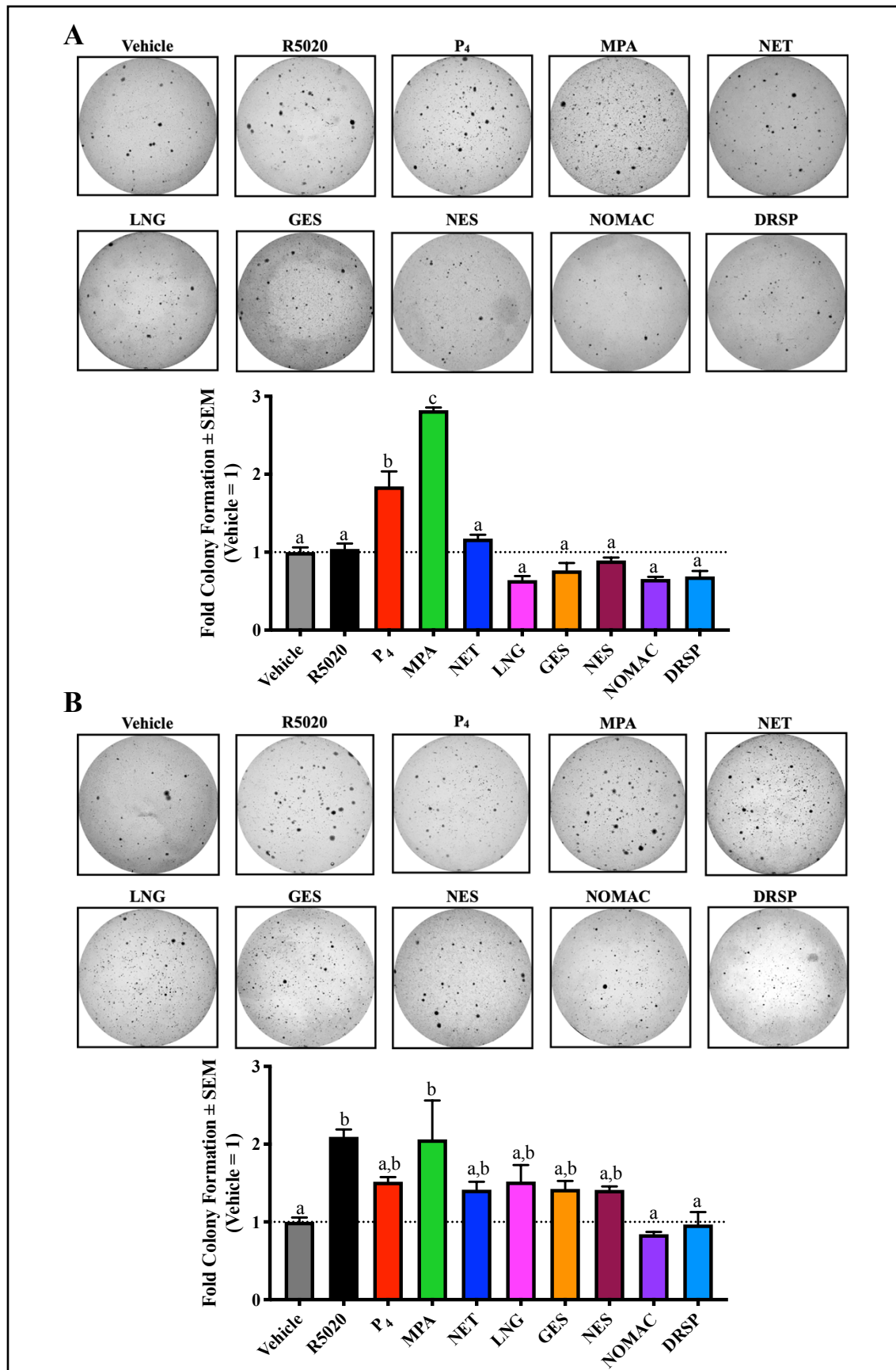
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Figure 4.8. Effects on anchorage-independent growth are progestin- and isoform-specific. The MDA-MB-231 cells were transiently transfected with 900 ng of either **(A)** pSG5-PR-A or **(B)** pSG5-PR-B. The cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for 21 days in total, with retreatment on day 7 and day 14. The colonies were fixed, stained with crystal violet and counted using the ImageJ software. Representative images are shown for PR-A and PR-B. The responses of all progestogens are set relative to the vehicle control which is set as 1. The results shown are averages (±SEM) of at least three biological repeats. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are shown by letters a, b and c, where the values that differ significantly from other values are assigned a different letter.

While it appears that R5020, P_4, MPA and GES increase colony formation when co-expressing PR-A and PR-B in the MDA-MB-231 cells at a 2:1 ratio, and that NET and LNG are decreasing colony formation, these responses are not statistically significant (Figure 4.9B). This is most likely due to large variability in the extent of responses by some progestins between experiments. When PR-A is expressed in 5-fold excess of PR-B, results show that colony formation is significantly increased by only R5020 and MPA (Figure 4.9C). Although P_4, NET, GES and NES are not significantly different compared to the vehicle, they are statistically similar to R5020. Thus, the probability that P_4, NET, GES and NES can in fact induce colony formation in MDA-MB-231 cells expressing both PR-B and excess PR-A, cannot be excluded. Interestingly, a ligand-independent decrease in colony formation was observed with the increased expression of PR-A relative to PR-B (Figure 4.9D). This result is in line with our results showing a ligand-independent decrease in proliferation (Figure 4.4) and increase in apoptosis (Figure 4.7) of the T47D cells exogenously expressing excess PR-A. These ligand-independent effects strengthens our argument for the increased expression of PR-A playing a positive role in breast cancer biology. Taken together, the results show that not all progestins increase colony formation in MDA-MB-231 cells expressing either PR-A or PR-B, or in cells co-expressing PR-A and PR-B at different ratios. It is noteworthy that MPA, the progestin most
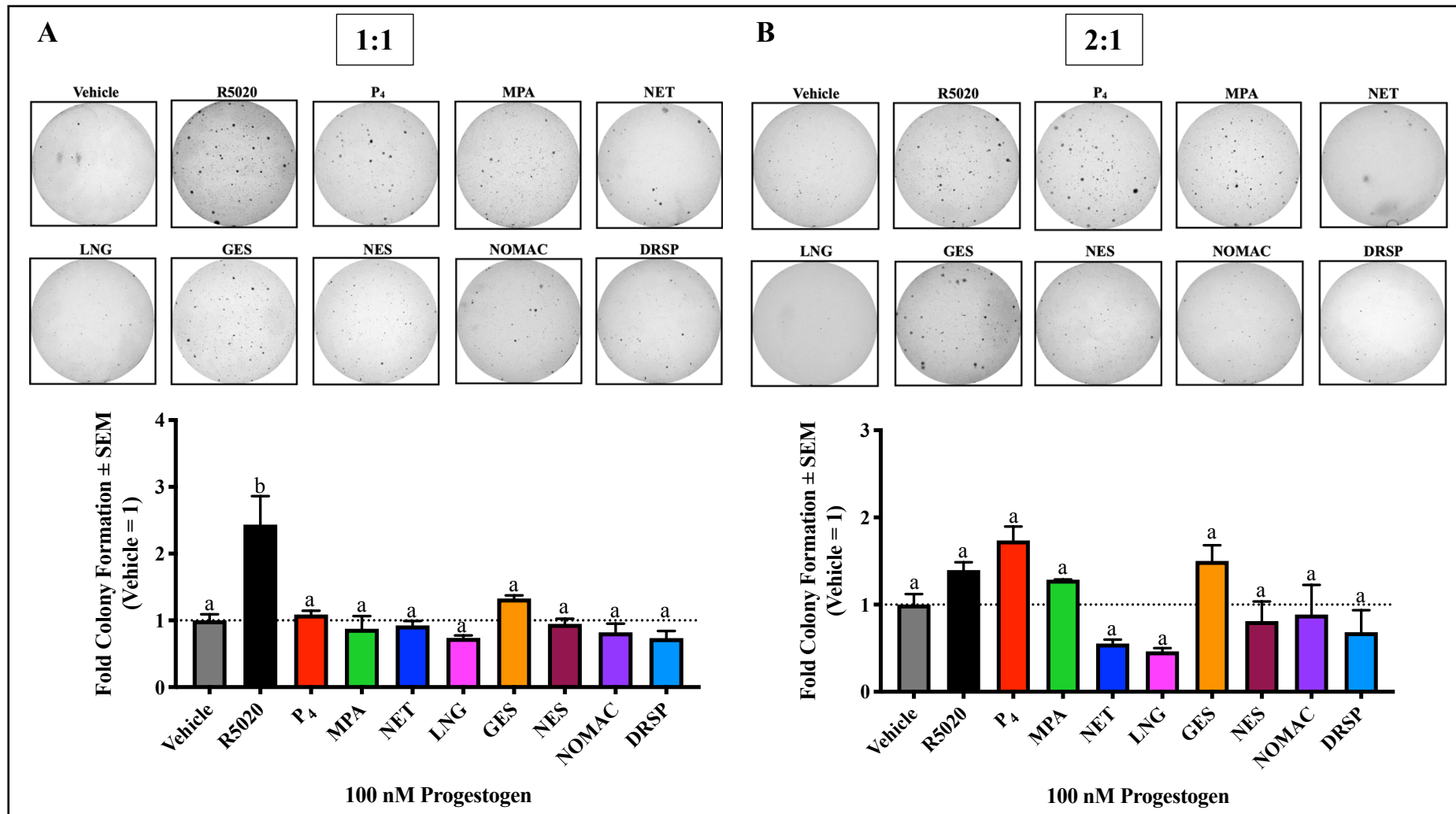
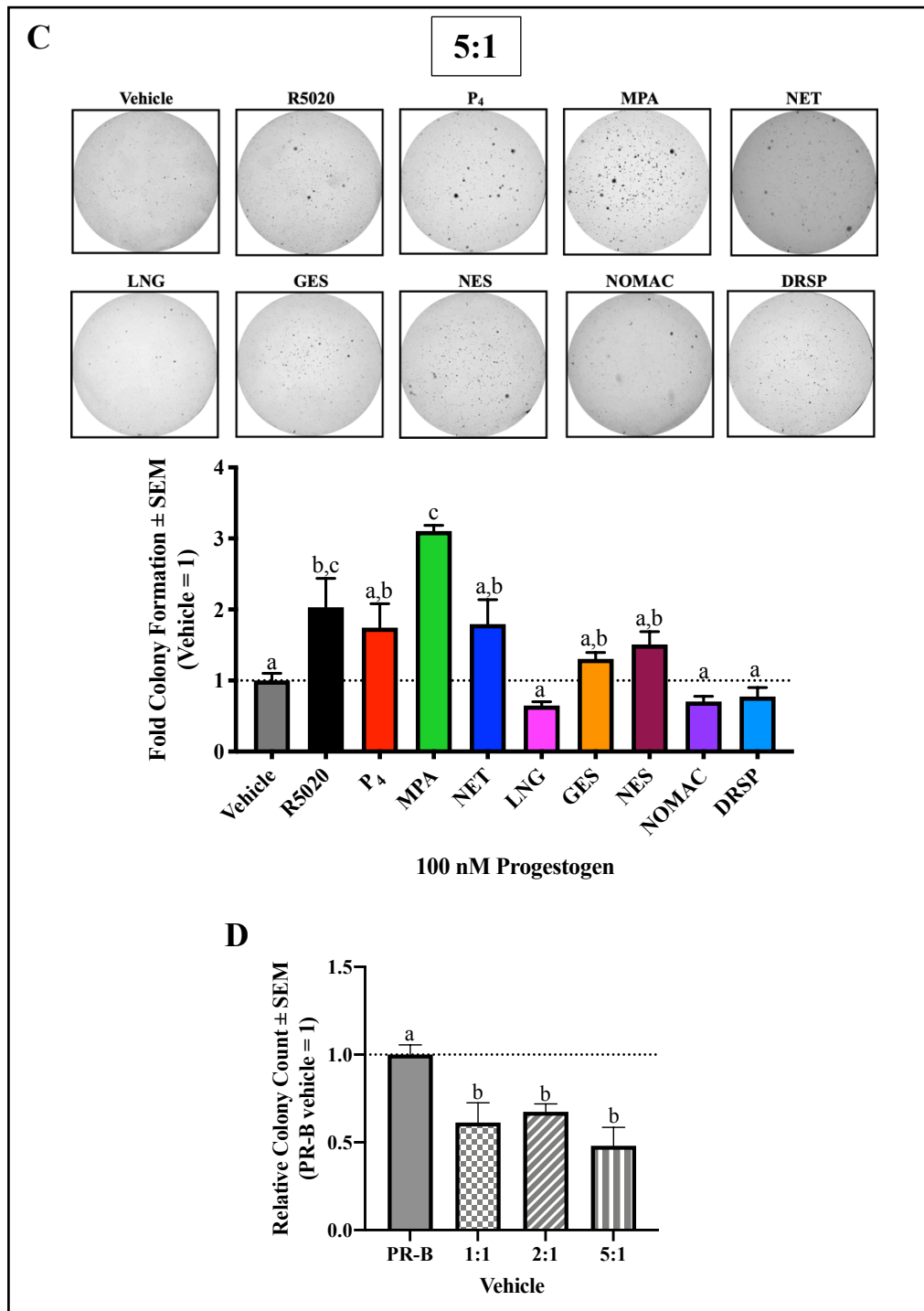**Figure 4.9. continues on the following page.**

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Figure 4.9. The effects on anchorage-independent growth of MDA-MB-231 cells transfected with varying ratios of PR-A:PR-B are progestogen- and PR-A:PR-B ratio-specific. The MDA-MB-231 cells were transiently transfected with 900 ng pSG5-PR-B and **(A)** 900 ng (1:1), **(B)** 1 800 ng (2:1) or **(C)** 4 500 ng (5:1) pSG5-PR-A. The cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for 21 days in total and retreated on day 7 and 14. The colonies were fixed, stained with crystal violet and counted using the ImageJ software. Representative images are shown for the co-expression of PR-A and PR-B. The responses of all progestogens are set relative to the vehicle control, which is set as 1. **(D) Irrespective of the PR-A:PR-B ratio, a ligand-independent decrease in colony formation is observed when PR-A and PR-B are co-expressed.** The number of colonies formed, in the presence of the vehicle, when PR-A and PR-B are co-expressed at 1:1, 2:1 and 5:1 was set relative to the vehicle of PR-B, set as 1. The results shown are averages (±SEM) of at least three biological repeats. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are shown by letters a, b and c, where the values that differ significantly from other values are assigned a different letter.

linked to an increased breast cancer risk, does in fact increase colony formation via both isoforms, and when PR-A is expressed 5x in excess of PR-B.

4.3.4. All progestogens, except R5020 and P₄, influence cell migration in an isoform-specific manner.

Next, we determined the effects of the progestogens on migration of MDA-MB-231 breast cancer cells transfected with PR-A or PR-B, and treated with 100 nM progestogen for 48 hours. The scratch wound-healing assay was used to assess migration, with images captured every 12 hours up to 48 hours. Representative images are shown in Figure 4.10A and B, respectively. The results show that 100 nM MPA, NOMAC and DRSP significantly increase cell migration (Figure 4.10A) via PR-A. Although migration is also increased in response to GES, this response is most likely not mediated by PR-A, as a similar response is observed in cells lacking PR-A (Addendum D, Figure D4). Interestingly, LNG also increases migration in the absence of transfected PR-A or PR-B (Addendum D, Figure D4), but not in the presence of either
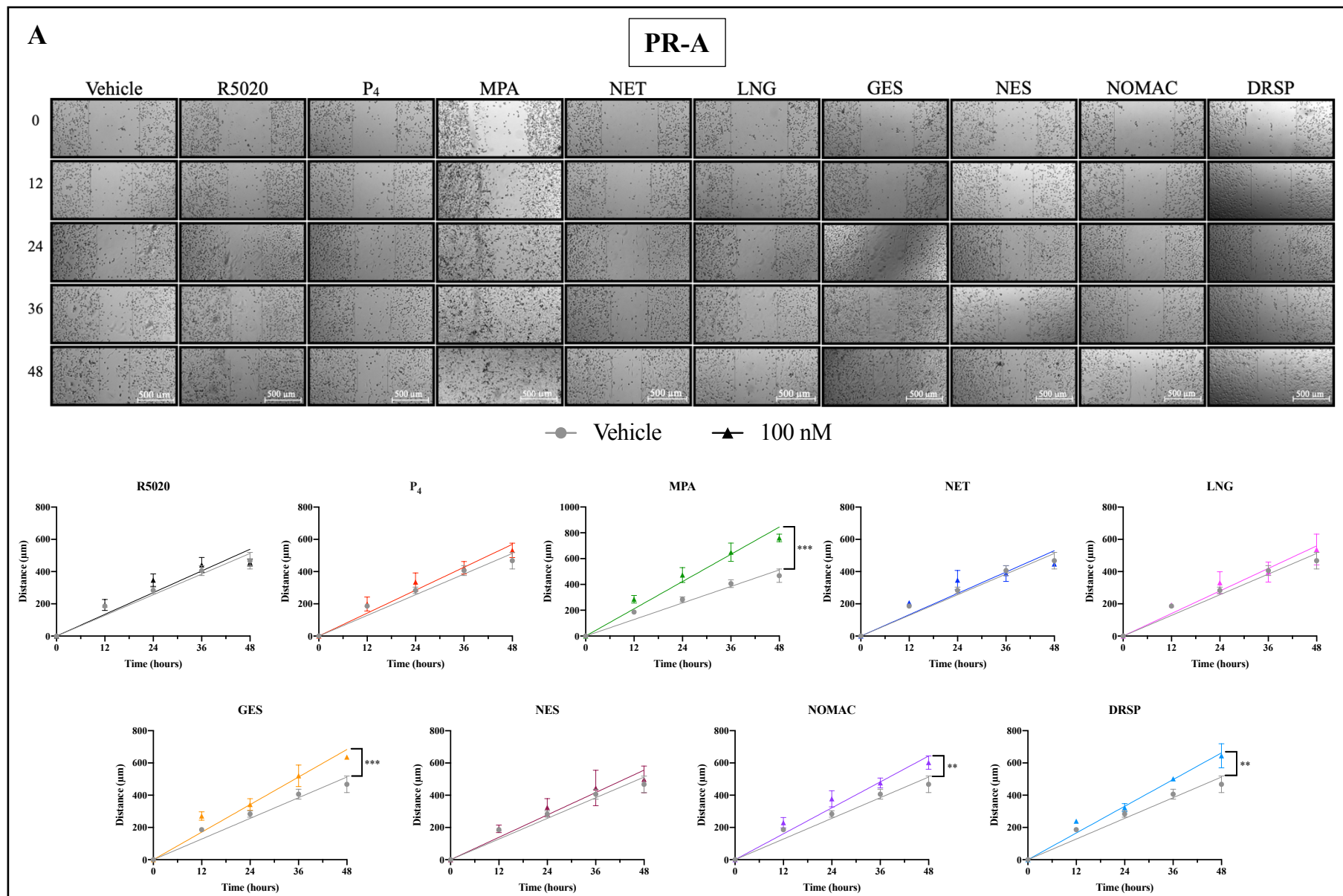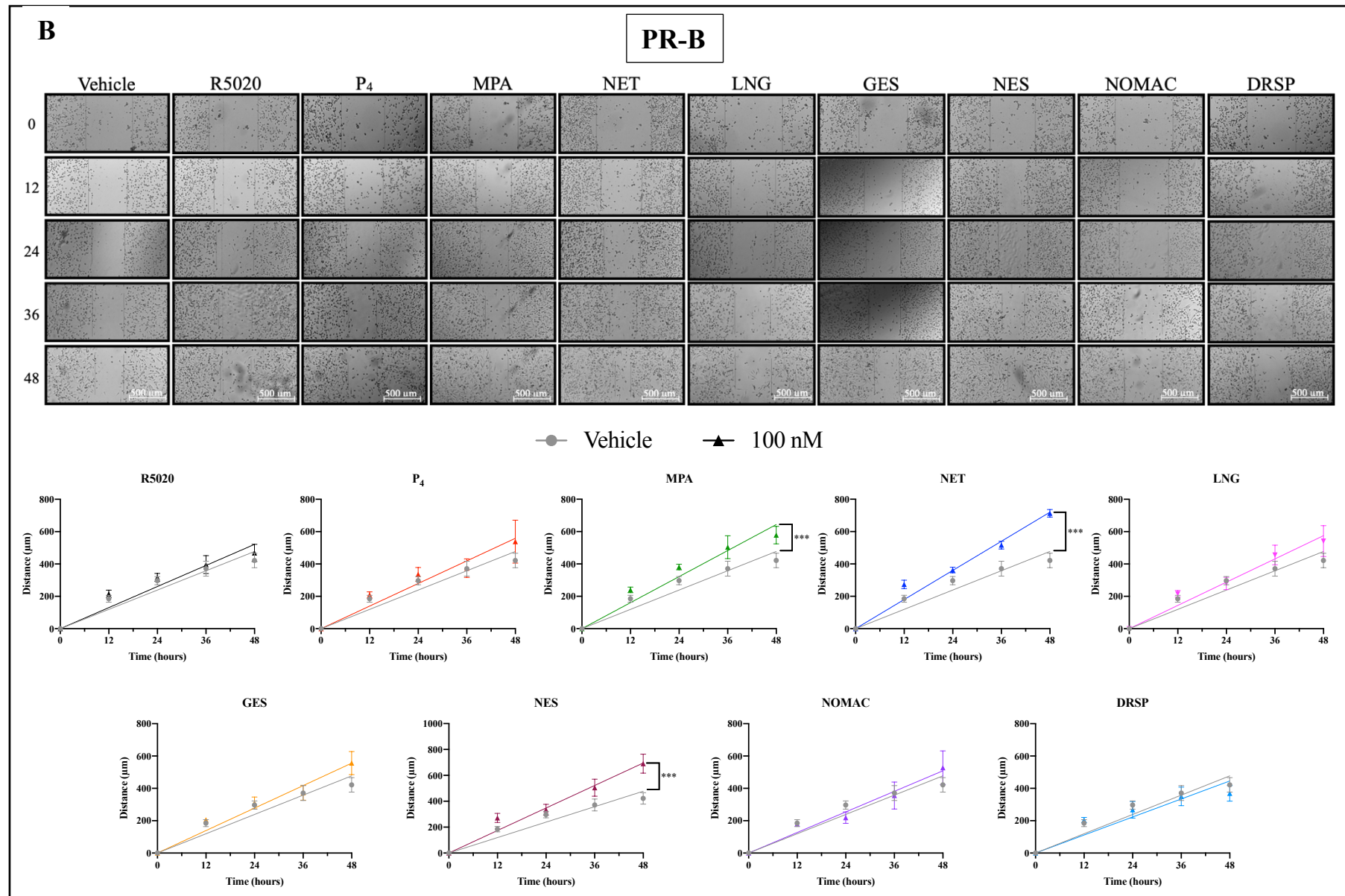
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Figure 4.10. While MPA, GES, NOMAC and DRSP increase cell migration via PR-A, MPA, NET and NES increase cell migration via PR-B. MDA-MB-231 cells were transiently transfected with 900 ng of either **(A)** pSG5-PR-A or **(B)** pSG5-PR-B. The cells were pre-treated for 2 hours with mitomycin C to inhibit proliferation, then treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for a total of 48 hours. Images were captured every 12 hours and migration quantified over time using ImageJ analysis software. Simple linear regression was used to fit the line and a two-way ANOVA with Dunnett's post-test was performed to determine statistical differences between the curves of the vehicle and treatment, with statistically significant differences shown using ** or ***, indicating $p < 0.01$ and $p < 0.001$, respectively. Absence of stars indicate no significant differences ($p > 0.05$).

isoform. In MDA-MB-231 cells transfected with PR-B, MPA, NET and NES increase migration (Figure 4.10B), which is consistent with activity via PR-B, as no migration of the MDA-MB-231 cells is observed in the absence of transfected PR-B (Addendum D, Figure D4). The aforementioned data suggest that migration of the MDA-MB-231 cells transfected with either PR-A or PR-B, in response to progestogens, is dependent on the specific progestin and PR isoform. To our knowledge, only one study has investigated isoform-specific effects in terms of breast cancer cell migration, and only for a single progestin (R5020) (Bellance *et al.*, 2013). Bellance and co-workers used a bi-inducible MDA-MB-231 cell line expressing either PR-A or PR-B, and showed that in contrast to our results, R5020 decreased cell migration after 10 hours in cells expressing PR-B, while similar to our results, the migration of cells expressing PR-A only (Figure 4.10A) was not influenced by R5020 (Bellance *et al.*, 2013). Collectively, our data showing progestin-specific effects, as well as isoform-specific differences between progestins, similarly observed in Chapter 3, once again highlight the importance of distinguishing between the individual isoforms, as well as assessing the activity of P_4 and that of individual progestins, when investigating progestogen activity in breast cancer. As differences in the effects of the progestogens on proliferation and anchorage-independent growth were observed with the co-expression of PR-A and PR-B in the MDA-MB-231 cells,

we next investigated migration of the MDA-MB-231 cells co-expressing equivalent levels of PR-A and PR-B (1:1), as well as that of cells expressing varying excess levels of PR-A relative to PR-B (2:1 and 5:1) (Figure 4.11). Results show that GES decreases cell migration when the isoforms are equally expressed (Figure 4.11A), while MPA increases migration at the 5:1 ratio, and DRSP decreases migration at both the 2:1 (Figure 4.11B) and 5:1 (Figure 4.11C) ratios. In line with our results at the 2:1 ratio of PR-A and PR-B showing no migration in the presence of 100 nM R5020, a previous study also showed no effect on migration of the bi-inducible MDA-MB-231 cells co-expressing PR-A and PR-B (2:1) with 10 nM R5020 (Bellance *et al.*, 2013). However, in contrast to our results, it has been shown that 10 nM R5020 (Holley *et al.*, 2009), 50 and 100 nM P_4 (Fu *et al.*, 2008a; Fu *et al.*, 2008b; Wang and Lee, 2016), 100 nM MPA (Fu *et al.*, 2008a) and DRSP (Fu *et al.*, 200b), as well as 1 nM NES (Fu *et al.*, 200b) increases migration of T47D cells. The discordance between these studies and ours may be due to progestogen concentration, differential PR expression levels or the involvement of other steroid receptors that are expressed in the T47D cells. These results show that while NOMAC increases migration via PR-A, and NET and NES increase migration via PR-B, none of these progestins have an effect on cell migration when the isoforms are co-expressed at any ratio. Interestingly, DRSP increases migration in cells expressing PR-A, has no effect on migration in cells expressing PR-B or co-expressing PR-A and PR-B equally, but decreases migration when PR-A is expressed in excess relative to PR-B. Furthermore, an increase in migration is observed in response to MPA when the PR isoforms are expressed individually, but no effect is observed when PR-A and PR-B are co-expressed at the 1:1 and 2:1 ratio.

Taken together, these results suggest that the response of progestins on migration of the PR-transfected MDA-MB-231 cells is ultimately dependent on the specific progestin, the particular PR isoform expressed and/or the ratio of the PR isoforms. Moreover, the fact that conclusions on progestin activity are often incorrectly drawn from observed effects of R5020 and P_4, is
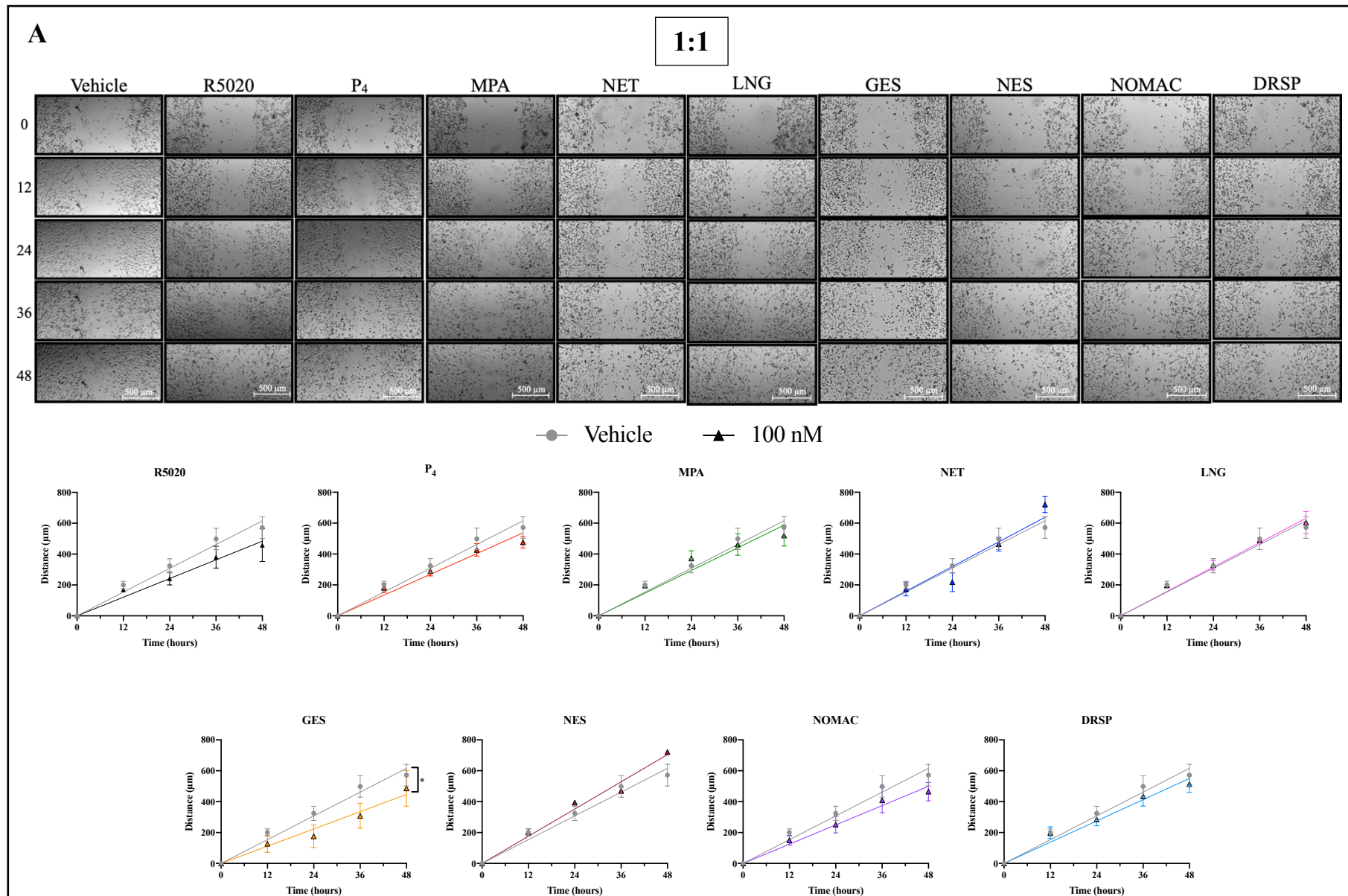
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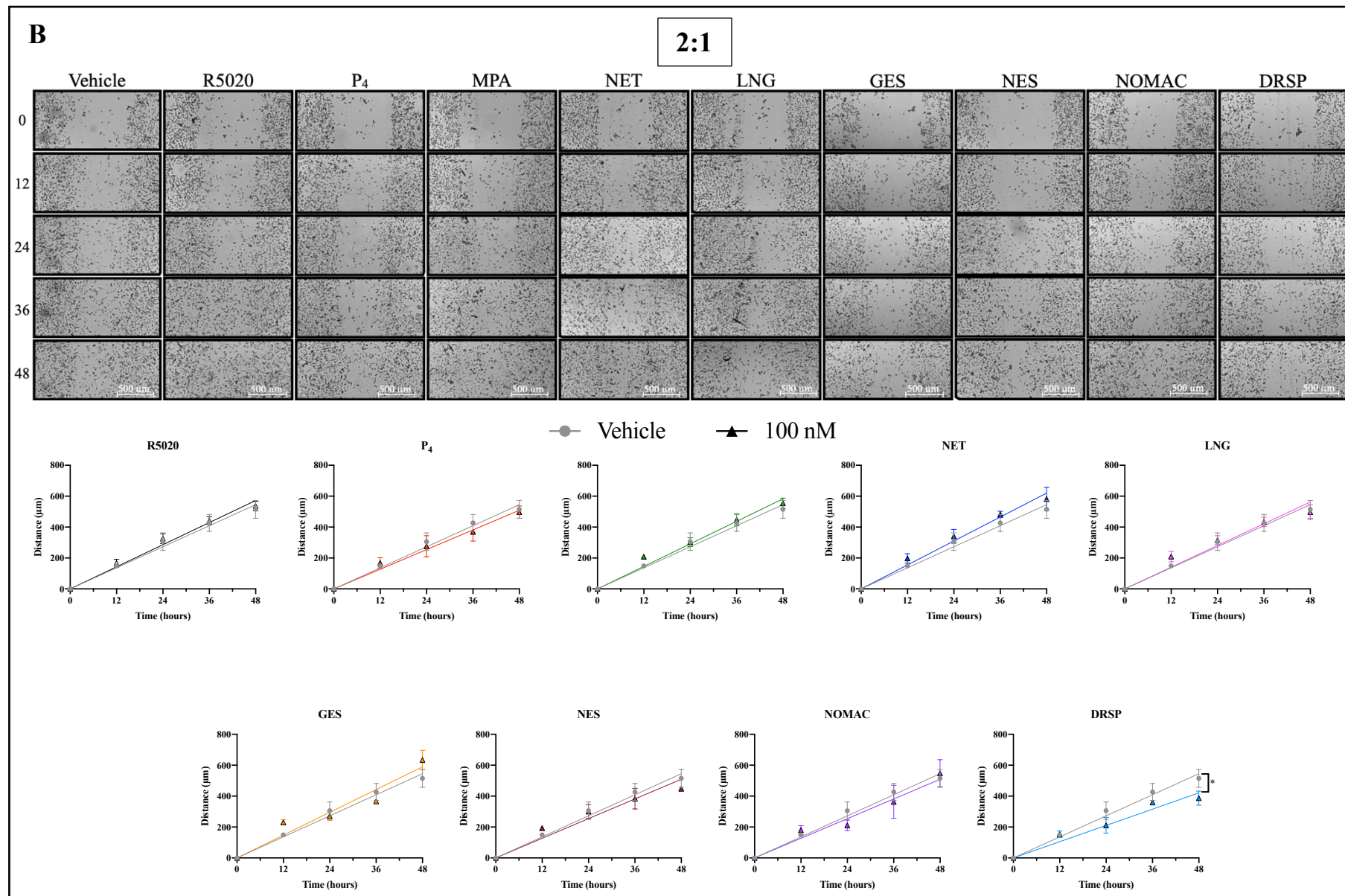**Figure 4.11. continues on the following page.**

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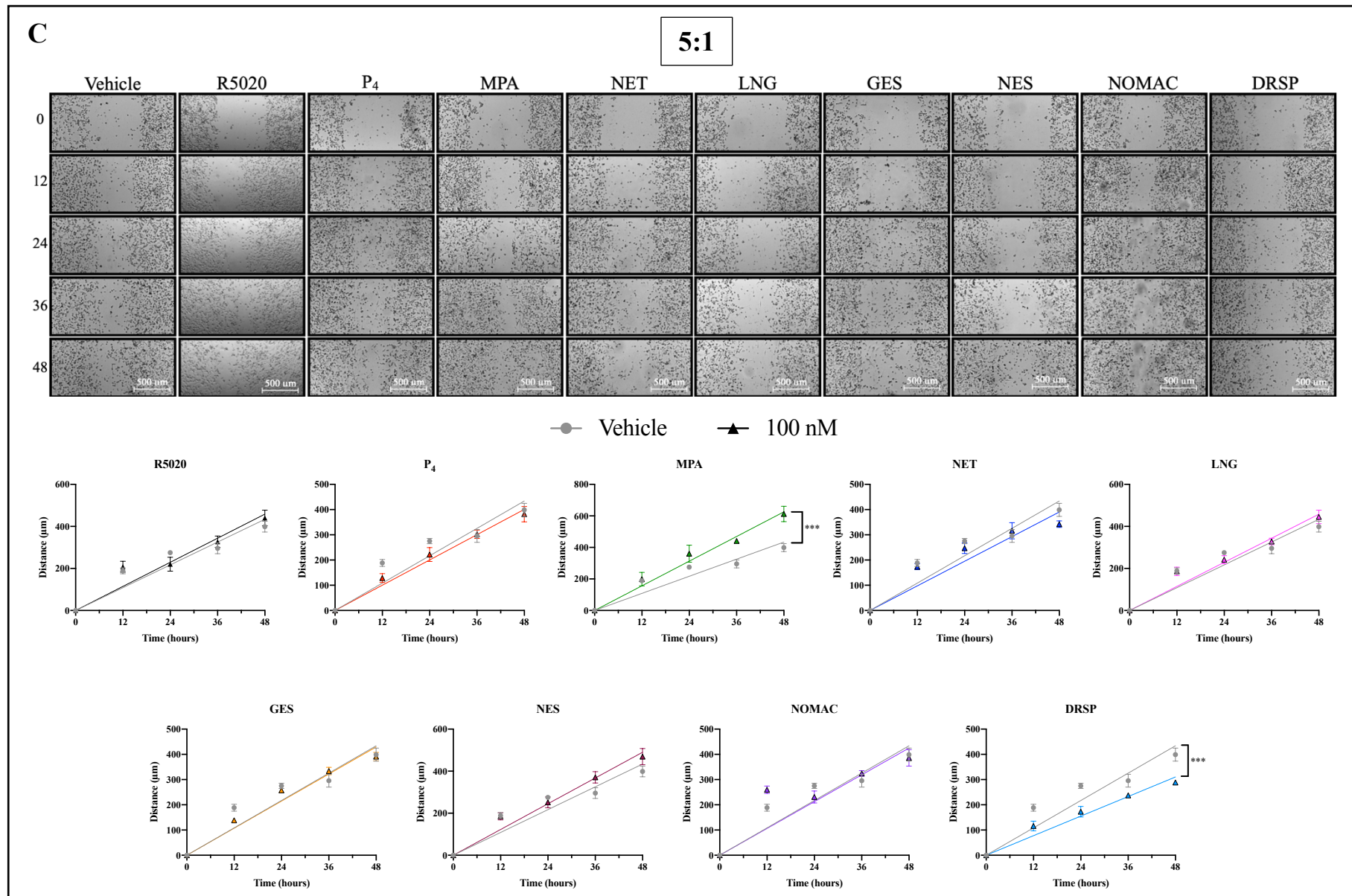**Figure 4.11. continues on the following page.**

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Figure 4.11. The effects on cell migration at the various PR-A:PR-B ratios are progestogen-specific. The MDA-MB-231 cells were transiently transfected with either 900 ng pSG5-PR-B and **(A)** 900 ng (1:1), **(B)** 1 800 ng (2:1) or **(C)** 4 500 ng (5:1) pSG5-PR-A. The cells were pre-treated for 2 hours with mitomycin C to inhibit proliferation, then treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for a total of 48 hours. Images were captured every 12 hours and migration quantified over time using ImageJ analysis software. Results shown are averages (±SEM) of at least three biological repeats and the distance travelled is plotted against time. Simple linear regression was used to fit the line and a two-way ANOVA, with Dunnett's post-test was performed to determine statistical differences between the curves of the vehicle and treatment, with statistically significant differences shown using * or ***, indicating $p < 0.05$ and $p < 0.001$, respectively. Absence of stars indicate no significant differences ($p > 0.05$).

highlighted by our results showing that both R5020 and P_4, unlike the other progestogens evaluated, have no effect on migration irrespective of whether PR-A and PR-B are expressed individually or co-expressed at the various ratios.

4.3.5. Progestins exhibit differential effects on invasion of the MDA-MB-231 cells transfected with PR-A or PR-B.

Cell invasion is related to migration, however, in this case the cells need to be able to penetrate the tissue barrier by moving through an extracellular matrix or basement membrane into neighbouring tissues (Kramer *et al.*, 2013). The transwell invasion assay was used to directly compare effects of the selected progestins and P_4 on the invasion of MDA-MB-231 breast cancer cells transfected with either PR-A or PR-B or both, and treated with 100 nM progestogen for 24 hours. Representative images of the invaded cells are shown in Addendum D, Figure D5. Results in Figure 4.12A show that MPA, LNG, NES and NOMAC increase invasion of MDA-MB-231 cells containing exogenously expressed PR-A, while NET decreases invasion, and R5020, P_4, GES and DRSP have no significant effect. The decrease in invasion in response to NET is consistent with a PR-A-dependent mechanism, as no invasion was observed with

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PR-A

A

PR-B

B

	P$_4$	MPA	NET	LNG	GES	NES	NOMAC	DRSP
R5020	ns	ns	*	ns	ns	ns	ns	ns
P$_4$		ns	**	*	ns	ns	ns	ns
MPA			*	ns	ns	ns	ns	ns
NET				**	*	*	**	ns
LNG					ns	ns	ns	*
GES						ns	ns	ns
NES							ns	ns
NOMAC								*

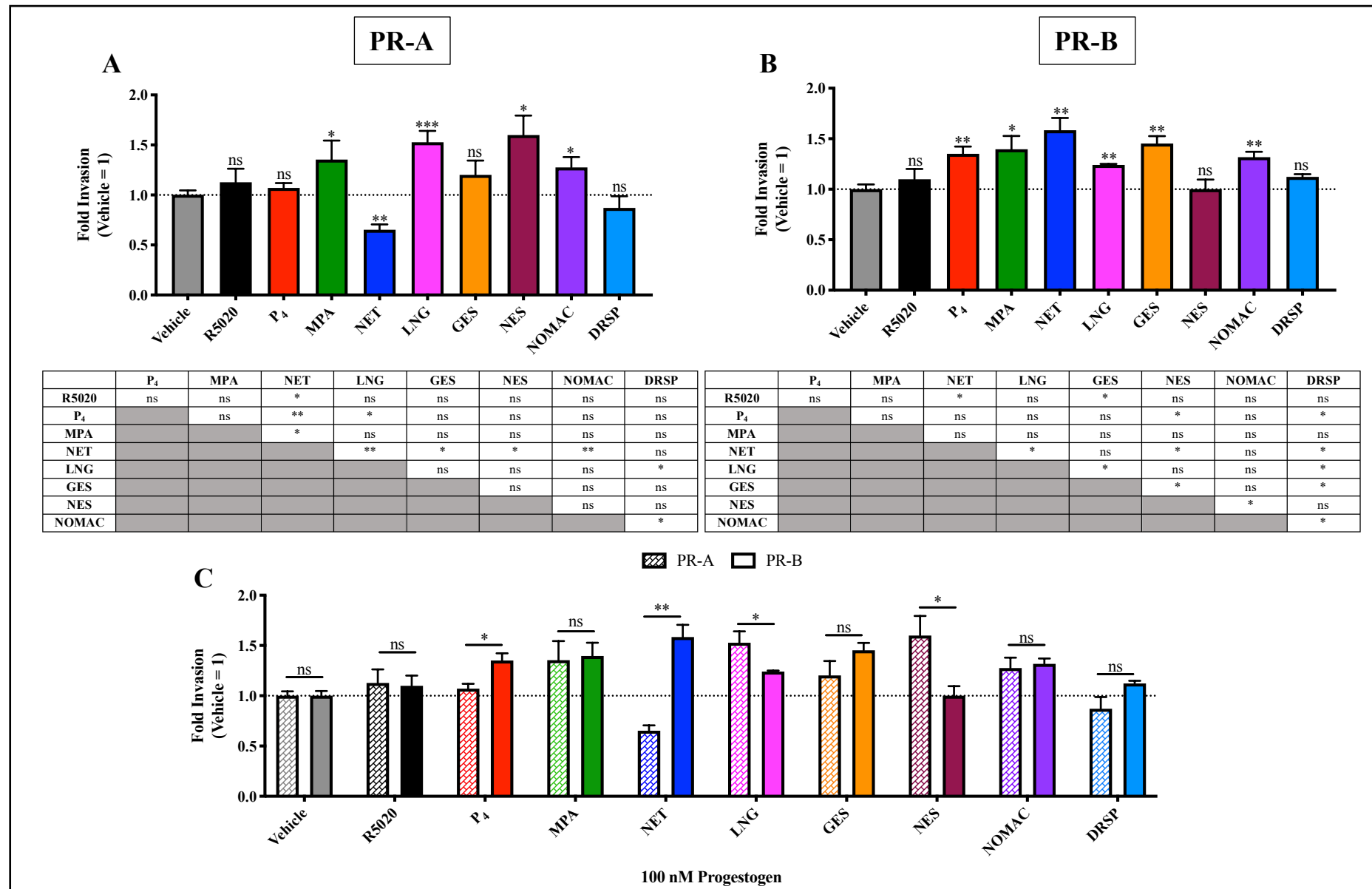|	P$_4$	MPA	NET	LNG	GES	NES	NOMAC	DRSP
R5020	ns	ns	*	ns	*	ns	ns	ns
P$_4$		ns	ns	ns	ns	*	ns	*
MPA			ns	ns	ns	ns	ns	ns
NET				*	ns	*	ns	*
LNG					*	ns	ns	*
GES						*	ns	*
NES							*	ns
NOMAC								*

C

100 nM Progestogen

Figure 4.12. Figure legend on the following page.

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Figure 4.12. While NET decreases cell invasion via PR-A and increases via PR-B, most progestogens either have no effect or increase invasion via either isoform. MDA-MB-231 cells were transiently transfected with 900 ng of either **(A)** pSG5-PR-A or **(B)** pSG5-PR-B. Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for 24 hours and invasion was assessed using Matrigel pre-coated Boyden chambers. Images were captured (Addendum C, Figure C5) and cells counted using the script in Mathematica (as described in the methods). **(C)** For a direct comparison of the progestogens between the two PR isoforms, the values for PR-A and PR-B were replotted as grouped data. Fold invasion was determined relative to the vehicle control set as 1. Results shown are averages (±SEM) of at least three biological repeats. Unpaired *t*-tests were used for statistical analysis and statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns. **(A and B)** Statistically significant differences relative to the vehicle are shown on the graph, while significant differences between progestogens are indicated in the tables below the graph.

NET in the absence of transfected PR-A (Addendum D, Figure D6). Due to financial constraints, cell invasion in the absence of transfected PR-A or PR-B was only evaluated for R5020, NET and DRSP, as representatives of progestin structures derived from P_4, testosterone and spironolactone, respectively (Addendum D, Figure D6). Thus, to confirm that the responses of the other progestogens are in fact due to PR-A or PR-B, we would need to conduct similar experiments in the absence of transfected PR-A and PR-B.

In MDA-MB-231 cells transfected with PR-B, R5020, NES and DRSP have no effect on cell invasion, while the rest of the progestogens all increase invasion (Figure 4.12B). Notably, one cannot exclude the possibility that the response of MPA in cells expressing either PR-A or PR-B, is via the endogenously expressed GR in the MDA-MB-231 cells, as invasion in response to MPA was not evaluated in the absence of PR expression, and MPA is known to bind to the GR (Ronacher *et al.*, 2009). Consistent with our finding that R5020 has no effect on invasion of MDA-MB-231 cells transfected with PR-A, a previous study has shown the same trend in T47D cells stably expressing PR-A (T47D-YA cells) (McFall *et al.*, 2015). McFall and co-

authors also showed that MPA induces invasion of T47D cells stably expressing PR-B (T47D-YB cells), which is in agreement with our results in the MDA-MB-231 cells transfected with PR-B (McFall *et al.*, 2015). However, in contrast to our study showing MPA-induced invasion of the MDA-MB-231 cells transfected with PR-A, these authors reported no invasion of cells expressing PR-A (T47D-YA cells) in response to MPA. Notably, our finding that R5020 does not induce invasion of MDA-MB-231 cells expressing PR-B, also contradicts the findings of McFall and colleagues showing an increase in invasion of T47D-YB cells, in response to 100 nM R5020.

When directly comparing the effects of the progestogens on invasion of cells expressing either PR-A or PR-B (Figure 4.12C), the results show that the effects on invasion of the MDA-MB-231 cells are similar for most progestogens, with the exception of P_4, NET, LNG and NES. While the response of LNG and NES is higher in cells expressing PR-A than those expressing PR-B, the response of P_4 is higher in cells expressing PR-B. NET, however, decreases invasion of cells expressing PR-A, while increasing invasion of cells expressing PR-B. These results again highlight that progestins are not all the same, as some increase cell invasion, some have no effect and at least one shows decreased cell invasion. Moreover, PR isoform-specific effects occur, again underscoring the importance of investigating responses via the individual isoforms.

As dysregulated isoform ratios, i.e. excess PR-A expression relative to PR-B, has been associated with a more invasive cancer phenotype (Hopp *et al.*, 2004; McGowan *et al.*, 2004; Jacobsen *et al.*, 2005; Pathiraja *et al.*, 2011; McFall *et al.*, 2015, 2018; Lamb *et al.*, 2018; Rosati *et al.*, 2020), we next determined the effects of co-expression of PR-A relative to PR-B, with PR-A in excess (5:1) on cell invasion. Notably, preliminary experiments comparing invasion of MDA-MB-231 cells transfected with different PR-A:PR-B ratios (1:1, 2:1 and 5:1) showed

no difference in response to progestogens at these ratios (Addendum D, Figure D7). Thus due to funding constraints, further invasion experiments only included co-expression at the 5:1 ratio. Results in Figure 4.13A show that while most of the progestins have no effect on cell invasion when PR-A and PR-B are co-expressed at a 5:1 ratio, P_4, NES and DRSP inhibit cell invasion. Previous studies have shown an increase in cell invasion after treatment with 10 nM R5020 (Holley *et al.*, 2009), 100 nM P_4, MPA, DRSP (Fu *et al.*, 2008a; Fu *et al.*, 2008b) and 1 nM NES (Fu *et al.*, 200b) in T47D breast cancer cells, presumably expressing either similar levels of PR-A and PR-B or 2x more PR-A (Figure 3.10B and D). Interestingly, we show a ligand-independent increase in invasion when directly comparing invasion of cells co-expressing the isoforms at a 5:1 ratio to that of cells expressing only PR-A or PR-B (Figure 4.13B). While the unliganded effects of the 5:1 ratio is in support of suggestions that excess PR-A is indicative of a more aggressive breast cancer phenotype, at this ratio P_4, NES and DRSP are able to counteract this increase in invasive properties. Indeed, it has been shown that a high PR-A:PR-B ratio in breast cancer leads to an increased sensitivity to anti-progestins (McFall *et al.*, 2015, 2018; Wargon *et al.*, 2015; Rojas *et al.*, 2017; Singhal *et al.*, 2018; Rosati *et al.*, 2020), strengthening the idea that the excess expression of PR-A relative to PR-B may have therapeutic benefits for breast cancer. Interestingly, the anti-progestin onapristone, is currently in a Phase II clinical trial in combination with fulvestrant for treatment of advanced breast cancer [reviewed in (Horwitz and Sartorius, 2020)].

4.4. Conclusion

As a class, progestins have been implicated in the increased incidence of breast cancer. It is, however, debatable whether all progestins would elicit similar risks, as only eight progestins, including R5020, MPA, NET and LNG used in this study, have been assessed for breast cancer risk in large scale clinical studies. At the cellular level, it is unclear whether the PR itself or

other members of the steroid receptor family are mediating the effects of the progestins. Indeed, the role of the PR in breast cancer is complex and dependent on multiple factors, including the relative expression of PR-A to PR-B.

	P₄	MPA	NET	LNG	GES	NES	NOMAC	DRSP
R5020	*	ns	ns	ns	ns	*	ns	**
P₄		*	ns	ns	*	ns	ns	ns
MPA			ns	ns	ns	*	ns	*
NET				ns	ns	ns	ns	ns
LNG					ns	ns	ns	ns
GES						*	ns	*
NES							*	ns
NOMAC								*

Figure 4.13. Figure legend on the following page.

Figure 4.13. While most progestins have no influence on cell invasion when PR-A is expressed in excess relative to PR-B (5:1), invasion is decreased by P₄, NES and DRSP. (A) The MDA-MB-231 cells were transiently transfected with 4 500 ng pSG5-PR-A and 900 ng pSG5-PR-B (5:1). Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for 24 hours and invasion was assessed using matrigel pre-coated Boyden chambers. Images were captured (Addendum D, Figure D8) and cells counted using the script in Mathematica (as described in the methods). Fold invasion was determined relative to the vehicle control set as 1. Statistically significant differences relative to the vehicle are shown on the graph, while significant differences between progestogens are indicated in the tables below the graph. **(B) In the absence of ligand, when PR-A is expressed in 5x excess compared to PR-B, cell invasion increases.** MDA-MB-231 cells were transiently transfected with 900 ng of either pSG5-PR-A or pSG5-PR-B, or 4 500 ng pSG5-PR-A and 900 ng pSG5-PR-B (5:1), and treated with 0.1% (v/v) ethanol (vehicle). Fold invasion was determined relative to the vehicle of PR-B, set as 1. Results shown are averages (±SEM) of at least three biological repeats. Unpaired *t*-tests were used for statistical analysis with statistically significant differences shown using * or **, indicating $p < 0.05$ and $p < 0.01$, respectively. No statistical significance ($p > 0.05$) is indicated by ns.

This is the first study to directly compare the *in vitro* effects of P₄ and multiple progestins on various cancer hallmarks, in breast cancer cell lines expressing either PR-A or PR-B or co-expressing different ratios of PR-A and PR-B. While neither P₄ nor the selected progestins induced apoptosis of the cell lines used in this study, we showed that P₄ and the progestins differentially influence anchorage-independent growth, migration and invasion of breast cancer cells, in not only a progestogen- and PR isoform-specific manner, but also in a manner dependent on the ratio of PR-A to PR-B. Although we show that progestin-induced proliferation occurs via the PR isoforms in the MDA-MB-231 cells, the contribution of the PR is less clear in T47D cells expressing multiple competing steroid receptors to which some progestins may bind. Notably, we show that R5020 and P₄ have no effect on the migration of MDA-MB-231 cells expressing PR-A or PR-B individually or with co-expression at any ratio, while some progestins increase and others decrease migration. Regarding the consequences of PR-A excess relative to PR-B in breast cancer, some results suggest a positive phenotype, such

as the ligand-independent decrease in proliferation and increase in apoptosis of T47D cells expressing PR-A in excess, while others suggest a negative phenotype such as the ligand-independent increase in invasion of MDA-MB-231 cells expressing excess PR-A relative to PR-B. However, this increase in invasion is inhibited in the presence of P4, NES and DRSP, supporting the idea that in addition to PR antagonists, PR agonists may also be beneficial in breast cancer treatment. Indeed, two window of opportunity trials are currently evaluating whether micronised P4 in combination with ER targeted endocrine therapies will enhance the effects of the latter therapies [reviewed in (Horwitz and Sartorius, 2020)]. Our results, however, suggest that not all PR agonists may be beneficial in this regard, as we show that some progestins increase anchorage-independent growth and migration when PR-A is expressed in excess. Strikingly, our results show that the notorious association of MPA and breast cancer may indeed have merit, as this is the only progestin to increase proliferation, anchorage-independent growth, migration and invasion of the PR-transfected MDA-MB-231 cells, as well as proliferation of the T47D cells, while having no effect on apoptosis. In contrast, the fact that DRSP, the newer progestin that has not yet been investigated in large scale clinical studies for breast cancer risk, has mostly positive effects on these cancer hallmarks, suggest little to no detrimental effects for breast cancer. For instance, DRSP either has no effect or decreases anchorage-independent growth, migration and invasion of MDA-MB-231 cells when PR-A and PR-B are co-expressed, and when PR-A is overexpressed relative to PR-B. Taken together, the results of this study provide support of our hypothesis that all progestins are not equal in terms of breast cancer and emphasize the importance of investigating the actions of the individual progestins in breast cancer biology. Furthermore, our data suggest that the PR isoform ratio dictates the response of certain progestins, and that the effects of excess expression of PR-A relative to PR-B, is not straightforward as both negative and positive effects are observed on the cancer hallmarks assessed. Further research on the role of

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dysregulated PR isoforms and the influence of the different progestins in breast cancer is thus warranted.

Chapter 5

Concluding Discussion and Future Perspectives

5.1. Introduction

Some progestins used in MHT have been associated with increased breast cancer risk (Africander and Louw-du Toit, 2020). However, only eight progestins out of the many that are available for therapeutic use, all differing in chemical structure, have been evaluated for breast cancer risk in large clinical studies. Although it is well known that progestins were designed to mimic the activity of the natural female hormone P_4 by acting via the PR [reviewed in (Stanczyk *et al.*, 2013)], it is unclear from the literature whether both PR isoforms were considered. Surprisingly, evidence for the pharmacological characterisations of progestins in terms of their efficacies and potencies via the individual PR isoforms are limited, especially studies that directly compare multiple progestins in the same model system. Characterising progestins in the same model system is crucial as there are a multitude of factors that could influence activity in a cell, including but not limited to, the expression and activity of multiple steroid receptors to which progestins can bind, the density of the steroid receptors, the ratio of receptor to reporter construct, and the differential expression of steroidogenic enzymes (Hapgood *et al.*, 2013). The latter may lead to differential progestin metabolism and result in the production of different metabolites that may or may not be active. Importantly, metabolism of progestins may confound the results from *in vitro* dose-response analyses used to evaluate their relative biological activities, and decrease the effective concentration of the progestin at the target cell *in vivo*. Surprisingly, differences in progestin metabolism are hardly ever considered when considering differences in progestin activity. In the first part of this thesis (Chapter 2) and in our recent publication (Skosana *et al.*, 2019) (Addendum A1), we investigated the metabolism of P_4 and selected progestins from four generations in three commonly used breast cancer cell lines, as well as six other commonly used laboratory cell lines. Additionally, the time-dependent metabolism of P_4 was compared in the three breast cancer cell lines (Chapter 2). In the second part of the experimental work in this thesis (Chapter

3), we compared the relative activity of P_4 and the selected progestins, in parallel, via exogenously expressed PR-A or PR-B in the MDA-MB-231 breast cancer cell line. As it has previously been shown that increasing concentrations of the GR and PR-B can influence progestin activity (Abdel-Hafiz *et al.*, 2009; Robertson *et al.*, 2013), we also investigated the influence of PR-A density on progestin activity. Lastly, as the ratio of PR-A and PR-B in breast cancer is often dysregulated, we also investigated how progestogen activity was influenced by the co-expression of PR-A and PR-B at various PR-A:PR-B ratios. Moreover, we investigated the role of the PR isoforms, as well as the influence of excess PR-A, relative to PR-B, on endogenous genes known to play a role in breast cancer progression and development in the T47D breast cancer cell line, endogenously expressing both PR-A and PR-B. In the third and final part of the experimental work in this thesis (Chapter 4), we directly compared the effects of P_4 and the progestins on specific cancer hallmarks via exogenously expressed PR-A or PR-B, or both PR-A and PR-B co-expressed at equimolar and dysregulated ratios in the MDA-MB-231 cell line. Similar studies were performed for proliferation in the T47D cells, endogenously expressing both isoforms, in the absence and presence of excess exogenously expressed PR-A.

5.2. Comparing the metabolism of P_4 and progestins in different breast cancer cell lines.

In Chapter 2 and in Skosana *et al*., (Skosana *et al.*, 2019), we showed differential metabolism of P_4 and a selected panel of progestins in nine commonly used laboratory cell lines using an UHPSFC-MS/MS method. Table 5.1 summarises the metabolism results for R5020, GES, NOMAC and DRSP (Chapter 2), MPA, NET, LNG and NES (Skosana *et al.*, 2019), as well as P_4 (Chapter 2 and (Skosana *et al.*, 2019)), in the MDA-MB-231, T47D and MCF-7 BUS breast cancer cell lines.

Table 5.1. Summary of the metabolism of P$_4$ and the selected progestins used in this thesis in three breast cancer cell lines[a].

Progestogen	Description/ Generation	Parent Structure	Percentage metabolism (%)		
			MDA-MB-231	T47D	MCF-7 BUS
R5020	PR-specific agonist	P$_4$	5.1^{ns}	7.6^{ns}	-
P$_4$	Natural Progestogen		80.9***	97.9***	79.0***
MPA[b]	1st	P$_4$	-	55.0***	10.9^{ns}
NET[b]		testosterone	7.7^{ns}	33.4*	12.9*
LNG[b]	2nd	testosterone	4.9^{ns}	22.8^{ns}	-
GES	3rd	testosterone	-	2.8^{ns}	3.2^{ns}
NES[b]	4th	P$_4$	10.0^{ns}	8.0^{ns}	40.3***
NOMAC			-	14.9^{ns}	17.9^{ns}
DRSP			25.9**	33.7**	10.2^{ns}

Statistically significant differences between the no cell control and progestogens are indicated by *, **, ***, representing $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; ns = not significant; -, indicates no metabolism.
[a]Results from Chapter 2, Figure 2.1. [b]Published in (Skosana *et al.*, 2019)*, Addendum A1.*

The MDA-MB-231 and T47D cell lines were the model systems used in Chapter 3 and 4, while the MCF-7 BUS cell line is also commonly used in our laboratory. Our results show that P$_4$ is metabolised in all three breast cancer cell lines at 24 hours. Indeed, it is well-known that the natural progestogen P$_4$ is metabolised in different cell lines and tissues [reviewed in (Wiebe, 2006; Stanczyk *et al.*, 2013; Trabert *et al.*, 2020a)], and this was also shown in all the commonly used laboratory cell lines investigated (Skosana *et al.*, 2019). Furthermore, the ratio of the 4-pregnenes:5α-pregnane P$_4$ metabolites has been shown to differ between the normal and cancerous breast (Wiebe and Lewis, 2003; Wiebe, 2006). In normal breast tissue, as well as the MCF-10A normal breast cell line, significantly more 4-pregnenes than 5α-pregnanes are produced due to higher expression levels and activities of 20α-HSD and 3α-HSD. In tumorous breast tissue and the MCF-7, MDA-MB-231 and T47D breast cancer cell lines on the other hand, the production of 5α-reduced metabolite production is favoured due to increased 5α-reductase expression and activity. Results from *in vitro* and *in vivo* studies indicate that 5α-

pregnanes promote tumour growth by stimulating cell proliferation, decreasing apoptosis and increasing detachment (Wiebe *et al.*, 2015), while 4-pregnenes, specifically $3\alpha HP_4$ and $20\alpha HP_4$, play an important role in preventing the development of cancer by decreasing cell proliferation, increasing apoptosis and decreasing detachment (Wiebe, 2006). Interestingly, the results from a recent study in postmenopausal women did not show an increased risk with the circulating ratio of $5\alpha P_4$:$3\alpha HP_4$ (Trabert *et al.*, 2020b). However, the authors did note an increased risk in women that have high $5\alpha P_4$ but low $3\alpha HP_4$ levels, suggesting that the involvement of these metabolites in breast cancer development cannot be completely excluded. In Chapter 2, we also showed that the rate of P_4 metabolism in the MDA-MB-231, T47D and MCF-7 BUS cells is cell line-specific, with the MDA-MB-231 and T47D cells metabolising P_4 at a similar, yet faster rate compared to the MCF-7 BUS cell line. P_4 and the progestin, R5020, are the ligands most often used in studies investigating PR mechanisms and unlike P_4, R5020 was not metabolised in any of the cells in this study. Although these results suggest that it would be more accurate to use the non-metabolisable R5020 when investigating PR action in these cell lines, rather than the rapidly metabolised P_4, no substantial differences were observed in the activity of P_4 and R5020 by us (Chapter 3 and 4) or others (Sathyamoorthy and Lange, 2020). Similar activities are, however, not always observed in our study between P_4 and all progestins or between different progestins (Chapter 3 and 4).

At 24 hours, we found that only DRSP was significantly metabolised in the MDA-MB-231 cells, while NET and NES were significantly metabolised in the MCF-7 BUS cells, and MPA, NET and DRSP in the T47D cells. Interestingly, the T47D breast cancer cell line is the most commonly used cell line in studies characterising the activities of progestins (Markiewicz and Gurpide, 1994; Attardi *et al.*, 2002, 2010; Austin *et al.*, 2002; Madauss *et al.*, 2004; Bray *et al.*, 2005; Zhang *et al.*, 2005), and also the main model for studying the role of the human PR in breast cancer (Jacobsen and Horwitz, 2012). Reported potencies of MPA, NET and DRSP in

the T47D cells are thus most likely underestimated as their metabolism would result in lower effective concentrations of these progestins. Indeed, various metabolites have been identified for MPA, NET and DRSP in human urine, faeces and serum [reviewed in (Bick *et al.*, 2021)]. While we show metabolism of NES in the MCF7-BUS cells, and metabolites of NES have been detected in rodents, there is currently no available evidence of NES metabolites in humans [reviewed in (Bick *et al.*, 2021)]. Although the activity of the metabolites of MPA are not known, and the metabolites of DRSP are reportedly inactive, various metabolites of NET have been shown to display activity via the AR, ER and PR isoforms (Larrea *et al.*, 2001; García-Becerra *et al.*, 2004). Specifically, it has been shown that 5α-NET has activity via both PR-A and PR-B, while $3\alpha,5\alpha$-NET has activity via only PR-B (Larrea *et al.*, 2001; García-Becerra *et al.*, 2004). While LNG, GES and NOMAC were not metabolised in the breast cancer cell lines, GES is known to be metabolised in the liver, while metabolites of LNG have been identified in serum [reviewed in (Bick *et al.*, 2021)].

Our results showing that some progestins are metabolised in breast cancer cell lines *in vitro* in a cell line-specific manner are insightful and relevant to studies using cell lines to investigate progestin mechanisms. Future work should include determining the half-life of the progestins metabolised in these model systems, as it will aid in choosing the appropriate timepoint in assays investigating progestins, as to avoid a decrease in the effective concentration of the progestin. To understand why cell-specific metabolism of these progestins is observed, identifying which steroidogenic enzymes are expressed in the cell lines would be a helpful starting point. Real-time qPCR and/or western blot analysis could thus be used to detect enzyme expression. Once specific enzymes have been identified, enzymatic assays could be performed to determine whether a progestin is a substrate for the enzyme and subsequently the rate of metabolism. Considering that differential P_4 metabolite formation has previously been shown between the T47D and MCF-7 cells (Fennessey *et al.*, 1986; Horwitz *et al.*, 1986), this

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may also be true for NET which is metabolised by both cell lines in this study. Mass spectrometry could be used to determine if different metabolites of NET would also be produced in these cell lines, and to identify the metabolites of DRSP in the MDA-MB-231 cells. Subsequently, transcriptional assays could be used to assess the putative activities of these metabolites via the PR isoforms and also other steroid receptors.

Identifying progestin metabolites in cell line models will be useful when investigating the mechanism of action of progestins at the cellular level. To understand the effects of progestins in women using these ligands clinically, it is important to investigate progestin metabolism *in vivo*. Although there is some information available regarding the serum levels of progestins and their metabolites, albeit with great variation between studies [reviewed in (Bick *et al.*, 2021)], studies measuring the actual levels of the progestins and/or their metabolites in tissue are surprisingly scarce. To our knowledge, only one study has measured the concentration of a progestin in breast tissue (Depypere *et al.*, 2019). Although the authors acknowledge that their findings are limited to a single time point investigated and a small sample size, their findings of a poor correlation between the levels of LNG measured in breast tissue and serum of women administered an LNG-containing intrauterine device, underscores the importance of measuring progestin levels in both serum and tissue. While our recent study showed metabolism of MPA and NET in endocervical tissue, to our knowledge, no studies have reported concentrations of these or other progestins, barring LNG, in breast tissue specifically. Metabolism of the selected progestins *in vivo*, may have major implications in therapeutic use, as this may lower the effective concentration of the progestins in the tissue. Moreover, it is possible that progestin metabolites may in fact be contributing to breast tumorigenesis. Indeed, it is known that metabolites of NET, LNG and GES can bind to ERα, and most display similar estrogenic activity compared to E_2 via ERα (Larrea *et al.*, 2001; García-Becerra *et al.*, 2002), the main etiological factors contributing to breast cancer. It is thus crucial to determine the actual

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concentration of the progestins and their metabolites in breast tissue and correlate this with the associated breast cancer risk reported in women using these progestins for contraception or MHT.

5.3. Comparing PR isoform-specific effects of progestins on gene expression and the influence of PR isoform co-expression at different ratios of PR-A:PR-B.

In the first part of Chapter 3, we characterised the activity of the selected progestins via the individual PR isoforms, relative to each other and P$_4$, on synthetic PRE and NFκB-containing reporters, and assessed the effects of co-expression of PR-A and PR-B at various ratios. We considered the MDA-MB-231 breast cancer line appropriate for these studies for two reasons: (1) none of the selected panel of progestins investigated in this thesis, except DRSP, was metabolised in the MDA-MB-231 cells (Chapter 2), and (2) this cell line expresses negligible levels of endogenous steroid receptors. The latter thus allowed for comparisons of the efficacies and potencies of the selected progestins via the specific transiently transfected receptor(s). In the second part of Chapter 3, we first investigated progestin regulation of a number of endogenous genes in the T47D breast cancer cell line endogenously expressing both PR-A and PR-B, and secondly assessed the role of the PR, as well as the effects of excess PR-A expression, in the progestin-mediated effects of the selected genes. The results for the characterisation of progestin activity in terms transactivation and transrepression are summarised in Tables 5.2 and 5.3, while the regulation of the endogenous genes is summarised in Table 5.4.

5.3.1. Reporter assays with minimal promoters and PR-transfected MDA-MB-231 cells.

We are the first to demonstrate that the transactivation activity of P$_4$ and selected progestins from all four generations on a synthetic minimal PRE-containing promoter, is PR isoform-,

PR-A density- and progestogen-specific (Table 5.2). We show that an increase in the density of PR-A results in a decrease in the efficacy of all progestins, but not P_4, while the potencies of P_4 and the progestins are differentially influenced and dependent on the level of PR-A expressed. We also show that compared to PR-B only, PR-A and PR-B co-expression at equivalent expression levels, and excess PR-A to PR-B, results in a decrease in the efficacies of all the progestogens for transactivation, while the potencies are either not influenced or increased in a ratio-dependent manner. As observed in the T47D cells, the role of excess PR-A on the expression of endogenous promoters is more complex and if PR-A is inhibiting the activity of PR-B, is difficult to determine from the current data. However, the reported data in Chapter 3 does suggest that the effects of P_4 and the progestins on transactivation are dependent on the ratio of PR-A to PR-B when the PR isoforms are co-expressed.

Our findings in Chapter 3 are also the first to show that all progestogens, except NES, are agonists for transrepression on a NFκB-containing promoter via both PR-A and PR-B (Table 5.3). We have previously shown promoter-specific effects for transrepression via the MR (Louw-du Toit *et al.*, 2020). For example, both MPA and NOMAC were shown to be agonists for transrepression, via the MR, on a NFκB, but not AP-1, reporter (Louw-du Toit *et al.*, 2020). To determine whether NES also induces a conformation in the PR that results in promoter-specific effects, or whether NES does in fact have dissociative characteristics in favour of transactivation, it would be interesting to investigate the transrepressive activity of NES on a different promoter such as that of AP-1. In contrast to our findings for transactivation, we show an increase in the repressive activity of all progestogens when the density of PR-A is increased, as well as when PR-A and PR-B are co-expressed at equivalent levels. To gain further insight into the transrepression activity of the selected progestins, experiments for dose-response analyses should be optimised to determine the biocharacter of the selected progestins for transrepression via PR-A and PR-B, as well as the effects of PR isoform co-expression at

Table 5.2. Transactivation activity of the progestogens via the PR transfected in MDA-MB-231 cells is isoform-, density and ratio-dependent.[a]

Progestogen	Efficacy (Maximal Response)						
	PR-A[b]	2x PR-A[c]	5x PR-A[c]	PR-B[d]	1:1[e]	2:1[e]	5:1[e]
R5020[f]	100%	n/c	↓	100%	↓	↓	↓
P4[f]	↓	n/c	n/c	n/c	↓	↓	↓
MPA	↓	↓	↓	↓	↓	↓	↓
NET	↓	↓	↓	↓	↓	↓	↓
LNG	n/c	↓	↓	n/c	↓	↓	↓
GES[f]	↓	n/c	↓	n/c	↓	↓	↓
NES[f]	↓	↓	↓	n/c	↓	↓	↓
NOMAC[f]	↓	↓	n/c	↓	↓	↓	↓
DRSP	↓	n/c	↓	↓	↓	↓	↓
	Potency (EC50)						
R5020	81.30 pM	↓	↓	18.03 pM	n/c	n/c	n/c
P4[g]	↑	↑	↓	↓	↑	↑	↑
MPA	n/c	↑	n/c	↑	n/c	n/c	n/c
NET[g]	n/c	↑	↑	n/c	n/c	↑	↑
LNG[g]	↑	n/c	↓	n/c	n/c	↑	↑
GES[g]	↑	↓	↓	n/c	n/c	n/c	n/c
NES[g]	↑	n/c	n/c	n/c	↑	↑	↑
NOMAC	↑	↑	n/c	↑	n/c	n/c	n/c
DRSP[g]	↑	n/c	n/c	↓	↑	↑	n/c

↑, increase in response (green); ↓, decrease in response (blue); n/c, indicates no change.
[a]Results from Chapter 3, Figure 3.1, 3.2, 3.3 and 3.4. [b]Statistically significant differences relative to the maximal response of R5020 via PR-A. [c]Statistically significant differences relative to PR-A[b]. [d]Statistically significant differences relative to the maximal response of R5020 via PR-B. [e]Statistically significant differences relative to PR-B[c]. [f]More efficacious via PR-B than PR-A. [g]More potent via PR-A than PR-B.

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various PR-A:PR-B ratios on progestin efficacies and potencies.

It is known that conformation of steroid receptors induced by ligand can influence activity (Raynaud *et al.*, 1980; Spilman *et al.*, 1986; Rayasam *et al.*, 2005), and thus to better understand the differential activities between the progestins, comparative molecular docking studies using the LBD of the PR could be conducted to determine how each progestogen fits into the ligand binding pocket of the PR. Since the full length crystal structure of the individual PR isoforms are currently unknown, this would be a good starting point to shed light on how a particular progestin could influence the conformation of the PR. Furthermore, it would be ideal to determine the binding affinities of the selected progestins for PR-A versus PR-B. As mentioned in Chapter 3, we were unable to perform such binding assays, as commercially available radio-labelled R5020 was discontinued by PerkinElmer, and the available radio-labelled P_4 is not ideal considering its rapid metabolism. Future studies could circumvent this limitation by having a custom radio-labelled R5020 manufactured. Since PR-A and PR-B are expressed at

Table 5.3. Transrepression activity via the PR transfected in MDA-MB-231 cells is isoform-, density-, ratio- and progestogen-specific.[a]

Progestogen	PR-A[b]	5x PR-A[c]	PR-B[b]	1:1[d]	5:1[d]
R5020	100%	↑	100%	↑	↑
P_4	*n/c*	↑	*n/c*	↑	*n/c*
MPA	↑	↑	↑	↑	↓
NET	*n/c*	↑	↓	↑	*n/c*
LNG	*n/c*	↑	*n/c*	↑	↑
GES	↑	↑	*n/c*	↑	*n/c*
NES	*n/e*	↑	*n/e*	↑	↑
NOMAC	↑	↑	↓	↑	↑
DRSP	↑	↑	↓	↑	↑

↑, *increase in response (green); ↓, decrease in response (blue); n/c, indicates no change; n/e, indicates no effect.*
[a]*Results from Chapter 3, Figure 3.5, 3.7, 3.8 and Addendum C, Figure C10;* [b]*Statistically significant differences relative to R5020;* [c]*Statistically significant differences relative to PR-A[b];* [d]*Statistically significant differences relative to PR-B[b].*

various levels in different tissues, it will also be useful to investigate whether the binding affinities of the progestogens change when the density of PR-A or PR-B differs. Indeed, it has been shown for the GR that the affinity of dexamethasone increases with an increase in GR expression (Robertson *et al.*, 2013).

Differential effects on gene expression could also be due to different ligands inducing different conformational changes of the PR, subsequently leading to differential recruitment of co-regulatory proteins (Rayasam *et al.*, 2005; Grimm *et al.*, 2016). A recent study using rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) coupled with liquid chromatography-electrospray mass spectrometry (LC-MS/MS) has shown that different sets of co-regulators are recruited by PR-A and PR-B in response to R5020 (Singhal *et al.*, 2018). Future studies could thus use this methodology to examine which co-regulators are recruited by the PR isoforms in response to different progestins.

It has been postulated that the PR can form three different molecular species, either PR-A/PR-A or PR-B/PR-B homodimers or PR-A/PR-B heterodimers, which influence transcriptional activity (Jacobsen *et al.*, 2002; Arnett-Mansfield *et al.*, 2007; Scarpin *et al.*, 2009). Arnett-Mansfield and co-workers have previously used fluorescence resonance energy transfer (FRET) to show the formation of both homo- and heterodimers of exogenously expressed PR isoforms in human U2OS bone osteosarcoma cells, in response to the progestin Org2058. These dimers localized in prominent foci within the nucleus, with the foci associated with active transcription (Arnett-Mansfield *et al.*, 2007). FRET measures the distance between two molecules within nanometers and is thus useful to show the presence of two adjacent PRs (Broussard *et al.*, 2013). As the influence of our panel of progestins on PR dimer formation has not been investigated, the experimental strategy of Arnett-Mansfield and colleagues could be followed to determine homodimer or heterodimer formation in the presence of different

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progestins, and whether dimer formation is affected by dysregulated PR-A:PR-B ratios. Such studies will aid in elucidating whether this is a mechanism contributing to the observed differences in progestin activity. Interestingly, while PR monomer assembly on palindromic PREs is reportedly more thermodynamically efficient than pre-formed PR dimers (Connaghan-Jones *et al.*, 2007), it has also been shown that the presence of authentic palindromic PREs in the promoters of PR-regulated genes are in actual fact limited *in vivo*, and that the PR isoforms may in fact be eliciting effects as monomers through interaction with PRE half-sites (Heneghan *et al.*, 2005; Jacobsen *et al.*, 2009; Jacobsen and Horwitz, 2012). Additionally, it has also been suggested that PR isoform-specific differences may be due to different DNA binding kinetics (Connaghan-Jones *et al.*, 2007). Connaghan-Jones and colleagues showed that, in response to P_4, PR-A has a weaker binding affinity and reduced DNA occupancy than PR-B. However, the authors also suggested that if PR-A is expressed in excess compared to PR-B, PR-A may monopolise the DNA binding sites on certain promoters which may result in displacement of PR-B. This mechanism may explain why the overexpression of PR-A relative to PR-B in our study results in such a drastic decrease in the efficacies of the progestins. Considering that the studies investigating DNA binding kinetics of the PR have only made used of R5020 and P_4, it would be interesting to investigate whether the binding kinetics of the PR to DNA would be influenced in response to our panel of progestins, and whether dysregulated isoform ratio would affect these kinetic parameters.

5.3.2. Endogenous genes expressed in T47D cells expressing endogenous PR-A and PR-B.

In Chapter 3, we showed that effects of P_4 and the progestins on endogenous gene expression in the T47D cells are ligand- and gene-specific (Table 5.4). We also show that the presence E_2 influences the activity of only of R5020, GES and NES, and in a gene-specific manner. On the other hand, P_4 and the progestins either have no effect, increase or inhibit the response of E_2

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only in a gene-specific manner. Although some investigators suggest that PR activity should always be investigated under estrogenic conditions, this has been contentious. However, the recent National Cancer Institute workshop report on the role of P_4 in breast cancer has suggested that interpretation of data would be facilitated by including all conditions i.e. P_4 only effects, E_2 only and $P_4 + E_2$ (Sathyamoorthy and Lange, 2020). Our results support the suggestion that it is best to always evaluate progestogens under both estrogenic and non-estrogenic conditions. The report by Sathyamoorthy and Lange (2020) has also recently highlighted the importance of the concentration of progestogens and E_2 used in the design of *in vitro* experiments investigating PR mechanisms. These authors specifically comment on the fact that it is difficult to interpret results of studies using concentrations of E_2 higher than 1 nM and progestogens higher than 1-10 nM (Sathyamoorthy and Lange, 2020). While we used a concentration of 100 nM progestogen and 100 nM E_2 in this thesis, we have observed similar results using 1 nM progestogen and 1 nM E_2 on at least Ki67 and BAX expression (Louw-du Toit, in preparation).

Investigating the role of the PR isoforms in the progestogen regulation of the selected genes using siRNA directed against total PR and PR-B was challenging and clear answers could not always be obtained. In future, it would be useful to design siRNA that specifically targets PR-A and include this in the experimental strategy. For FOXO1 we showed that PR-B mediates the effects of all progestins, while neither PR-A nor PR-B were involved in activity on Ki67 expression, and the R5020- and P_4-induced upregulation of BAX was mediated via PR-A. Truong and colleagues have shown that R5020 can upregulate FOXO1 expression via both PR-A and PR-B (Truong *et al.*, 2019). In the latter study, using total RNA extracted from T47D 3D tumorspheres, it was shown that R5020 had a greater effect on FOXO1 expression in T47D cells stably expressing PR-A than cells stably expressing PR-B. Although both 2D and 3D culture techniques have their advantages and limitations, 2D techniques are based on the

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Table 5.4. Summary of endogenous gene regulation by progestogens in T47D cells [a].

Treatment	NSC[b]	+ E$_2$[c]	siPR-B[c]	siPR-A/B[c]	+PR-A[c]
	GATA3				
Vehicle	1		*n/c*	↑	*n/c*
R5020	↓		*n/c*	↑	↑
P$_4$	↓		↑	↑	↑
MPA	↓	*n/c*	↑	↑	↑
NET	↓		*n/c*	↑	↑
LNG	↓		*n/c*	↑	↑
GES	↓		↑	↑	*n/c*
DRSP	↓		↑	↑	↑
	TGFβ1				
Vehicle	1	↑	*n/c*	↑	↑
R5020	↓	↑	↑	↑	↑
P$_4$	↓			↑	↑
MPA	↓	*n/c*		↑	↑
NET			*n/c*	↑	*n/c*
LNG	*n/e*			↑	*n/c*
GES		↓		*n/c*	↓
DRSP		*n/c*		↑	*n/c*
	FOXO1				
Vehicle	1		*n/c*	*n/c*	
R5020	↑		↓	↓	
P$_4$	↑	*n/c*	↓	↓	*n/c*
MPA	↑		↓	↓	
NET	↑	↑	↓	↓	↓
LNG	↑	*n/c*	↓	↓	*n/c*
GES	↑	*n/c*	↓	↓	*n/c*
DRSP	↑	*n/c*	↓	↓	↑

↑, *increase (green); ↑, increase but to a lesser extent (light green) ↓, decrease (blue); n/e, indicates no effect; n/c, indicates no change.*
[a]*Results from Chapter 3, Figure 3.9 and 3.11.* [b]*Statistically significant differences compared to the vehicle for T47D cells transfected with the non-silencing control (NSC).* [c]*Statistically significant differences compared to the response of the treatments of T47D cells transfected with the NSC.*

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Table 5.4. continued[a].

Treatment	NSC[b]	E₂[c]	siPR-B[c]	siPR-A/B[c]	+PR-A[c]
	Ki67				
Vehicle	1	↑	*n/c*	↑	
R5020	↑	↑	*n/c*	*n/c*	
P₄	↑	*n/c*	↑	↑	
MPA	↑	*n/c*	↑	↑	*n/c*
NET	↑	↑	*n/c*	*n/c*	
LNG	↑	*n/c*	↑	↑	
GES	↑	*n/c*	↑	↑	
DRSP	↑	*n/c*	*n/c*	*n/c*	
	BAX				
Vehicle	1	↑	*n/c*	*n/c*	
R5020	↑	*n/c*	↑	↓	*n/d*
P₄	↑	*n/c*	*n/c*	↓	
MPA		↑			
NET		↑			
LNG	*n/e*	↑	*n/d*		
GES		*n/c*			
DRSP		↑			
	SOX4				
Vehicle	1	↓			
R5020	↑	*n/c*			
P₄	↑	*n/c*			
MPA	↑	*n/c*	*n/d*		
NET	↑	↑			
LNG	*n/e*	*n/c*			
GES	*n/e*	↑			
DRSP	↑	*n/c*			

↑, *increase (green); ↑, increase to a lesser extent (light green) ↓, decrease (blue); n/e, indicates no effect; n/c, indicates no change; n/d, indicates not determined.*
[a]*Results from Chapter 3, Figure 3.9, 3.11 and Addendum C, Figure C12.* [b]*Statistically significant differences compared to the vehicle for T47D cells transfected with the NSC.* [c]*Statistically significant differences compared to the response of the treatments of T47D cells transfected with the NSC.*

adherence of a cell to a flat surface, while 3D culture attempts to recreate a more physiologically relevant tumour micro-environment, mimicking a more complex *in vivo* setting [reviewed in (Duval *et al.*, 2017)]. It would thus be interesting to investigate gene expression

in response to progestins, other than R5020, using total RNA extracted from 3D tumorsphere cultures. Work in this area is currently in progress in our laboratory (Hayley Jackson, PhD). Moreover, as PR isoform-specific recruitment to promoters of genes, including FOXO1, has been reported in the presence of R5020 (Diep *et al.*, 2016b; Singhal *et al.*, 2018; Truong *et al.*, 2019), similar experiments could be conducted to assess whether isoform-specific recruitment would also be observed with our panel of progestins.

The possible mechanism behind the differences in progestin activity on endogenous gene expression and the actual role of the PR was not easy to untangle. The lack of PR involvement on some genes suggest that the progestins are either acting via another steroid receptor expressed in the T47D cells or possibly mediating their effects via the activation of the membrane PR (mPR) (Price *et al.*, 2005; Valadez-Cosmes *et al.*, 2016). The mPR has been shown to be expressed in some breast cancer cell lines [reviewed in (Valadez-Cosmes *et al.*, 2016)], and it has been shown that at least P_4 and R5020 can bind and elicit effects via the mPR (Smith *et al.*, 2008; Kelder *et al.*, 2010; Pang and Thomas, 2011), while NET cannot bind the mPR (Thomas *et al.*, 2007).

Gene regulation by steroid receptors, including the PR, is complex and involves many different mechanisms that will contribute to differences in PR activity in response to various progestins. These include ligand binding to the receptor, receptor conformation, dimerization, as well as DNA binding discussed earlier, but also the context of the promoter. All of the endogenous genes investigated in this study have a complex array of *cis*-regulatory elements which are important in the regulation of gene expression, and with which the PR can interact, such as Sp1 and AP-1 (Tseng *et al.*, 2003). In addition, post-translational modifications of the PR isoforms including, but not limited to, phosphorylation, ubiquitination and SUMOylation at various sites are also important, as these modifications have been shown to influence nuclear localization,

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dimerization, protein stability, DNA binding, hormone sensitivity as well as co-regulator interactions [reviewed in (Abdel-Hafiz and Horwitz, 2014; Grimm *et al.*, 2016). For example, phosphorylation of PR-B at S294 is associated with the upregulation of genes linked to proliferation and survival (Knutson *et al.*, 2012), while phosphorylation of PR-A at S294 is associated with increased cancer stem cell-like cell behaviours (Truong *et al.*, 2019). On the other hand, SUMOylation of PR-A and PR-B has been shown to not only decrease the activity of the R5020-bound receptor, but also to determine whether the PR up- or downregulates certain genes involved with breast cancer progression (Abdel-Hafiz and Horwitz, 2014; Abdel-Hafiz *et al.*, 2018). Western blot analysis using antibodies targeting the S294 phosphorylated PR and the SUMOylated PR could be used to investigate whether these modifications of PR-A and PR-B occur in a progestin- and/or cell line-specific manner.

In summary, we have shown that P_4 and the progestins have both similar and different activities on gene expression, and that it is dependent on the gene investigated, which isoform is expressed as well as the density of PR-A relative to PR-B. The many unanswered questions remaining on the PR-mediated activity of the selected progestins, as well as the role of the PR isoforms in progestin-mediated gene regulation in breast cancer, warrant further investigations into these mechanisms.

5.4. Comparing progestin effects on hallmarks of cancer.

Since biological responses are a culmination of effects at the transcriptional level, we also investigated the effects of the progestins on processes involved in breast cancer development and progression in the last results chapter of this thesis (Chapter 4). Specifically, we compared the effects of P_4 and the selected progestins on cell proliferation and apoptosis (Table 5.5), anchorage-independent growth (Table 5.6), as well as migration and invasion (Table 5.7). An interesting observation was that, across all of the hallmarks investigated, when PR-A was

expressed in excess, relative to PR-B, ligand-independent effects occurred. Specifically, there was a ligand-independent decrease in proliferation and increase in apoptosis in the T47D cells expressing PR-A in excess. Considering that the essential balance between cell proliferation and apoptosis in the normal breast cell is often disrupted in breast cancer, with proliferation usually being increased and apoptosis inhibited, the latter results could indicate that the expression of excess PR-A in some breast cancers may have a positive effect. In support of this, a decrease in anchorage-independent growth was also observed with the excess expression of PR-A relative to PR-B. Invasion, on the other hand, increased in the absence of ligand when PR-A was expressed in excess relative to PR-B, supporting previous reports of PR-A in excess contributing to metastatic behaviours (McFall *et al.*, 2015, 2018; Truong *et al.*, 2019; Rosati *et al.*, 2020). Although the mechanism behind the ligand-independent effects of the PR isoforms was not investigated in our study, ligand-independent effects of both PR-A and PR-B have previously been reported (Jacobsen *et al.*, 2002, 2005; Khan *et al.*, 2012; Bellance *et al.*, 2013; Daniel *et al.*, 2015; Truong *et al.*, 2019).

In the presence of ligand, we showed that P_4 and all the progestins increased proliferation of the T47D breast cancer cells, as well as the MDA-MB-231 cells expressing either PR-A or PR-B, or both PR isoforms at various ratios (Table 5.5). Although the increase in proliferation in the MDA-MB-231 cells in response to the progestins was mostly to a similar extent, DRSP was less potent via PR-B and NOMAC was less potent via PR-A. When PR-A was expressed in excess relative to PR-B, the efficacies and potencies of the progestogens were mostly unchanged, with few progestins being influenced in a ratio-dependent manner. On the other hand, in the T47D cells, we observe that the effects of P_4 and NES are mediated via PR-A, while PR-A only partially mediated the effects of R5020, MPA, NET, NOMAC and DRSP. Interestingly, the proliferative effects of LNG and GES were regulated by either PR-A or PR-B.

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Table 5.5. Summary of progestogen regulation of proliferation in the MDA-MB-231 cell line, as well as proliferation and apoptosis in the T47D cell line.[a]

MDA-MB-231 cells					
Treatment	*Efficacy (Maximal Response)*				
	PR-A[b]	**PR-B[c]**	**1:1[d]**	**2:1[d]**	**5:1[d]**
R5020	↑	↑	*n/c*	*n/c*	
P$_4$			*n/c*	*n/c*	
MPA			↑	*n/c*	
NET			*n/c*	↓	*n/c*
LNG[e]	*n/c*	*n/c*	*n/c*		
GES			*n/c*		
NES			↑	*n/c*	
NOMAC			*n/c*		↑
DRSP			*n/c*		*n/c*
	Potency (EC$_{50}$)				
R5020	0.045 pM	0.021 pM		*n/c*	
P$_4$				*n/c*	
MPA				↓	
NET		*n/c*			
LNG	*n/c*		*n/c*		*n/c*
GES				*n/c*	
NES					
NOMAC[f]		↓		↑	
DRSP[g]	↓	*n/c*	↓	*n/c*	

↑, *increase (green); ↑↑, increase but to a greater extent (green) ↑, increase but to a lesser extent (light green) ↓, decrease (blue); - effect is abrogated; n/c, indicates no change.*
[a]*Results from Chapter 4, Figure 4.1, 4.2, 4.4, 4.5 and 4.7.* [b]*Statistically significant differences relative to the maximal response of R5020 via PR-A.* [c]*Statistically significant differences relative to the maximal response of R5020 via PR-B.* [d]*Statistically significant differences relative to PR-B[c].* [e]*More efficacious via PR-B compared to PR-A.* [f]*More potent via PR-A compared to PR-B.* [g]*More potent via PR-B compared to PR-A.*

Table 5.5. continued.

T47D cells					
Proliferation					*Apoptosis*
Treatment	**NSC[h]**	**siPR-B[i]**	**siPR-A/B[i]**	**+PR-A[i]**	**+PR-A[j]**
Vehicle	1	*n/c*	↓	↓	↑
R5020	↑	*n/c*	↑	↓	↑
P$_4$	↑	↑↑	-	↓	↑
MPA	↑		↑	↓	↑
NET	↑		↑	↓	↑
LNG	↑		*n/c*	↓	↑
GES	↑	*n/c*	*n/c*	↓	↑
NES	↑		-	↓	↑
NOMAC	↑		↑	↓	↑
DRSP	↑		↑	↓	↑

↑, increase (green); ↑↑, increase but to a greater extent (green) ↑, increase but to a lesser extent (light green) ↓, decrease (blue); - effect is abrogated; n/c, indicates no change.
[h]Statistically significant differences compared to the vehicle for T47D cells transfected with the non-silencing control (NSC). [i]Statistically significant differences compared to the response of the treatments for T47D cells transfected with the NSC. [j]Statistically significant difference in apoptosis of T47D cells transfected with PR-A compared to the effects of the treatments in pSG5 transfected T47D cells.

In contrast, neither P$_4$ nor the progestins had an effect on apoptosis of the above-mentioned cells (Table 5.5).

In the MDA-MB-231 cells, R5020 increased colony formation via PR-B, but not via PR-A. This result is in agreement with a recent study in T47D cells stably expressing either PR-A or PR-B (Truong *et al.*, 2019), where the authors suggest that PR-B drives the proliferative response in luminal breast cancer cells. Interestingly, these authors also showed that PR-A drives CSC expansion. However, we also show that colony formation increases in response to P$_4$ via PR-A, as well as MPA via both PR-A and PR-B (Table 5.6), suggesting that PR-A may also influence the proliferative response of breast cancer cells in a progestogen-dependent manner. Furthermore, our findings indicate that the effects of the selected progestins on cell migration and invasion were not limited to PR-A (Table 5.7), suggesting that both PR-A and PR-B may play a role in metastasis.

Table 5.6. Summary of progestogen regulation of anchorage-independent growth (colony formation) of MDA-MB-231 cells.[a]

Treatment	PR-A[b]	PR-B[b]	1:1[b]	2:1[b]	5:1[b]
Vehicle	1	1	↓[c]	↓[c]	↓[c]
R5020	n/c	↑	↑	n/c	↑
P$_4$	↑	n/c	n/c	n/c	n/c
MPA	↑	↑	n/c	n/c	↑
NET	n/c	n/c	n/c	n/c	n/c
LNG	n/c	n/c	n/c	n/c	n/c
GES	n/c	n/c	n/c	n/c	n/c
NES	n/c	n/c	n/c	n/c	n/c
NOMAC	n/c	n/c	n/c	n/c	n/c
DRSP	n/c	n/c	n/c	n/c	n/c

↑, increase (green); ↓, decrease (blue); n/c, indicates no change.
[a]Results from Chapter 4, Figure 4.8 and 4.9. [b]Statistically significant differences compared to the vehicle.
[c]Statistically significant differences compared to the vehicle of MDA-MB-231 cells expressing PR-B.

Notably, R5020 had no effect on migration or invasion via any of the PR isoforms, whether expressed individually or at different ratios, with the same being true for the effects of P$_4$ on migration. However, when the isoforms are co-expressed at the various ratios, only one or two progestogens differentially influenced migration and invasion in a ratio-specific manner. Two progestins that stood out were MPA and the structurally unique, DRSP. MPA was the only progestin that increased both cell migration and invasion via both isoforms and when the isoforms were co-expressed, while DRSP, despite increasing migration via PR-A, decreased both migration and invasion in cells co-expressing excess PR-A relative to PR-B. As it has been suggested that PR-A, in the presence of R5020 and MPA, is the driver of metastatic behaviour (McFall *et al.*, 2015, 2018; Truong *et al.*, 2019; Rosati *et al.*, 2020), it would be interesting to determine if the same is true for the other progestins via PR-A. However, as the liganded PR-B does influence both migration and invasion, it would also be important to assess whether the involvement of PR-B in metastatic characteristics is progestin-dependent. In light

of our data, the reported role of PR-B on proliferation or PR-A in CSC expansion and

metastasis cannot be assumed for all progestins.

Table 5.7. The progestogens differentially influence migration and invasion of the MDA-MB-231 cells exogenously expressing PR-A and/or PR-B.[a]

Treatment	PR-A[b]	PR-B[b]	1:1[b]	2:1[b]	5:1[b]
	Migration				
Vehicle					
R5020	*n/c*	*n/c*			*n/c*
P4	*n/c*	*n/c*			*n/c*
MPA	↑	↑	*n/c*		↑
NET	*n/c*	↑		*n/c*	
LNG	*n/c*	*n/c*			*n/c*
GES	↑	*n/c*	↓		
NES	*n/c*	↑			
NOMAC	↑	*n/c*	*n/c*		
DRSP	↑	*n/c*		↓	↓
	Invasion				
Vehicle	1	1			↑[c]
R5020	*n/c*	*n/c*			*n/c*
P4	*n/c*	↑			↓
MPA	↑	↑			*n/c*
NET	↓	↑	*n/d*		*n/c*
LNG	↑	↑			*n/c*
GES	*n/c*	↑			*n/c*
NES	↑	*n/c*			↓
NOMAC	↑	↑			*n/c*
DRSP	*n/c*	*n/c*			↓

↑, increase (green); ↓, decrease (blue); n/c, indicates no change; n/d, indicates not determined.
[a]Results from Chapter 4, Figure 4.10, 4.11, 4.12 and 4.13. [b]Statistically significant differences compared to the vehicle. [c]Statistically significant differences compared to the vehicle of PR-B expressing MDA-MB-231 cells.

Various hypotheses have been put forward to explain the detrimental actions of progestins in

the breast, including the possibility that progestins may not be carcinogenic, but rather enhance

the growth of tumours already present in the breast [reviewed in (Horwitz and Sartorius,

2020)]. In fact, like P_4, MPA has been shown to reactivate stem cell-like properties in pre-existing breast CSCs, suggesting that MPA-containing treatments may be activating the growth of pre-existing tumours (Horwitz and Sartorius, 2008). In support of this, it has been suggested that the involvement of the liganded PR in CSC expansion, occurs at early disease stages and promotes a more invasive phenotype [reviewed in (Horwitz and Sartorius, 2020)]. It has also been shown that MPA increases the activity of aldehyde dehydrogenase in T47D cells, which is another characteristic of CSCs (Goyette *et al.*, 2017). Whether the reactivation of stem cells is observed in response to other progestins, has not yet been investigated.

Although we did not investigate the mechanisms underlying the progestin- and isoform-dependent increase in migration, some non-genomic mechanisms have been proposed for progestogen-induced migration (Fu *et al.*, 2008b). For example, it has been shown that MPA, acting via either PR-A or PR-B, increases migration of T47D cells, through a phosphoinositide 3-kinase (PI3K)-dependent pathway (Fu *et al.*, 2008b). Whether the isoform-dependent increase in migration observed for the other progestins in our panel is mediated through similar non-genomic mechanisms, has not been investigated.

Two limitations of the current study is that, firstly, we did not compare the effects of the progestins in the absence and presence of E_2 on any of the cancer hallmarks, and secondly, we did not compare the effects of the progestins on apoptosis, migration and invasion, in a model system containing a more physiologically relevant milieu of steroid receptors. Work in this area is, however, currently being addressed by other projects in our laboratory. Lastly, an investigation into the possible oncogenic effects of different progestins on breast explant tissue would be advantageous, as these tissues are more physiologically relevant, and are often used to validate findings from cell line experiments (Mohammed *et al.*, 2015; Singhal *et al.*, 2016).

Although there is a spotlight on the role of P_4, progestins and the PR in breast cancer, the precise role of the PR isoforms is still understudied. Our data show that the activity of the selected progestins on the various hallmarks of breast cancer not only differs between each other, but are also mostly different to both P_4 and R5020. We also show that PR-A and PR-B differentially contribute to progestin effects on all the investigated cancer hallmarks in a progestin-specific manner, while both positive and negative effects were observed with the excess expression of PR-A relative to PR-B. These results emphasize that more investigation is needed into the role of the PR isoforms, as well as dysregulated isoform ratio in progestin activity in breast cancer.

5.5. Conclusion

Collectively, the findings presented in this thesis show that P_4 and the selected progestins from all four generations are differentially metabolised in a cell line- and progestin-specific manner. This suggests that reported efficacies and potencies of P_4 and some progestins may be underestimated in some cell line models. Moreover, these parameters may also be skewed by the presence of active progestogen metabolites. These findings highlight the importance of considering metabolism in the experimental design of *in vitro* experiments investigating the activity of progestins. These results also underline the importance of identifying progestin metabolites and investigating their possible biological activities. All progestins, except NES, displayed progestogenic activity via PR-A and PR-B for both transactivation and transrepression, with some progestins showing a preference for a specific PR isoform. Given that progestins are PR ligands, it was surprising to find that not all progestins displayed full agonist activity for transactivation via PR-A and PR-B. Furthermore, as the expression of the PR isoforms differs across various tissues, these results suggest that the physiological outcomes in response to progestins will most likely be tissue-specific.

As observed for gene expression, the effect of the progestins on the cancer hallmarks investigated in this study were progestin- and PR isoform-specific. All of the aforementioned findings emphasise the importance of comparing multiple progestins in parallel in the same model system, and not drawing conclusions on progestin action from data on one or two progestins. These results are important given the widespread use of progestins, as well as the fact that it is unclear which progestins may or may not increase breast cancer risk. Appreciating the fact that progestins are not all the same, may aid clinicians and women using progestin-containing MHTs to make a more informed choice when it comes to choosing the best progestin to use with the least possible associated risks in terms of breast cancer. Additionally, this study showed that the activity of P_4 and the selected progestins on gene expression, as well as their effects on the cancer hallmarks, are often dependent on the specific PR isoform present and the ratio of PR-A:PR-B. Notably, these isoform- and ratio-specific differences in progestin potencies are likely to occur *in vivo*, as the potencies of the progestins reported in this study are well below the serum levels reported in women. Furthermore, in both the absence and presence of progestin, our results showed that the excess expression of PR-A relative to PR-B has both positive and negative effects on the specific cancer hallmarks investigated. In addition to observing a ligand-independent decrease in proliferation and increase in apoptosis, we also found that a ligand-independent increase in invasion is inhibited by P_4 and some progestins, supporting the potential therapeutic use of some, but not all, progestins in combination with endocrine therapies for tumours expressing a high PR-A:PR-B ratio.

Overall, the findings of this study contribute to an outstanding question in the field of the PR and breast cancer raised in the recent review by Horwitz and Sartorius (2020): "are progesterone and synthetic progestins similar or not?". Our findings contribute to the knowledge and understanding of progestins used in MHT by showing that P_4 and the selected progestins are mostly not similar, and in fact, the progestins themselves often display

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differential activities. This suggests that some progestins may be safer than others in terms of breast cancer risk. Finally, although the role of P_4, progestins and the PR isoforms in breast cancer is far from resolved, the findings in this study add a small piece to the completion of a huge puzzle.

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Addendum A

Contributions to publications and conference

Outputs of the PhD study

A1: Differential metabolism of clinically-relevant progestogens in cell lines and tissue: Implications for biological mechanisms

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The candidate is the third author on this publication, contributing the experimental work and analysis of the data for the MDA-MB-231 cells, as well as contributing to the critical evaluation and editing of the study alongside the other authors.

Differential metabolism of clinically-relevant progestogens in cell lines and tissue: Implications for biological mechanisms

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ABSTRACT

Steroid hormones regulate a variety of physiological processes, including reproductive function, and are widely used in hormonal therapy. Synthetic progestogens, or progestins, were designed to mimic progesterone (P_4) for use in contraception and hormonal replacement therapy in women. Medroxyprogesterone acetate (MPA) and norethisterone (NET) are the most widely used injectable contraceptives in the developing world, while other progestins such as levonorgestrel (LNG), etonogestrel (ETG) and nestorone (NES) are used in or being developed for other forms of contraception. As concerns remain about the most appropriate choice of progestin and dosage, and the associated side-effects, the mechanisms and biological effects of progestins are frequently investigated in various *in vitro* mammalian cell line and tissue models. However, whether progestogens are differentially metabolised in different cell types *in vivo* or *in vitro* is unknown. For nine mammalian cell lines commonly used to investigate progestogen mechanisms of action, we developed and validated an ultra-high performance supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS) protocol for simultaneously quantifying the metabolism of the above-mentioned steroids. We show for the first time that, while 50–100% of P_4 was metabolised within 24 h in all cell lines, the metabolism of the progestins is progestin- and cell line-specific. We also show that MPA and NET are significantly metabolised in human cervical tissue, but to a lesser extent than P_4. Taken together, our findings suggest that differential progestogen metabolism may play a role in cell-specific therapeutic and side-effects. Relative affinities for binding to steroid receptors as well as potencies, efficacies and biocharacters for transcriptional activity of progestins, relative to P_4, are most frequently determined using some of the cell lines investigated. Our results, however, suggest that differential metabolism of progestins and P_4 may confound these results. In particular, metabolism may under-estimate the receptor-mediated intrinsic *in vitro* binding and dose-response values and predicted endogenous physiological effects of P_4.

1. Introduction

Choice of hormonal contraception and hormone replacement therapy (HRT) in women is an important public health issue, especially regarding possible side-effects relevant to cancer, metabolic disorders, cardiovascular complications, bone mineral density and susceptibility to infectious diseases [1,2,3]. Synthetic steroids are commonly utilised in contraceptive treatments and HRT. These synthetic steroids, known as progestins or synthetic progestogens, are intended to mimic the actions of the endogenous hormone P_4 [1–3] and are classified into two groups. The first class of progestins, which includes medroxyprogesterone acetate (MPA) and nestorone (NES), is structurally related to P_4, while the second class is structurally related to testosterone (T), and includes norethisterone (NET), etonogestrel (ETG) and levonorgestrel (LNG) [2,3]. Injectable progestins, which are especially popular in the developing world as contraceptives due to their discreet

nature, include MPA and NET, the latter administered in its enanthate form (NET-EN) [2,3]. Other progestins such as LNG and ETG are widely used in combined oral contraceptives and implants and, together with NES, are currently being investigated for use intravaginally or in multipurpose prevention technologies [3].

Progestin research relies extensively on model systems using well-established laboratory cell lines [2–6] or *in vitro* experiments with primary cells, tissue or tissue extracts [7–12]. In such experiments, specific concentrations of the steroids are used and these concentrations are assumed to remain constant over the incubation period. Differences in activity between steroids is thought to be due to their different biocharacters, and metabolism is not taken into account. Differential metabolism may confound the results of concentration-dependent experiments such as dose-response analyses and binding studies [2–4]. It is well established that progestins act intracellularly via binding to and activating the progesterone receptor (PR) [2,3], which is a ligand-activated transcription factor. Evidence is emerging that some of the side-effects of progestins may occur by off-target effects via binding to and activating steroid receptors other than the PR [3,5]. However, very little is known about the metabolism of progestins; in particular, whether this metabolism is cell-specific, which metabolites are produced, what the role is of metabolites and whether metabolism may confound interpretation of the results when investigating relative biological activities.

The aim of this work was therefore to investigate the metabolism of P_4 and selected progestins in nine commonly used laboratory cell lines, and to validate select findings in endocervical tissue. To this end, we developed and validated an ultra-high-performance supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS) method for the separation and quantification of these progestogens in the nanomolar range, as detected in the serum of women. We included the synthetic glucocorticoid dexamethasone (DEX) in our panel of steroids, since the activity of progestins is often investigated in parallel with DEX, given the established glucocorticoid activity of MPA [10,13,14]. Results showed that P_4 was substantially metabolised in all cells lines and the endocervical tissue after 24 h, while cell line- and steroid-specific metabolism were observed for the different progestins.

2. Materials and methods

2.1. Steroids and solvents

LNG was obtained from the United Stated Pharmacopoeia (USP, Rockville, MD, USA) and Sigma-Aldrich (South Africa). P_4, MPA, NES, DEX, NET, ETG, T, UHPLC-grade methanol, absolute ethanol, formic acid and methyl *tert*-butyl ether (MTBE) were all purchased from Sigma-Aldrich (South Africa).

2.2. Cell lines and endocervical tissue

Human embryonic kidney cells (HEK293T), human epithelial cervical cancer cells (HeLa), human endocervical cells (END-1), human bone osteosarcoma epithelial cells (U2OS) and monkey kidney fibroblast cells (COS-1) were purchased from American Type Culture Collection (ATCC, USA). Human cervical cells (TZM-bl) were procured from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr John C. Kappes, Dr Xiaoyun Wu and Tranzyme Inc. (ARP, NIH, USA). The human MDA-MB-231 breast cancer cell line was originally acquired from ATCC, but was received from Prof Adrienne Edkins at Rhodes University, South Africa, while Prof Ana Soto at Tufts University, Boston, USA provided the human MCF-7 BUS breast cancer cells. The human T47D breast cancer cell line was donated by Prof Iqbal Parker at the University of Cape Town, South Africa.

Endocervical tissue was obtained after informed consent from HIV-1 negative, post-menopausal women undergoing hysterectomies for benign reasons. Ethical permission was obtained from the Human

Research Ethics Committee (University of Cape Town) for the duration of this study (HREC 258/2017). Fresh tissue was supplied from two sites in the Western Cape, South Africa; namely, Groote Schuur and Tygerberg Hospitals.

2.3. Cell line culture

HEK293T, HeLa, U2OS, TZM-bl, T47D and COS-1 were all cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 1 mM sodium pyruvate (Sigma-Aldrich, South Africa), 44 mM sodium bicarbonate (Sigma-Aldrich, South Africa), 10% (v/v) fetal bovine serum (FBS) (Thermo Scientific, South Africa), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, South Africa). Culture medium for MDA-MB-231 cells was as described above, with the addition of 2 mM ʟ-glutamine (Sigma-Aldrich, South Africa). Culture medium for MCF-7 BUS cells was as described above, except that 5% heat-inactivated FBS was used. END-1 cells were maintained in keratinocyte serum-free (KSF) medium (Sigma-Aldrich, South Africa) supplemented with the provided keratinocyte growth supplement, 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco Invitrogen). Cells were maintained at 37°C in a water-jacketed incubator (90% humidity and 5% CO_2). All cells were routinely tested and found to be mycoplasma-free.

2.4. Cervical tissue experiments

Cervical tissue was processed as previously described by Fletcher et al. (i.e. between one to three hours post-operation) [15]. Excess underlying stromal tissue was removed from the epithelial layer of the endocervical tissue. The epithelial layer was then diced into 3 mm³ explant pieces that were randomly placed into separate wells of 96-well round-bottomed plates. Non-polarised explants were cultured in 200 μL Roswell Park Memorial Institute medium (RPMI) (Lonza, Switzerland) supplemented with 10% (v/v) charcoal stripped FBS (Thermo Scientific, USA), 2 mM ʟ-glutamine (Sigma-Aldrich, South Africa), 10 μg/mL Fungizone (Sigma-Aldrich, South Africa), 10 U/mL interleukin-2, 100 IU/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, South Africa). Cervical tissue explants were incubated in quadruplicate with steroids in RPMI and incubated at 37°C in a water-jacketed incubator (90% humidity and 5% CO_2) for 24 h.

2.5. Cell line and tissue incubations with steroids

Cells were seeded at 5×10^4 cells per well (T47D, MCF-7 BUS, MDA-MB-231) or 1×10^5 cells per well (END-1, U2OS, TZM-bl, HEK293T, COS-1 and HeLa) in full phenol red-containing media in a 24-well Greiner Bio-One CELLSTAR tissue culture plate. Tissue was processed and plated as described above. Following a 24-hour incubation period, T47D, MCF-7 BUS and MDA-MB-231 cell media was replaced with phenol red-free media. For analysis of extent of metabolism, the cells and no-cell controls were washed with pre-warmed media then treated with 100 nM steroid or vehicle (0.1% v/v ethanol) in serum-free media. The U2OS, TZM-bl, HEK293T, COS-1, END-1 and HeLa cells were treated in phenol red-containing DMEM, while T47D, MCF-7 BUS and MDA-MB-231 cells were treated in phenol red-free DMEM. Upon treatment, 500 μL of the steroid- and vehicle-containing media was aliquoted into a glass tube and stored at −20°C; this served as the T_0 control. After 24 h, 500 μL aliquots of media were removed from the cells (or no-cell control) and transferred into clean glass tubes and stored at −20°C prior to extraction

2.6. Preparation of standards and samples

Individual stock solutions of the seven steroids (P_4, MPA, NES, NET, LNG, ETG and DEX) plus internal standard T were prepared in absolute ethanol (1 mg/mL) and stored at −20°C until use. These individual

stock solutions were later used to prepare two standard master mixes (1 000 ng/mL and 1 ng/mL) containing all of the above-mentioned steroids in ethanol. These standard master mixes were subsequently used to prepare standards (1 mL, 0.01–100 ng/mL) by the addition of the appropriate volume of the standard master mix to either (i) DMEM containing 1% penicillin-streptomycin and 10% FBS ("supplemented DMEM"), (ii) DMEM without penicillin-streptomycin or FBS ("un-supplemented DMEM"), (iii) KSF without penicillin-streptomycin or FBS, (iv) RPMI 1640 without penicillin-streptomycin or FBS or (v) 50% methanol (no matrix). Samples used for method validation (1 mL) were prepared by spiking the matrix with the appropriate volume of the master mixes. 100 μL of internal standard prepared in distilled water to a final concentration of 1 ng/mL was added to all samples and standards.

2.7. Steroid extractions

Samples and standards were extracted using a 1:3 ratio of sample to MTBE (v/v). The samples were shaken at 1 000 rpm for 15 min before being placed at $-80°C$ for an hour to allow the aqueous phase to freeze. The MTBE layer containing steroids was transferred to a pyrolyzed glass test tube and the MTBE was evaporated at room temperature in a fume hood overnight, or under a stream of nitrogen gas. Samples were subsequently reconstituted in 150 μL 50% methanol and stored at $-20°C$ prior to analysis.

2.8. Instruments and chromatographic conditions for UHPSFC-MS/MS

Steroids were separated using an Acquity Ultra High Performance Convergence Chromatography (UPC²) system (Waters Corporation, Milford, USA) with an Acquity UPC² Ethylene Bridged Hybrid (BEH) column (3 mm × 100 mm, 1.7 μm particle size). The mobile phase consisted of liquid CO_2 (Mobile phase A) and methanol [Mobile phase B (MPB)]. A 2.5-minute gradient inlet method was used to separate the steroids using a constant flow rate of 1.9 mL/min according to the following protocol: 4% MPB from 0 to 1 min; 10% MPB from 1 to 1.5 min; 25% MPB from 1.5 to 2.5 min and back to 4% MPB at 2.5 min for re-equilibration.

The column temperature and automated back pressure regulator were set to 60°C and 1700 pounds-force per square inch (psi), respectively. The injection volume was 2.0 μL. Quantitative mass spectrometric detection was carried out using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA). A make-up pump fed 1% formic acid in methanol into the mixer preceding the MS line at a constant flow rate of 0.2 mL/min. All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray probe in the positive ionisation mode (ESI+). The following settings were used: capillary voltage of 3.8 kV, desolvation temperature 350°C, desolvation gas 900 L/h and cone gas 150 L/h. MRM transitions are included in Supporting Table 1. Data collection and analysis were performed using MassLynx 4.1 (Waters Corporation).

2.9. UHPSFC-MS/MS method validation

Standard curves were generated for each steroid metabolite using standards prepared in either of the four matrices listed above or 50% methanol (no matrix), and included the following concentrations: 0, 0.01, 0.1, 0.25, 0.5, 1.0, 5.0, 10, 25, 50 and 100 ng/mL. The limit of detection (LOD) for each steroid was defined as the lowest concentration at which a signal-to-noise (S/N) ratio greater than three was measured for the quantifier ion. The lower limit of quantification (LLOQ) for each steroid was defined as the lowest concentration for each steroid at which: a S/N ratio greater than ten was measured for the quantifier ion; a S/N ratio greater than three was measured for the qualifier ion; an acceptable precision [% relative standard deviation (% RSD) < 20] could be measured. The upper limit of quantification

(ULOQ) was defined as the maximum concentration at which the % RSD values did not exceed 20. Precision was defined as the % RSD from the average calculated concentrations following the repeated injection (n = 6) of a simple sample. Accuracy was defined as the % RSD from the analysis of independent replicate samples (n = 6).

2.10. Statistical analysis

Results were analysed using GraphPad Prism 7 from GraphPad Software, Inc. (La Jolla, California, USA). Data are expressed as mean ± SEM. To evaluate whether the metabolism of a steroid within a cell line/tissue was statistically significant, a paired *t*-test was performed to compare results in the absence and presence of cells. Statistical significance is denoted by the relevant p-value. Multiple paired *t*-tests were used to compare the metabolism of the seven steroids within a cell line to each other, and to compare the metabolism of a specific steroid across different cell lines. (ANOVA was not used since these experiments were not all performed in parallel.) Where statistical significance was determined, it is denoted by *, **, ***, or **** to indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, or $p < 0.0001$, respectively.

3. Results

3.1. Validation and performance of UHPSFC-MS/MS method

A UHPSFC-MS/MS method was developed for the quantification of six clinically-relevant, commercially-available progestogens and DEX. Their chemical structures are depicted in Fig. 1. Molecular ion species, MRM mass transitions and retention time for each steroid are given in Supporting Table 1. Comprehensive method validation was performed, the results of which are listed in Table 1.

As most of the cell lines were treated with unsupplemented DMEM, accuracy and precision were determined only for this medium at a minimum of three concentrations within the calibration range of each steroid as shown in Table 1, i.e. 1, 10 and 100 ng/mL. Acceptable % RSDs were obtained for all concentrations for both accuracy and precision, which were less than 20 at concentrations of 1, 10 and 100 ng/mL. Accuracy at low concentrations ranged from 11 to 18% at 1 ng/mL, 11–18.9% at 10 ng/mL and 8–17% at 100 ng/mL. Precision at low concentrations ranged from 14–19% at 1 ng/mL, 4–16% at 10 ng/mL and 9–19% at 100 ng/mL. LLOQs ranged from 0.01 ng/mL for P_4 to 5.00 ng/mL for LNG, ETG and DEX, allowing for the quantification of steroids at levels at the low nanomolar range. For each of unsupplemented DMEM, KSF and RMPI media, the ULOQ was 100 ng/mL (the highest concentration measured), while ULOQ in supplemented DMEM was 50 ng/mL.

3.2. Effects of adsorption and hydrophobicity

Following the development and validation of the UHPSFC-MS/MS method we first considered the potential effects of adsorption of the steroids to the cell culture plates, before measuring the metabolism of the steroids. To do this we assessed the differences in steroid concentration between the T_0 and media from no-cell control plate incubations for all the steroids in the different experiments (Supporting Fig. 1). There was 0%–40% adsorption of the steroids to the cell culture plates across experiments (Fig. 2 and Supporting Fig. 1). We further investigated whether there was a correlation between hydrophobicity and adsorption of steroids. Results showed a positive correlation suggesting that the adsorption of steroids to the cell culture plates increases with increasing hydrophobicity (Fig. 2). We investigated whether retention of the steroids occurred within the cell pellets. We found that this was negligible (Supporting Fig. 2) and hence was not taken into account when calculating percentage metabolism. Based on these results, metabolism (Section 3.3–3.5) was calculated relative to a no-cell control parallel incubation in cell culture plates and hence was

Fig. 1. The chemical structures of the seven commercially-available steroids described in this work. MPA and NES are structurally related to P$_4$ while NET, LNG and ETG are structurally related to T. DEX is a synthetic glucocorticoid.

independent of adsorption to the cell culture plates.

3.3. Differential metabolism of steroids between cell lines

Next, we incubated nine common laboratory cell lines, along with a parallel plate without any cells ("no-cell control"), with 100 nM of each steroid for 24 h. We subsequently measured the concentration of steroid present in the cell and no-cell supernatants. The percentage metabolism of each individual steroid in a cell line was calculated as the difference between the steroid remaining in the absence (no-cell) and presence of cells. When percentage metabolism of a ligand was less than zero, it was represented as zero. It should be noted that steroid incubations were performed in the absence of serum for 24 h as is frequently done for experiments in cell lines incubated with steroids [16–18]. It is possible that the presence of serum may affect metabolism.

When comparing metabolism effects, it was noted that the error bars were in general much greater for some cells than for others, as well as

between some progestins for a particular cell line. Assessment of no-cell samples indicates that this reflects variations in technical error during experiments. Only differences that were found to be statistically significant are highlighted and discussed below. It should, however, be noted that there may be other differences that are significant, but beyond the statistical power of the experiments. The most striking result was that P$_4$ was extensively metabolised by all the cell lines, although the extent of metabolism varied from 50 to 97%, showing some cell line-specific effects (Figs. 3 and 4). Although the metabolism of the progestins and DEX was also cell line-specific, it ranged from 0 to 50%, with the rank order for most to least metabolised steroid different for each cell line. These did not appear to be related to the anatomical source or type of cell line, as shown in Fig. 4. However, some trends were apparent (Figs. 3 and 4). Of the cervical cell lines, HeLa exhibited a higher percentage of significant metabolism for most progestogens than observed in TZM-bl and END-1 cells, except for NET and ETG where END-1 cells exhibited more metabolism. The T47D cell line

Table 1
Comprehensive method validation data: r^2, LOD (ng/mL and nM), LLOQ (ng/mL and nM), accuracy (% RSD, n = 6) and precision (% RSD, n = 6). LOD, LLOQ, accuracy and precision are shown for unsupplemented DMEM only. (-) indicates that the concentration is below the LLOQ for that steroid and is therefore not included.

Steroid	r^2 (Unsupplemented DMEM)	LOD, ng/mL (nM)	LLOQ, ng/mL (nM)	Accuracy % RSD, 1 ng/mL	Accuracy % RSD, 10 ng/mL	Accuracy % RSD, 100 ng/mL	Precision % RSD, 1 ng/mL	Precision % RSD, 10 ng/mL	Precision % RSD, 100 ng/mL
P$_4$	0.9928	0.01 (0.03)	0.01 (0.03)	18.2	12.6	8.3	16.9	9.5	9.5
MPA	0.9939	0.5 (1.3)	1 (2.6)	10.9	11.1	9.7	14.0	6.7	9.3
NES	0.9955	0.5 (1.3)	1 (2.7)	16.5	18.9	16.9	19.0	16.1	18.8
NET	0.9951	0.5 (1.7)	1 (3.3)	14.3	11.5	9.8	14.1	4.4	9.7
LNG	0.9739	0.01 (0.03)	5 (16.0)	–	4.7	11.2	–	7.4	9.7
ETG	0.9960	1 (3.18)	5 (15.4)	–	13.1	8.0	–	10.9	8.7
DEX	0.9951	5 (12.7)	5 (12.7)	–	12.8	16.9	–	12.5	11.8

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Fig. 2. Predicted steroid hydrophobicity correlates with adsorption. Regression analysis was performed to determine whether there was a correlation between the adsorption and hydrophobicity of the seven clinically-relevant steroids. The percentage adsorption of each steroid to the tissue culture plates was determined from the difference between the time-zero (T_0) measurement and its corresponding no-cell control. CLogP values were predicted using ChemDraw Professional Version 16.0.1.4 (PerkinElmer Informatics, Inc.). Non-linear regression using GraphPad Prism Version 7 (GraphPad Software, Inc.) revealed a correlation with $r^2 = 0.5394$.

exhibited the highest significant metabolism within the breast cancer cell lines, except for NES where MCF-7 BUS showed greater significant metabolism. END-1, T47D, MCF-7 BUS and COS-1 cells were the most metabolically diverse cells in the panel, with all four of these cell lines displaying significant metabolism of P_4 and ETG; and three of these four cell lines showing metabolism of DEX (Fig. 3). Interestingly, COS^{-1} cells displayed a similar high degree of significant metabolism for both P_4 (60%) and DEX (53%). These cell lines were followed by U2OS cells, which significantly metabolised three steroids. TZM-bl and MDA-MB-231 cells had significant metabolism of P_4 and one other steroid, namely, MPA and ETG respectively. HeLa and HEK293 T cells displayed the lowest metabolic activity of progestogens with significant metabolism observed only for P_4 (Fig. 3). Taken together, there was no significant difference in the overall metabolism of the progestins structurally related to P_4 (MPA and NES) compared to those steroids structurally related to T (NET, LNG and ETG) (Fig. 4). There was an average of four times more metabolism observed for P_4 than for the other steroids.

3.4. Differential metabolism of steroids within cell lines

Upon comparison of the metabolism of each steroid within each cell line, it was observed that, apart from P_4, all steroids were metabolised in a cell-specific manner (Fig. 3 and Supporting Fig. 3). T47D, END-1 and HeLa cells had the highest percentage metabolism of P_4 with over 90% metabolism in all three cell lines. HEK293 T and COS^{-1} cells displayed the least metabolism of P_4 with only 50% and 60% metabolism, respectively. P_4 demonstrated the greatest differences in metabolism compared to other steroids within each cell line (Fig. 4 and Supporting Fig. 3).

ETG was the second most metabolised steroid, with significant metabolism in five cell lines ranging between 14% and 43% (Figs. 3 and 4). HeLa cells showed a high percentage of metabolism of ETG with 28% metabolism; this, however, was not significant (Fig. 4). MPA and DEX were significantly metabolised in three cell lines each. Significant metabolism of MPA was observed in U2OS, TZM-bl and T47D cells, ranging between 19% and 55% (Fig. 3). Significant metabolism of DEX was observed in END-1, MCF-7 BUS and COS^{-1} cells with metabolism ranging from 8% and 52%. As shown in Fig. 4, metabolism of DEX in HeLa, T47D and U2OS cells, although not significant, ranged from 21% to 37%. TZM-bl, HEK293 T and MDA-MB-231 cells exhibited less than 10% metabolism of DEX. NET and NES were significantly metabolised only in two cell lines each (Fig. 3). NET was significantly metabolised only in U2OS and MCF-7 BUS cells (Fig. 3). NES was metabolised in END-1 and MCF-7 BUS cell lines by 9% and 40%, respectively (Fig. 4). There was 41% metabolism of NES in COS^{-1} cells, and between 0% and

20% in the remaining cell lines; however, these effects were not statistically significant (Supporting Fig. 3). LNG was significantly metabolised only in COS^{-1} cells with 23% metabolism which was comparable to the metabolism in T47D and HeLa cells which exhibited 22% and 20% metabolism, respectively. However, these latter effects were not statistically significant. The metabolism of LNG in the remaining six cell lines was less than 10% (Supporting Fig. 3).

3.5. Metabolism in endocervical tissue

To determine if the metabolism observed in cell lines would be similar in a more physiologically-relevant system, we investigated the metabolism of three progestogens in post-menopausal endocervical tissue explants following a 24-hour incubation. P_4 was investigated due to the high rate of metabolism in all cell lines, whilst MPA and NET were chosen as representative of progestins structurally related to P_4 and T, respectively.

P_4 was completely metabolised in the endocervical tissue explants after 24 h (Fig. 5). The results for P_4, but not MPA and NET, are comparable to those observed in the three cervical cell lines (Fig. 3). There was between approximately 87% and 96% metabolism of P_4 in the cervical cell lines, which is similar to the 100% metabolism in tissue (Fig. 5). As depicted in Fig. 5, 38% of MPA was metabolised in tissue. This is different from the pattern observed in cervical cell lines in which TZM-bl cells exhibited significant, but less, metabolism of MPA, (Fig. 3) while no significant metabolism was observed in the other cervical cell lines. While NET was not significantly metabolised in the cervical cell lines, endocervical tissue metabolised 43% of available NET.

4. Discussion

Previous research into progestogen metabolism has been limited and has typically focused on measuring the serum or urine concentrations of progestogens and/or their metabolites in a clinical setting [19–26]. In this work we investigated, for the first time, the cell- and steroid-specific metabolism of a range of clinically-relevant steroids using *in vitro* cell line models. These cell line models are widely used to investigate the biocharacter and mechanisms of action of these steroids, which themselves are commonly used in hormonal therapy and steroid receptor-based studies. We developed and validated a UHPSFC-MS/MS method of measuring the concentration of seven steroids in cell culture media, which allowed for the determination of the metabolism of these steroids by these cell lines. Our experimental design corrected for adsorption (up to 40%) of the steroids to the cell culture plates, where we found a positive correlation between adsorption and increasing hydrophobicity. The extent of metabolism could be measured from analysing the medium (supernatant) alone, since we found no significant retention of parent steroid in the cell pellets.

We found that individual progestins and DEX are differentially metabolised within the same cell line, and amongst different cell lines. For example, over 24 h, some progestins are not significantly metabolised in a particular cell type (< 20%), while others display a high degree of metabolism (20–50%) in the same cell type. A particular progestin can be metabolised to a vastly different degree in different cell lines (e.g. MPA metabolised by 55% in T47D and less than 10% metabolism in HEK293 T and MDA-MB-231 cells). We detected no correlation between extent of metabolism and whether progestins were structurally related to P_4 or T.

Taken together, these results may have important physiological and pharmacological implications. Progestins used in hormonal contraception and HRT are known to exert several side-effects, such as effects on bone mineral density, metabolism, cardiovascular effects and reproductive cancers [2–4,6]. Progestins also exert their contraceptive effects at several levels at different target tissues. If these *in vitro* effects of metabolism are translated *in vivo*, this suggests that different progestins may exert very different side-effects and may be more or less

Fig. 3. Differential metabolism of seven clinically-relevant steroids following incubation at 100 nM for 24 h in nine different cell lines. Medium containing the steroids was added to a 12-well plate, containing no cells, as a negative control for metabolism. Steroids were extracted and analysed by UHPSFC-MS/MS. The amount of steroid 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%. These data show the mean ± SEM of a minimum of three independent biological repeats. Statistical analysis via paired *t*-tests was performed on each steroid and statistically significant differences relative to its no cell control are indicated by the p-value above bar. To quantify whether the relative metabolism of two steroids within a cell line was significantly different, multiple *t*-tests were performed between steroids. Significant differences in tables below the histograms are indicated by asterisks where *, **, ***, **** represent $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

efficacious for contraception due not only to their inherent biocharacters, potencies and efficacies, but also due to differential metabolism in different target cells and tissues. This metabolism could both selectively lower their effective concentrations at the target cells in a cell-specific manner and may, also result in different metabolites with different off-target effects. The tissue findings are particularly

Fig. 4. Heat map summarising differential metabolism of seven clinically-relevant steroids. Data are from Fig. 3 and cell lines and steroids are grouped according to anatomical and structural similarities, respectively. Pregnanes (P) have a methyl group at C10 position, while norpregnanes (NP) have a hydrogen group at C10; estranes (E) have a methyl group at C13, while gonanes (G) have an ethyl group at C13. Data are represented as % metabolism relative to the no-cell control which was set to 100%. Statistical significance is indicated by asterisks.

Fig. 5. Differential metabolism of three clinically-relevant progestogens in post-menopausal endocervical tissue. Tissue explants were incubated with 100 nM progestogens for 24 h. The supernatant was removed and extracted before quantification via UHPSFC-MS/MS, as described in methods. Results were normalised to the progestogen concentration detected in a corresponding no-tissue control experiment to account for adsorption loss. The data show the mean ± SEM of three to five independent biological repeats. Statistical analysis via paired *t*-tests was performed comparing the progestogen concentration in the supernatant of the tissue condition to the no-tissue control; significant results are indicated by the p-value above the bar. To determine whether the relative metabolism of the progestogens was significantly different, an unpaired *t*-test with Welch's correction was performed. Statistical significance indicated with * represents $p < 0.05$.

interesting since they suggest that doses and types of progestins used for intravaginal delivery need to be carefully considered, taking into account that the progestins may be significantly metabolised in the tissues. The results also suggest that different cell types contain different steroid metabolising enzymes which discriminate between the progestins, despite some of their structures being similar. Although, the identification of the enzymes that metabolise DEX and the progestins in our models was beyond the scope of the present study, it would be an interesting avenue to explore in future studies.

Our results with P_4 showing the rapid and substantial metabolism of this steroid in all the cells lines and in the cervical tissue, are particularly interesting. These results suggest that enzymes that metabolise P_4 are widely expressed in most cell types, including the female genital tract, bones and breast tissue, and that its rapid turnover may be a mechanism required physiologically to fine-control PR responses to

endogenous P_4. Our results are consistent with those of Arici et al. who reported 90% metabolism of P_4 in isolated primary endometrial stromal and gland cells after 24 h [27]. However, it should be noted that only one time point was assessed and that the temporal dynamics of P_4 metabolism may differ between cell lines. Moreover, the metabolism of P_4 appears to be independent of PR expression since a similar extent of metabolism was seen in all three breast cancer cell lines even though T47D and MCF-7 BUS cells are PR-positive whilst MDA-MB-231 cells are PR-negative [28]. Several researchers have reported that 20α-(*S*)-hydroxyprogesterone is a major metabolite of P_4 via the actions of AKR1C1 [28–30]. Whether AKRIC1 or its isozymes are involved in metabolism of P_4 in our model systems remains to be investigated.

Wiebe and Lewis found that breast cancer cell lines express higher levels of SRD5A1 and lower levels of AKR1C enzymes [31]. Therefore, breast cancer cells have a higher conversion of P_4 to 5α-pregnane metabolites as opposed to other systemic cells and tissues. This has major implications, as 5α-pregnanes modify the growth of tumour cells within breast tissue. This highlights the importance of examining the metabolism of steroids, as some metabolites are active and may be a confounding factor in receptor-based studies comparing P_4-activity to other progestins [28,32,33]. HEK293 T (human embryonic kidney cells) and COS-1 cells (monkey kidney cells) had the lowest metabolism of P_4, which may mean that kidneys have lower turnover of P_4.

An important implication of our findings is that the detected differential and rapid metabolism of P_4, progestins and DEX may confound the interpretation of results when investigating mechanisms of action and biological responses via steroid receptors using *in vitro* and pre-clinical models. This would be particularly relevant to the determination of relative binding affinities and potencies (EC_{50} values), which are highly relevant to drug efficacy, specificity and design. We have previously proposed that the determination of progestogen binding affinities and potencies via a specific receptor are dependent on a number of factors, including metabolism of the progestogen [4]. Several researchers have investigated binding affinities of one or more progestogens and/or DEX in COS-1 cells or cytosols prepared from MCF-7 cells [16–18,34,35] or in cytosols prepared from tissue [11,12]. Given that COS-1 cells show high metabolism of P_4 and DEX relative to progestins such as MPA and NET, which show no metabolism in these cells, the reported relative binding affinities may be underestimated for P_4 and DEX [16–18]. If metabolising activity is retained in cytosols, our results suggest that relative binding affinities for NET, but not LNG or ETG, from MCF-7 cytosols [34,35] may also be underestimated. Similarly, potencies (EC_{50}) and/or efficacies (maximal activities) have been reported for transcriptional activity using one or more progestogens and/or DEX in either COS-1, T47D, MCF-7 BUS, END-1, or HEK293 cell lines

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investigated in this study [11,16–18,36–39,40], or in primary cell models [8,9]. The reported potencies and efficacies may also be underestimated, particularly for P_4, compared to some progestins, and to different degrees, depending on the cell model. For example, the relative potency of P_4 via the androgen receptor (AR) and PR in HEK293 T cells may be greater than that reported relative to LNG or NES [39], while the potency of P_4 and DEX may be greater than that reported for other progestins in COS-1 cells for a particular receptor such as the PR, androgen, glucocorticoid and mineralocorticoid receptors [16–18,39]. Potentially further complicating the interpretation, are different metabolites of these steroids produced in different cells that may also confound the results. Clearly, further investigation into the steroid- and cell-specific effects of metabolism of these clinically-relevant compounds, and the biological activities of their metabolites, is urgently required.

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2019.02.010.

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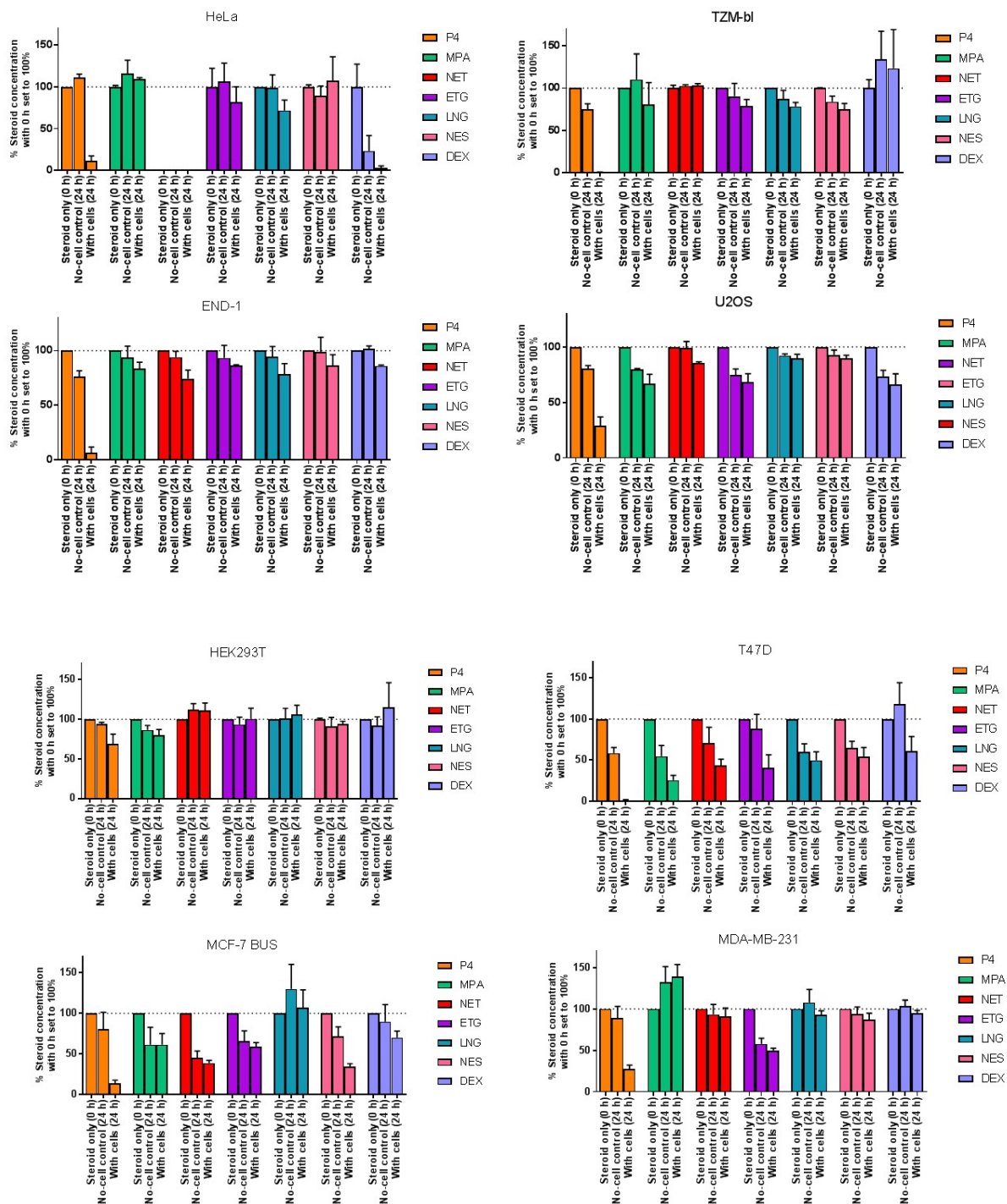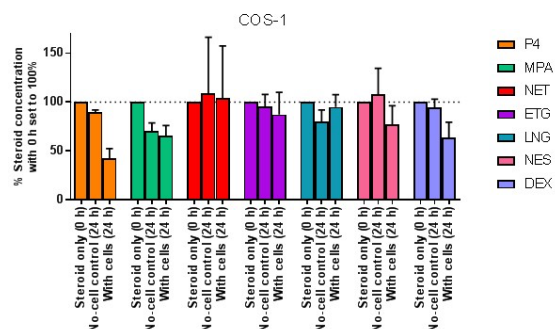
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Supporting Information

Supporting Table 1. Molecular ion species, MRM mass transitions and retention time for each steroid.

Name	Abbreviation	Mass transition: Quantifier ion	Mass transition: Qualifier ion	Retention time
Progesterone	P$_4$	315.2 > 97.1	315.2 > 109.1	0.89 min
Medroxyprogesterone acetate	MPA	387.3 > 123.3	387.3 > 285	1.00 min
Nestorone	NES	371.3 > 253.2	371.3 > 269	1.20 min
Testosterone	T	289.2 > 97.2	289.2 > 109	1.58 min
Norethisterone	NET	299 > 109	299 > 231	1.46 min
Levonorgestrel	LNG	313.1 > 109.4	313.1 > 131.1	1.30 min
Etonogestrel	ETG	325.2 > 109.1	325.2 > 147.1	1.28 min
Dexamethasone	DEX	393.4 > 236.95	393.4 > 147	1.71 min

Supporting Figure 1. Complete data set for all cells lines and steroids investigated. Individual panels show results for each cell-line, with results for each steroid for that cell line, showing the percentage concentration of each steroid at T_0 (the initial amount of steroid added to the media and here set to 100%), the no-cell control result after 24 hours (note that the difference between this bar and T_0 indicates the amount of steroid adsorbed onto the plate) and the percentage metabolism of the steroid after incubation with the cell line at 100 nM for 24 hours, as indicated on the *x-axes*. NET was not investigated in HeLa cells.

Supporting Figure 2. Percentage of 100 nM P$_4$ remaining in supernatant and cell pellets following a 24 hour incubation, with six different cell lines quantified via UHPSFC-MS/MS. The percentage P$_4$ remaining is shown relative to the no-cell control which was set to 100% to account for adsorption loss. These data show the mean ± SEM of a minimum of three independent biological repeats.

P$_4$

	HeLa	TZM-bl	END-1	U2OS	HEK293T	T47D	MCF-7 BUS	MDA-MB-231	COS-1
HeLa		ns	ns	*	*	ns	*	**	****
TZM-bl			ns	*	*	ns	*	*	***
END-1				ns	*	ns	ns	ns	ns
U2OS					*	*	ns	ns	ns
HEK293T						*	*	*	ns
T47D							*	**	****
MCF-7 BUS								ns	**
MDA-MB-231									**
COS-1									

MPA

	HeLa	TZM-bl	END-1	U2OS	HEK293T	T47D	MCF-7 BUS	MDA-MB-231	COS-1
HeLa		ns	ns	ns	ns	**	ns	**	ns
TZM-bl			ns	ns	ns	**	ns	**	ns
END-1				ns	ns	*	ns	ns	ns
U2OS					*	*	ns	**	ns
HEK293T						**	ns	ns	ns
T47D							ns	ns	**
MCF-7 BUS								ns	ns
MDA-MB-231									ns
COS-1									

NES

	HeLa	TZM-bl	END-1	U2OS	HEK293T	T47D	MCF-7 BUS	MDA-MB-231	COS-1
HeLa		ns	ns	ns	ns	ns	ns	ns	ns
TZM-bl			ns	ns	ns	ns	****	ns	ns
END-1				ns	ns	ns	****	ns	ns
U2OS					ns	ns	*	ns	ns
HEK293T						ns	*	ns	ns
T47D							ns	ns	ns
MCF-7 BUS								*	ns
MDA-MB-231									ns
COS-1									

NET

	HeLa	TZM-bl	END-1	U2OS	HEK293T	T47D	MCF-7 BUS	MDA-MB-231	COS-1
HeLa		*	ns	ns	ns	**	ns	**	ns
TZM-bl			ns	***	ns	ns	ns	ns	ns
END-1				ns	ns	ns	ns	ns	ns
U2OS					ns	ns	ns	ns	**
HEK293T						ns	ns	ns	ns
T47D							ns	ns	ns
MCF-7 BUS								ns	ns
MDA-MB-231									ns
COS-1									

LNG

	HeLa	TZM-bl	END-1	U2OS	HEK293T	T47D	MCF-7 BUS	MDA-MB-231	COS-1
HeLa		ns	***	**	**	ns	ns	ns	ns
TZM-bl			ns	ns	ns	ns	ns	ns	ns
END-1				ns	*	ns	ns	ns	*
U2OS					ns	ns	ns	ns	*
HEK293T						ns	ns	ns	ns
T47D							ns	ns	ns
MCF-7 BUS								ns	ns
MDA-MB-231									ns
COS-1									

ETG

	HeLa	TZM-bl	END-1	U2OS	HEK293T	T47D	MCF-7 BUS	MDA-MB-231	COS-1
HeLa		ns	ns	*	ns	ns	ns	ns	ns
TZM-bl			ns	ns	ns	ns	ns	ns	ns
END-1				**	*	ns	ns	*	ns
U2OS					ns	ns	ns	ns	*
HEK293T						ns	ns	ns	ns
T47D							ns	ns	ns
MCF-7 BUS								ns	ns
MDA-MB-231									ns
COS-1									

DEX

	HeLa	TZM-bl	END-1	U2OS	HEK293T	T47D	MCF-7 BUS	MDA-MB-231	COS-1
HeLa		ns	ns	ns	ns	ns	ns	ns	**
TZM-bl			ns	ns	ns	ns	ns	ns	*
END-1				ns	ns	ns	ns	ns	**
U2OS					ns	ns	ns	ns	*
HEK293T						ns	ns	ns	ns
T47D							ns	ns	ns
MCF-7 BUS								ns	ns
MDA-MB-231									ns
COS-1									

Supporting Figure 3. Differential metabolism of seven clinically-relevant steroids. Quantification was by UHPSFC-MS/MS, following incubation at 100 nM for 24 hours with nine different cell lines. The percentage metabolism is shown relative to a corresponding no-cell control which was set to 100% to account for adsorption loss. These data represent the mean ± SEM of a minimum of three biological repeats. To determine whether the metabolism

of a ligand was statistically significant across two cell lines, multiple paired *t*-tests were performed across all cell lines for that ligand. Significant differences are indicated by asterisks where * and ** represent $p < 0.05$ and $p < 0.01$, respectively.

A2: Characterisation of progestins used in hormonal contraception and progesterone via the progesterone receptor

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The candidate is shared first author on this publication, contributing the planning, experimental work and analysed data for the MDA-MB-231 and COS-1 cells. The candidate was responsible for writing the first draft of the introduction and discussion, as well as compiling the tables in the manuscript. The candidate also contributed to the critical evaluation and editing of the manuscript.

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Characterisation of progestins used in hormonal contraception and progesterone via the progesterone receptor

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ABSTRACT

Different progestogens are widely used in hormonal therapy and mediate their therapeutic actions via the progesterone receptor (PR). Little published data exist on their relative efficacies and potencies via the PR, while those available may be confounded by off-target receptors, different methodologies and model systems. We performed dose-response analysis to investigate the efficacies and potencies for transcription of progesterone and several progestins widely used in contraception via the B isoform of human PR (PR-B). We compared responses using three different cell lines and two different transient transfection conditions. Results show that *in vitro* biological responses via PR-B for the select progestogens can vary significantly in biocharacter, rank order and absolute values for efficacies and potencies, depending on the cell line and transfection condition. Progestogen rank orders for published relative binding affinities are mostly different to those for relative efficacies and potencies. These *in vitro* differences suggest that rank orders and absolute values of the efficacies and potencies of the progestogens are likely to vary *in vivo* in a cell-specific and progestogen-specific manner, and cannot easily be extrapolated from *in vitro* data, as is usually the practice. While obtaining such data *in vivo* is not possible, these *in vitro* data show proof of concept for likely significant cell- and progestogen-specific PR-B effects.

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1. Introduction

Synthetic progestogens (progestins) are used for hormonal therapy to mimic the actions of progesterone (P_4), by binding to and activating the progesterone receptor (PR), with the B isoform of the PR (PR-B) being the predominant and most transcriptionally active isoform [1]. However, some progestins are associated with side-effects such as increased risk of breast cancer, cardiovascular disease and HIV-1 acquisition [2].

Development of therapeutic progestins requires determination of affinities, efficacies (maximal response a progestin can elicit) and potencies (EC_{50}; the concentration that can elicit half the maximal response) for transcriptional regulation in cell line models expressing the PR, or potential off-target steroid receptors (SRs). This provides important information predictive of clinical relevance and side-effects [1,3]. The PR regulates transcription of specific target genes via multiple mechanisms including direct binding to progesterone response elements (PREs) in the promoter region of these genes or tethering to various DNA-bound transcription factors [4]. To determine potencies and efficacies for transcription via a particular receptor, dose-response analysis is usually performed using promoter-reporter constructs in cell lines overexpressing that receptor, where the promoter contains a SR binding site such as a PRE, ideally in a cell line deficient in competing receptors [5,6]. While the physiological relevance of such models could be

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2.2. Reporter assays

Promoter-reporter assays were performed essentially as previously described [9], with a few modifications. Briefly, COS-1 or MDA-MB-231 cells were seeded at a density of 2×10^6 cells, while U2OS cells were seeded at 1.5×10^6 cells into 10 cm dishes. Cells were transiently transfected, using XtremeGene HP (Roche Molecular Biochemicals), as follows: Transfection condition #1: 900 ng of the pSG5 empty vector or pSG5-hPR-B and 9000 ng of the pTAT-2xPRE-E1b-luciferase construct; Transfection condition #2: 3500 ng pSG5 or pSG5-hPR-B and 1410 ng pTAT-2xPRE-E1b-luciferase. The following day, the transfected cells were reseeded into 96-well plates at a density of 1×10^4 cells per well and subsequently treated with vehicle (0.1% EtOH) or increasing concentrations of the test compounds for 24 h in either serum-free medium (COS-1 and U2OS), or medium containing charcoal-stripped fetal calf serum (FCS) (MDA-MB-231). Luciferase activity was measured and normalized as previously described [9].

2.3. Immunoblotting

Protein samples were prepared as previously described [26] and 20 μg separated on a 10% SDS-polyacrylamide gel before transfer to nitrocellulose membranes (Amersham) and blocking in 10% fat-free milk powder. Membranes were probed with anti-PR (PGR-312-L-CE, Leica Biosystems, UK) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 0411, Santa Cruz Biotechnology, USA) (loading control) followed with the HRP-conjugated secondary goat anti-mouse antibody (Santa Cruz Biotechnology, USA). Proteins were visualised using enhanced chemiluminescence (Bio-Rad Laboratories, Inc. USA) and a MyECL Imager (Pierce Thermo Scientific Inc. USA), and expression levels quantified using ImageJ (Version 1.49).
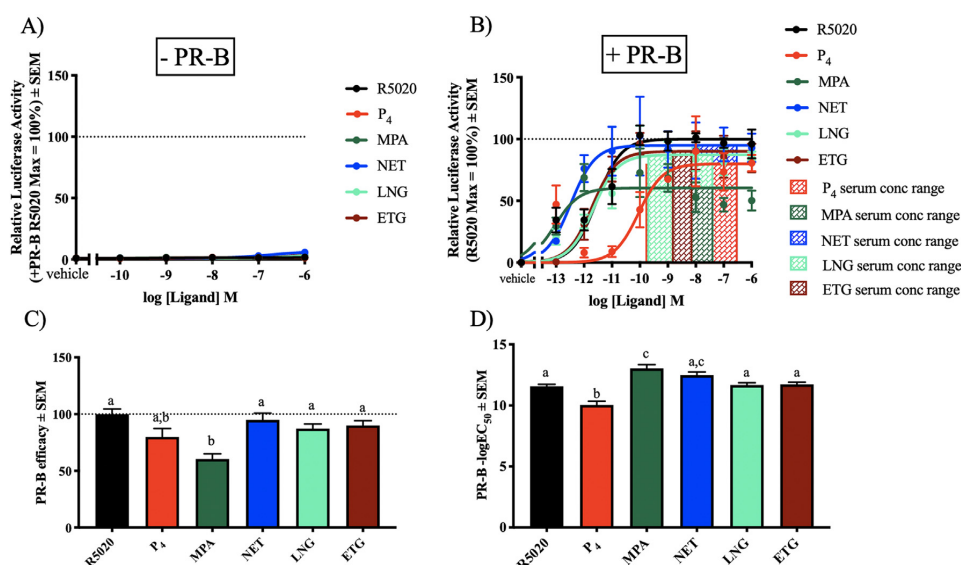
2.4. Data and statistical analysis

Graph Pad Prism® software version 7 was used for data analysis. Non-linear regression and sigmoidal dose-response were used with the slope set to one. One- or two-way ANOVA (analysis of variance) and the Bonferroni (compares all pairs of columns) post-test were used for statistical analysis when multiple ligands were tested in parallel, while unpaired *t*-tests were used when ligands were not tested in parallel. The error bars represent the standard error of the mean (SEM) of at least three independent experiments, each performed in triplicate.

3. Results

3.1. Some progestogens have different efficacies and potencies via PR-B

To compare the efficacies and potencies of select progestogens, promoter-reporter and dose-response analyses were performed in COS-1 cells exogenously expressing human PR-B (Fig. 1). This cell line was selected due to negligible endogenous expression of SRs [3]. Relative to the R5020 response in PR-B transfected cells, negligible transactivation by all the progestogens was observed in the absence of transfected PR-B (Fig. 1A). In PR-B-transfected cells, all progestogens except MPA were full agonists for transactivation (Fig. 1B and C). MPA was significantly less efficacious than all progestogens investigated except P_4 (Fig. 1C), suggesting it is a partial agonist relative to R5020, NET, ETG and LNG. Interestingly, although MPA displayed a similar potency to NET, it was significantly more potent than all the other progestogens investigated, while P_4 was the least potent ligand (Fig. 1D).

Fig. 1. Some progestogens display different efficacies and potencies via PR-B. COS-1 cells transiently transfected with (A) 900 ng pSG5-empty vector or (B−D) pSG5-hPR-B expression vector and 9000 ng pTAT-2xPRE-E1b luciferase reporter (transfection condition #1), were treated with 0.1% EtOH (vehicle) or increasing concentrations of each ligand for 24 h. Luciferase activity was measured and normalized to protein concentration. (A−B) Relative luciferase activity is shown with PR-B R5020 (maximal response) set as 100% and all other response relative to this. The shaded bars in B indicate the reported serum concentrations of progestogens in women. (C) Efficacy and (D) -logEC$_{50}$ values ± SEM of the ligands via PR-B were plotted and analysed using one-way ANOVA with a Bonferroni post-test. Different letters denote statistically significant differences while the same letters do not.
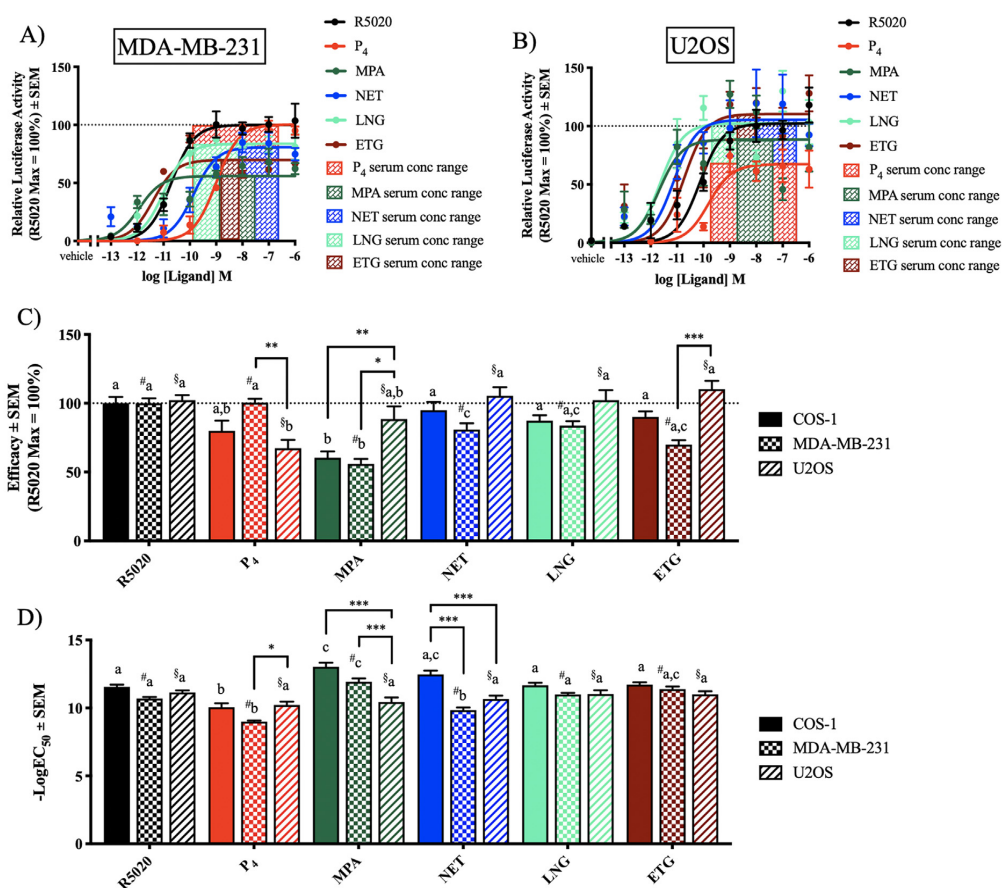
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3.2. Relative and absolute efficacies and potencies are cell-specific

To investigate whether the efficacies and potencies of the progestogens via human PR-B are influenced by the model system, we also performed experiments in the MDA-MB-231 breast cancer (Fig. 2A) and U2OS bone osteosarcoma (Fig. 2B) cell lines. These cell lines do not express endogenous PR, while low endogenous GR or AR levels are sometimes detectable [28,29]. However, we observed negligible reporter transactivation in the absence of exogenous PR-B (Supplementary Fig. 1). When the efficacies of the progestogens via PR-B were compared between the cell lines, the only statistically significant differences were seen with P_4, MPA and ETG (Fig. 2C). Both ETG and MPA were significantly more efficacious in U2OS cells compared to MDA-MB-231 cells, with MPA also being more efficacious compared to the COS-1 cells, while P_4 was significantly less efficacious in U2OS cells compared to MDA-MB-231 cells (Fig. 2C). The efficacy and potency rank orders were not conserved between cell lines (Supplementary Fig. 2). A greater number of statistically significant differences was observed for potencies compared to efficacies across the three cell lines (Fig. 2D).

All the progestogens investigated via PR-B in the three cell lines had potency and efficacy values falling within the serum concentration range found in contraceptive users (Figs. 1B, 2A-B, Supplementary Table 2). Although all cell lines were transfected with 900 ng PR-B (transfection condition #1), the MDA-MB-231 cells expressed much less PR-B compared to both COS-1 and U2OS cells, while U2OS cells expressed the most PR-B (Supplementary Fig. 3G).

3.3. Relative and absolute efficacies and potencies are mostly not significantly affected by transfection conditions

Next, we investigated whether the efficacies and potencies of the progestogens via PR-B are influenced by the transfection conditions. When comparing the first (Fig. 1B—D) to the second (Fig. 3A—C) transfection condition, very few significant differences were detected. Only NET displayed a significantly lower efficacy via condition 2 (Fig. 3B), while only MPA was more potent via condition 1 (Fig. 3C). The progestogen responses via PR-B using condition 2 were also investigated in MDA-MB-231 and U2OS cells (Supplementary Figs. 3A—B). No significant differences in efficacy were observed in

Fig. 2. Progestogen efficacies and potencies via PR-B are cell line-specific. (A) MDA-MB-231 and (B) U2OS cells were transfected and treated as for Fig. 1B—D, while results were analysed as in Fig. 1A and B. (C) The bar graph shows the efficacies ± SEM for the COS-1 (Fig. 1), MDA-MB-231 and U2OS cell-lines, while (D) shows the -logEC50 values ± SEM. Relative efficacies and -logEC$_{50}$ were analysed using unpaired t-tests with *, **, *** denoting $p < 0.05$, 0.01 and 0.001, respectively. One-way ANOVA with a Bonferroni post-test was performed to determine statistical differences within each cell line (COS-1, no symbol, MDA-MB-231 # and U2OS §) where different letters denote statistically significant differences while the same letters do not.

Fig. 3. Progestogen efficacies and potencies via PR-B are minimally influenced by transfection conditions. COS-1 cells, transiently transfected as for Fig. 1 (transfection condition #1) or 3500 ng pSG5-hPR-B expression vector and 1410 ng pTAT-2xPRE-E1b-luciferase (transfection condition #2), were treated with the 0.1% EtOH (vehicle) or increasing concentrations of each ligand for 24 h. Luciferase activity was measured and normalized to the protein concentration. (A) Relative luciferase activity is shown with R5020 set as 100% and all other responses relative to this. (B) Efficacy and (C) -logEC$_{50}$ values ± SEM of the ligands via PR-B using transfection condition #1 vs #2 were plotted. (D) Total protein was harvested and a representative Western blot of the PR-B expression levels between the two different transfection conditions is shown. Two-way ANOVA with a Bonferroni post-test (B and C) and unpaired t-tests (D) were performed to determine statistical differences * denoting p < 0.05.

MDA-MB-231 and U2OS cells (Supplementary Figs. 3C–D), while similar to COS-1 cells, MPA was more potent via condition 1 in the MDA-MB-231 cells, however more potent via condition 2 in the U2OS cells (Supplementary Figs. 3E–F). NET was more potent via condition 2 in the MDA-MB-231 cells (Supplementary Fig. 3E). Interestingly, about 4-fold more PR-B was expressed under condition 2 compared to condition 1 in COS-1 cells (Fig. 3D). A similar increase in PR-B expression under condition 2 was observed for MDA-MB-231 cells, while the increase for U2OS cells was negligible (Supplementary Fig. 3G).

4. Discussion

Our study is the first to determine efficacies and potencies of these progestogens in parallel within the same model system. We show that all progestogens, except MPA, are full agonists for transactivation via human PR-B in the COS-1 cell line. While MPA displays similar potency to NET, it is significantly more potent than R5020, LNG, ETG and P$_4$. Only two studies have previously investigated the potencies of P$_4$, NET, LNG and ETG in parallel [13,14], one of which included MPA [14], via the human PR. Our results are not directly comparable since Bain and co-workers performed experiments in U2OS cells stably expressing multiple copies of the PRE and did not specify the PR isoform or include R5020 and MPA [13], while the study by Bray and co-workers included MPA and was conducted in T47D cells expressing both PR isoforms [14]. The absolute EC$_{50}$ values for PR-B (Supplementary Table 1) are lower than those reported in both studies [13,14]. However, in agreement with our study, P$_4$ is the least potent progestogen and the rank order is very similar to that determined for PR-B in the U2OS cells. Other studies obtained similar potencies with overexpressed PR-B for R5020 in HeLa cells [16] and P$_4$ and LNG in HEK293 cells [6], to

those we obtained for those progestins in COS-1 cells, while other studies have reported greater potencies for P$_4$ [5,12]. In terms of MPA and NET, much lower potencies were obtained for human PR-B in some other studies [5,6] compared to our study. Clearly the absolute and relative values obtained for progestogen potencies in different studies are highly dependent on the model system used. Thus, while data are consistent across studies, differences in absolute and relative progestogen potencies between studies could be due to multiple factors, including differences in cell type, promoter-reporter constructs, expression levels of the PR, method of dose-response analysis or the PR isoform(s) investigated.

Given these apparent discrepancies, we investigated whether efficacies and potencies of the progestogens for human PR-B are sensitive to the model system used. We found that MPA acts as a full agonist only in the U2OS cells, but a partial agonist in the other 2 cell lines, while most other progestogens are full agonists in all 3 cell lines. We show that relative potencies of the progestogens are more sensitive than efficacies to the cell line model system used. A possible explanation could be differential metabolism [22], either to decrease the effective concentration of select progestogens, and/or production of a metabolite that is active via the PR. Different cell lines could express different types and/or expression levels of co-regulators which may play a role in PR-B-mediated transcriptional regulation, as different ligands may cause differential recruitment of coregulators [30], and are sensitive to which progestogen is bound to the PR. Indeed, it has been shown that the ratio of coactivators and corepressors could modulate the inhibitory or stimulatory effects of the PR antagonist, RU486 [31]. All the cell line models used contain the downstream factors necessary to support PR-mediated transcription as evident from the potent responses. It is possible that some *in vivo* cofactors in cells that express endogenous PR are not present in some or all of our cell line

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models which may result in different relative potencies and efficacies *in vivo*. When investigating the effects of changing both PR-B levels and DNA reporter template levels in all three cell lines, our results show that both efficacies and potencies of most progestogens are not significantly affected by the change in transient transfection conditions. However, the biocharacter of NET changes from being a full to a partial agonist, suggesting that NET in complex with PR-B is particularly sensitive to changing the concentrations and/or ratio of PR-B to DNA template.

In summary, our results show that biological responses via the PR for different progestogens *in vitro* can vary in rank order, biocharacter, and absolute values for efficacies and potencies, depending mainly on the cell line and to only a limited extent on transient transfection conditions. One of the key findings of our work is that it is difficult to establish statistically significant differences by dose-response analysis for efficacy and potency even *in vitro*, when multiple ligands are investigated in parallel. Thus caution should be used when drawing conclusions about differences between ligands without any statistical analysis of significance of difference. We established that significant differences are detected in our assays between the efficacies and potencies of several progestogens. Moreover, we show that the rank order for progestogen efficacies and potencies sometimes but not always correlate with rank order for relative binding affinities for PR-B (Supplementary Table 2 and Supplementary Fig. 4), confirming that affinity is not proportional to biological activity [8]. While steroid efficacy and potency are affected by affinity for the receptor, they are also affected by precise conformation induced by the steroid, as well as cell-specific cofactors and SR expression levels [32,33]. Our results suggest several of these factors may play a role in the progestogen-specific and cell-specific efficacies and potencies of the progestins via the PR-B.

The physiological significance of our results is to suggest that rank order and absolute values for efficacy and potency, and even biocharacter, of progestogens are likely to vary in different cells and tissues *in vivo* and cannot easily be predicted from *in vitro* dose-response assays or receptor binding affinities. Nevertheless, the *in vitro* results show valuable proof of concept effects and present viable strategies to further directly investigate mechanisms of such effects. Our findings showing that the EC$_{50}$ values of the progestogens are well below the reported serum levels found in women using contraceptives containing these progestogens (Supplementary Table 2). Although this suggests that these progestogens are likely to elicit similar effects *in vivo*, we show that absolute potency values change depending on assay conditions, suggesting that these may vary *in vivo* in a cell-specific manner. Further studies to understand mechanisms of progestogens *in vitro* on endogenous genes via PR-B, as well as clinical studies investigating specific biological responses to progestogens, would allow a more comprehensive understanding of the benefit/side-effect profiles of these clinically-significant steroids, and facilitate choice and dose of progestin for use in hormonal therapy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.09.058.

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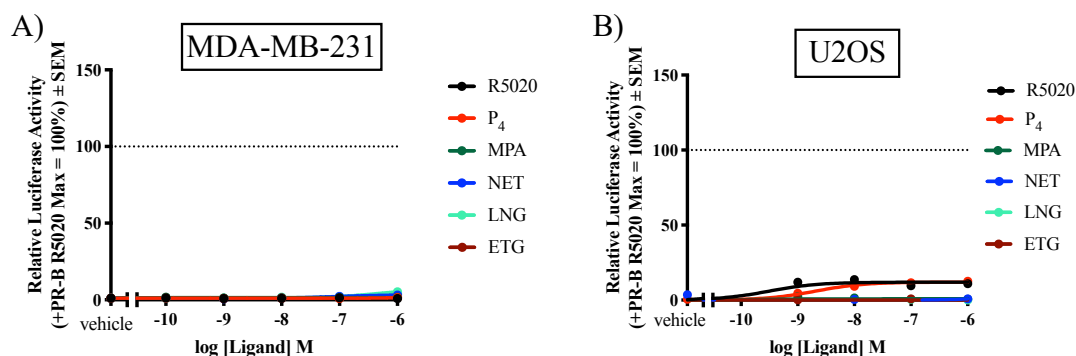
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Supplementary data

Supplementary Table 2: EC$_{50}$ values determined for different progestogens via PR-B compared to published relative binding affinities (RBAs) and serum progestogen concentrations.
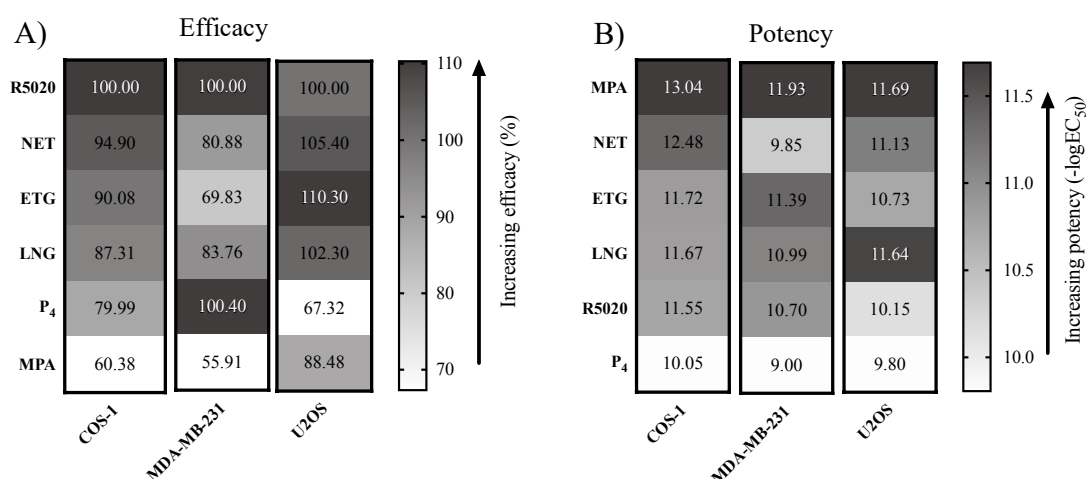
Progestogen	EC$_{50}$ (pM ± SEM)			Relative EC$_{50}$ (% ± SEM)			RBA (%)[a*]	Serum concentrations (pM)[a]
	COS-1	MDA-MB-231	U2OS	COS-1	MDA-MB-231	U2OS		
R5020	2.84 ± 1.10	18.03 ± 6.19	71.5 ± 32.61	100 ± 38.94	100 ± 34.33	100 ± 45.58	100	-
P$_4$	202.0 ± 115.74	791.0 ± 264.82	157.0 ± 110.06	7121.69 ± 57.33	4387.13 ± 33.48	219.45 ± 70.48	50	650-600 000
MPA	0.33 ± 0.29	0.71 ± 0.57	2.03 ± 1.67	12.16 ± 84.88	79.95 ± 3.94	2.84 ± 82.56	33-49	3 300-21 000
NET	0.38 ± 0.21	339.7 ± 180.41	7.47 ± 4.87	13.60 ± 55.25	1884.08 ± 53.11	10.44 ± 65.23	14-17	10 000-50 000
LNG	2.66 ± 1.02	8.22 ± 3.82	18.4 ± 10.23	93.69 ± 38.39	45.61 ± 46.50	25.78 ± 52.53	12-48	300-28 000
ETG	4.40 ± 1.72	7.68 ± 2.58	2.29 ± 1.29	155.13 ± 39.03	42.61 ± 33.64	3.21 ± 56.47	75	2 500-7 500

[a][1,2]; [*]RBAs for the human PR (isoforms not specified).

EC$_{50}$ concentrations (pM) and relative EC$_{50}$ values (%) were calculated using the EC$_{50}$ values obtained from three independent experiments for COS-1 (Fig. 1), MDA-MB-231 and U2OS cells (Fig.2). Relative EC$_{50}$ values and published RBAs were expressed as % relative to R5020 set at 100%. Progestin serum concentrations are given in ranges as different values were obtained from the literature depending on the route of administration, while P$_4$ concentration ranges are those for premenopausal women not on contraception.
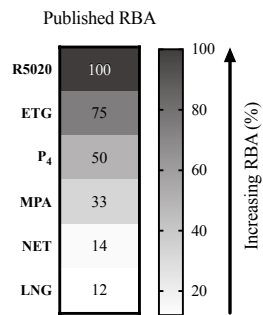
Supplementary Figure 1. Negligible basal activity of progestogens in the absence of PR-B in MDA-MB-231 and U2OS cells. Cells transiently transfected with 900 ng of the pSG5 empty expression vector and 9000 ng of the pTAT-2xPRE-E1b-luc reporter plasmid were treated with vehicle (0.1% EtOH) or increasing concentrations of each ligand for 24 hours. Cells were harvested and the Luciferase and Bradford assays were performed. Results are shown as relative Luciferase activity for which the maximal response with R5020 via PR-B was set to 100% and all other responses were calculated relative to this. The means and SEM values were calculated based on three biological repeats each comprising three technical repeats of each condition.

Supplementary Figure 2. Rank order for progestogen efficacies and potencies are not conserved between cell lines. Heatmap displaying the (A) efficacies and (B) potencies via PR-B in COS-1 (Fig. 1C-D), MDA-MB-231 and U2OS cells (Figure 2C-D). The colour gradient indicates rank order for the mean values, without taking into account statistical analysis or p-values, where the darker the colour, the greater the detected mean response.

Supplementary Figure 3. Relative efficacies and potencies are cell-specific and are generally not significantly affected by transfection condition. Cells transiently transfected with either 900 ng of the pSG5-PR-B as well as 9000 ng of the pTAT-2xPRE-E1b-luc reporter plasmid (transfection condition #1) or with 3500 ng of the pSG5-PR-B vector as well as 1410 ng of the pTAT-2xPRE-E1b-luc reporter plasmid (transfection condition #2) were treated with vehicle (0.1% EtOH) or increasing concentrations of each ligand for 24 hours. Cells were harvested and the Luciferase and Bradford assays were performed. A-B) Relative luciferase activity is shown for PR-B with the R5020 maximal response set as 100% and all other responses expressed relative to this. The shaded bars in B indicate the reported serum concentrations of progestogens in women. A comparison between the efficacies (C-D) and potencies (E-F) of each progestogen is shown. (G) Shows PR-B protein levels for each transfection condition in the three cell lines. Relative efficacies, potencies and PR-B expression levels were analysed using two-way ANOVA with a Bonferroni post-test where ns indicates no significant difference, while *, ** and *** and denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively. The means and ± SEM values were calculated based on three biological repeats with each condition conducted in triplicate.

Published RBA

Supplementary Figure 4. Rank order for progestogen relative binding affinities (RBAs) for PR-B: Heatmap displaying the published RBAs taken from Table 2. For values where a range was given, the lowest value is shown. The colour gradient indicates rank order for the RBAs where the darker the colour, the greater the value.

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A3: Progestins used in endocrine therapy and the implication for the biosynthesis and metabolism of endogenous steroid hormones

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For this publication, the candidate was responsible for research and creation of Tables 1 and 2, writing the first draft of the section on glucocorticoids, and the critical proofread of the manuscript.

Review

Progestins used in endocrine therapy and the implications for the biosynthesis and metabolism of endogenous steroid hormones

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A B S T R A C T

Steroidogenesis refers to the *de novo* synthesis of steroid hormones from cholesterol by a number of sequential enzyme catalysed reactions in the adrenal and the gonads. In addition, circulating steroid hormone precursors are further metabolised in selected peripheral tissues. It has been suggested that the biosynthesis of endogenous steroid hormones can be modulated by progestins, used widely by women in female reproductive medicine. However, as a number of structurally diverse progestins with different pharmacological properties are available, it is possible that these synthetic compounds may vary in their effects on steroidogenesis. This review summarises the evidence indicating that progestins influence the biosynthesis of steroid hormones in the adrenal and gonads, as well as the metabolism of these endogenous hormones in the breast, highlighting the limitations to the current knowledge and directions for future research.

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1. Introduction

Progestins are a class of synthetic compounds that were developed to mimic the biological action of the endogenous sex steroid hormone progesterone (Prog), which plays a pivotal role in female reproduction (Geller et al., 1999; Graham and Clarke, 1997). These progestins have many therapeutic applications in female reproductive medicine, and are used instead of Prog as they have better bioavailabilities and half-lives (Hapgood et al., 2004; Speroff, 1996; Stanczyk et al., 2013). Therapeutic applications include contraception, hormone replacement therapy (HRT), cancer therapy as well as the treatment of gynaecological disorders such as endometriosis (Africander et al., 2011; Schindler, 2014; Stanczyk et al., 2013). In addition to these beneficial effects, a number of side-effects have been reported with the clinical use of progestins. However, since progestins differ in their chemical structures and their biological activities, it is likely that not all progestins will display beneficial effects and side-effects to the same extent. It is thus crucial to improve our understanding of the risk/benefit profile of progestins used in hormone therapy. Progestins were

designed to elicit their intracellular actions via the progesterone receptor (PR), but many progestins also bind to other members of the steroid receptor family such as the glucocorticoid, androgen and mineralocorticoid receptors (MR). Although these actions of progestins via steroid receptors are suggested to be the main mechanism of the differential intracellular actions, other factors such as metabolism, pharmacokinetics, bioavailability and effects on steroidogenesis cannot be excluded. In contrast to the numerous studies on progestin actions via steroid receptors, not much research has been devoted to the effects of progestins on endogenous steroid hormone biosynthesis. While it is known that the first-generation progestin medroxyprogesterone acetate (MPA) (Van Veelen et al., 1984) suppresses steroidogenesis by inhibiting the hypothalamic-pituitary-adrenal (HPA)-axis, studies investigating the direct inhibition of specific steps in the steroidogenic pathway by MPA, and other progestins, are limited. Furthermore, since it has been suggested that progestins do not always act in a similar manner, an important question is whether, and to what extent, progestins from different generations modulate these steps. A number of reviews have described the molecular mechanisms of action of progestins via steroid receptors (Africander et al., 2011; Kuhl, 1990; Schindler et al., 2003; Stanczyk et al., 2013) and will thus not be replicated in this review. Instead, we will describe the different generations of progestins and the enzymes involved in

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Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase
5α-dione	5α-androstanedione
11βHSD	11β-hydroxysteroid dehydrogenase
17βHSD	17β-hydroxysteroid dehydrogenase
11OH-A4	11β-hydroxyandrostenedione
16OH-Prog	16α-hydroxyprogesterone
17OH-Preg	17α-hydroxypregnenolone
17OH-Prog	17α-hydroxyprogesterone
A4	androstenedione
Ald	aldosterone
CBG	corticosteroid binding globulin
CMA	chlormadinone acetate
COC	combined oral contraceptive
CORT	corticosterone
CPA	cyproterone acetate
CYB5A	cytochrome *b*5
CYP11A1	cytochrome P450 side-chain cleavage
CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	cytochrome P450 aldosterone synthase
CYP17A1	cytochrome P450 17α-hydroxylase/17,20-lyase
CYP19A1	cytochrome P450 aromatase
CYP21A2	cytochrome P450 21-hydroxylase
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DNG	dienogest
DOC	11-deoxycorticosterone
DRSP	drospirenone
DSG	desogestrel
E1	estrone
E1-S	estrone sulfate
E2	estradiol
E2-S	estradiol sulfate
EE	ethinyl estradiol
FSH	follicle-stimulating hormone
GES	gestodene
HRT	hormone replacement therapy
HPA	hypothalamic-pituitary-adrenal
LH	luteinizing hormone
LNG	levonorgestrel
MA	megestrol acetate
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
MWS	Million Women Study
NES	nestorone
NET	norethisterone
NET-A	norethisterone acetate
NET-EN	norethisterone enanthate
NoMAC	nomegestrol acetate
PCOS	polycystic ovary syndrome
PR	progesterone receptor
Preg	pregnenolone
Prog	progesterone
SRD5A	steroid 5α-reductase
StAR	steroidogenic acute regulatory protein
STS	sulfatase
SULT1E1	sulfotransferase family 1E member 1
SULT2A1	sulfotransferase family 2A member 1
TMG	trimegestone
WHI	Women's Health Initiative
WISDOM	Women's International Study of Long Duration Oestrogen after Menopause

steroidogenesis, while highlighting the effects of progestins on steroid biosynthesis likely via the regulation of expression and/or enzyme inhibition.

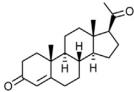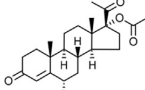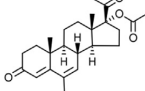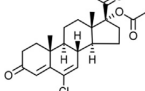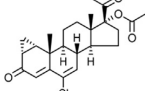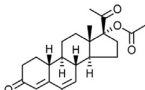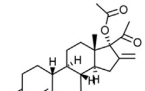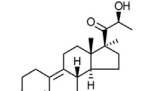## 2. Progesterone and the classification of progestins

Prog and progestins are generally referred to as progestogens; compounds known to exhibit progestational activity. Prog is a natural progestogen, while progestins are synthetic progestogens developed to mimic the activity of Prog. The *de novo* synthesis of Prog occurs in various steroidogenic tissues, including the ovaries, adrenal gland and central nervous system (Capper et al., 2016; Hu et al., 2010; Miller and Auchus, 2011; Payne and Hales, 2004; Schumacher et al., 2012), with the ovary being the major site of biosynthesis in females (Graham and Clarke, 1997; Norman and Litwack, 1987). The physiological roles of Prog are dependent on the particular target tissue where it exerts its physiological effects by binding to the PR (Li et al., 2004; Scarpin et al., 2009). In the uterus and ovary, for example, Prog is crucial for the development and release of oocytes, support of implantation of a fertilised ovum and the maintenance of pregnancy (Gellersen et al., 2009; Graham and Clarke, 1997). In the mammary gland, Prog is required for the development of lobular-alveolar and the inhibition of milk protein synthesis during pregnancy (Graham and Clarke, 1997; Savouret et al., 1988), while in the brain Prog regulates signals required for sexual responsiveness and elicits neuroprotective effects (Brinton et al., 2008).

The list of available progestins has grown substantially since their first appearance more than five decades ago (Greenblatt, 1958; Inhoffen and Hohlweg, 1938; Kuhl, 2011; Mansour, 2005), and they are currently classified into four consecutive generations (Schindler, 2014; Sitruk-Ware, 2006). The newer, fourth-generation progestins have been developed to be closer in activity to Prog than progestins from the first three generations. It should be noted that although a large number of progestins have been developed, many are no longer commercially available. Tables 1 and 2 list examples of progestins commonly used. Most progestins are structurally related to either Prog (Table 1) or testosterone (Table 2) (Schindler et al., 2003; Sitruk-Ware, 2004; Stanczyk et al., 2013), with only one, the fourth-generation progestin drospirenone (DRSP), being derived from the MR antagonist, spironolactone (Table 2) (Fuhrmann et al., 1996; Krattenmacher, 2000). Those progestins structurally related to Prog can be subdivided into compounds with and without a methyl group at carbon 10. Progestins containing the methyl group are referred to as 17α-hydroxyprogesterone (17OH-Prog) derivatives, while 19-norprogesterone derivatives lack the methyl group (Sitruk-Ware, 2004; Stanczyk et al., 2013). The first-generation progestins MPA, megestrol acetate (MA), chlormadinone acetate (CMA) and cyproterone acetate (CPA) are examples of 17OH-Prog derivatives, while the fourth-generation progestins nestorone (NES), nomegestrol acetate (NoMAC) and trimegestone (TMG) are examples of 19-norprogesterone derivatives. Progestins structurally related to testosterone, the 19-nortestosterone derivatives, include the first-generation progestin norethisterone (NET), second-generation progestin levonorgestrel (LNG), third-generation progestins desogestrel (DSG), norgestimate and

Table 1
Progestins structurally related to progesterone and examples of their therapeutic applications.

Structural derivation	Progestogen (Abbreviation)	Description/Generation	Structure	Therapy
Progesterone and structurally related progestins				
—	Progesterone (Prog)	Natural		Micronised: HRT; prevention of preterm labour
17α-Hydroxyprogesterone	Medroxyprogesterone acetate (MPA)	1st		HRT; PO injectable contraceptive; cancer therapy
	Megestrol acetate (MA)	1st		cancer therapy
	Chlormadinone acetate (CMA)	1st		COC
	Cyproterone acetate (CPA)	1st		COC; HRT; cancer therapy
19-Norprogesterone	Nomegestrol acetate (NoMAC)	4th		COC; PO contraceptive implant; HRT
	Nestorone (NES)	4th		PO injectable contraceptive; HRT
	Trimegestone (TMG)	4th		HRT; COC

COC: combined oral contraceptive; POP: progestin-only pill; PO: progestin only; HRT: hormone replacement therapy.

gestodene (GES), and the fourth-generation progestin dienogest (DNG) (Sitruk-Ware, 2006; Stanczyk et al., 2013). Norethisterone acetate (NET-A) and norethisterone enanthate (NET-EN) are derivatives of NET, while DSG and norgestimate are prodrugs that are converted to active progestogenic compounds. DSG is converted to etonogestrel (3-ketodesogestrel), while norgestimate is metabolised to LNG and norelgestromin (Stanczyk et al., 2013).
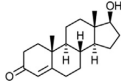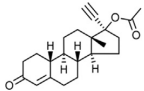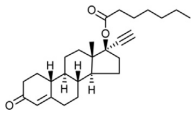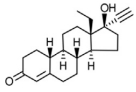
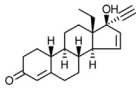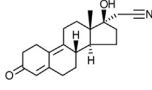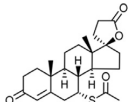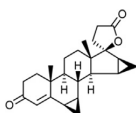Although Tables 1 and 2 indicate that some progestins are used as treatment for cancer, the two most common uses of progestins include contraception (either progestin alone or combined with an estrogen) and HRT (combined with an estrogen). The latter is administered to women with an intact uterus so as to counteract the proliferative effects of estrogen on the uterine epithelium, thereby preventing estrogen-induced endometrial hyperplasia (Campagnoli et al., 2005; Gambrell et al., 1980; Greendale et al., 1999). However, studies such as the Women's Health Initiative (WHI) and the Million Women Study (MWS) indicate a relationship between increased breast cancer risk in postmenopausal women and progestin treatment as part of HRT (Anderson et al., 2004; Beral et al., 2003; Rossouw et al., 2002). The progestin investigated in the WHI study was MPA, while MPA, NET and LNG were investigated in the MWS. The fact that MPA has therapeutic effects in the treatment of metastatic cancer, but has been shown to increase breast cancer risk, indicates that the role of progestins in breast cancer development is not straightforward (Stanczyk and Bhavnani, 2014). Interestingly, the contraceptive use of MPA has also been associated with increased risk of invasive breast cancer (Li et al., 2012). Furthermore, results from both the WHI study and the Women's International Study of Long Duration Oestrogen after Menopause (WISDOM) investigation (Rossouw et al., 2002; Vickers et al., 2007) indicated increased cardiovascular and thromboembolic risk when HRT (estrogen plus MPA) was started a considerable time after menopause. Despite the effectiveness of progestins in preventing pregnancy, there are also several minor side-effects associated with

Table 2
Clinical applications of drospirenone and progestins structurally related to testosterone.

Structural derivation	Progestogen (Abbreviation)	Description/Generation	Structure	Therapy
Testosterone and structurally related progestins				
_	Testosterone	Natural		HRT
	Norethindrone acetate (NET-A)[a]	1st		HRT; POP; COC
	Norethindrone enanthate (NET-EN)[a]	1st		PO injectable contraceptive
	Levonorgestrel (LNG)	2nd		HRT; COC; POP; IUD; cancer therapy
	Desogestrel (DSG)[b]	3rd		POP; COC
	Norgestimate[b]	3rd		COC
	Gestodene (GES)	3rd		COC
	Dienogest (DNG)	4th		COC
Spironolactone and structurally related progestins				
	Spironolactone	MR antagonist		Primary aldosteronism
	Drospirenone (DRSP)	4th		HRT; COC

COC: combined oral contraceptive; POP: progestin-only pill; PO: progestin only; HRT: hormone replacement therapy; IUD: intrauterine device.
[a] Metabolically converted to NET in order to become biologically active.
[b] Inactive prodrugs are metabolised to biologically active forms.

their contraceptive use. The side-effect profile includes amenor-rhea, breast tenderness, headaches, weight gain, acne, fatigue, bloating of the abdomen, behavioural changes, reduced libido, decreased bone mineral density and vaginal discharge (Africander et al., 2011). It should be noted that these side-effects have mostly been reported for MPA, and that the side-effect profile of most other progestins is not well-defined.

Progestins generally mediate their contraceptive action by inhibiting the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland, thereby preventing follicular maturation and suppressing ovulation (Benagiano et al., 2004; Doren et al., 2001; Duijkers et al., 2016; Jeppsson and Johansson, 1976; Mishell, 1996; Rice et al., 1999). However, some progestins also exert contraceptive action by thickening the cervical mucus, thus limiting sperm penetration into the uterus (Benagiano et al., 2004; Landgren and Diczfalusy, 1980;

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Rice et al., 1999). Inhibition of FSH and LH release results in decreased production of androgens and estrogens (Craig et al., 2011). For example, the contraceptive use of MPA has been shown to cause a decrease in the plasma levels of estradiol (E_2) (Aedo et al., 1981). Interestingly, Prog and 17OH-Prog levels were also decreased. In cancer therapy, high doses of MPA are used and it has been shown that these doses cause significant inhibition of adrenal function (Blossey et al., 1984; Lang et al., 1990; Papaleo et al., 1984; Van Veelen et al., 1984). For example, significantly lower cortisol, androstenedione (A4) and dehydroepiandrosterone sulfate (DHEA-S) levels were observed in postmenopausal breast cancer patients treated with MPA (Van Veelen et al., 1984). There are thus indications that progestins such as MPA can modulate steroid hormone levels by eliciting effects on the HPA-axis. Considering that some progestins have chemical structures similar to that of Prog, 17OH-Prog and testosterone, the possibility exists that progestins may also decrease steroid biosynthesis by competing with endogenous steroid metabolites for binding to steroidogenic enzymes. Indeed, it has previously been shown that MPA, which is structurally similar to 17OH-Prog, binds to and competitively inhibits the activity of human 3βHSD2 (Lee et al., 1999).

3. Biosynthesis of endogenous steroid hormones

Steroid hormones are all derived from cholesterol via a sequential series of enzyme catalysed reactions, collectively referred to as steroidogenesis. These steroidogenic enzymes are divided into two main classes, namely the heme-containing cytochrome P450 enzymes (CYP's) and the hydroxysteroid dehydrogenases (HSD's) (Capper et al., 2016; Hu et al., 2010; Miller and Auchus, 2011; Payne and Hales, 2004; Sanderson, 2006). Generally, CYP's catalyse irreversible reactions, while the reactions catalysed by the HSD's are reversible.

3.1. Adrenal steroidogenesis

The adrenal gland is primarily responsible for the synthesis of glucocorticoids, mineralocorticoids and androgen precursors, while low levels of the sex steroids (progestogens, androgens and estrogens) are also synthesised (Abdel-Rahman, 2015; Capper et al., 2016; Miller and Auchus, 2011). Fig. 1 illustrates the major enzymes involved in the steroidogenic pathways in the adrenal. For the purpose of this review, pregnenolone (Preg), Prog and their intermediate metabolites have been grouped as progestogens and are indicated in white boxes in Fig. 1. These progestogens serve as precursors for the biosynthesis of mineralocorticoids (light grey), glucocorticoids (dark grey), androgens and their precursors (grey) as well as estrogens (black) (Fig. 1).
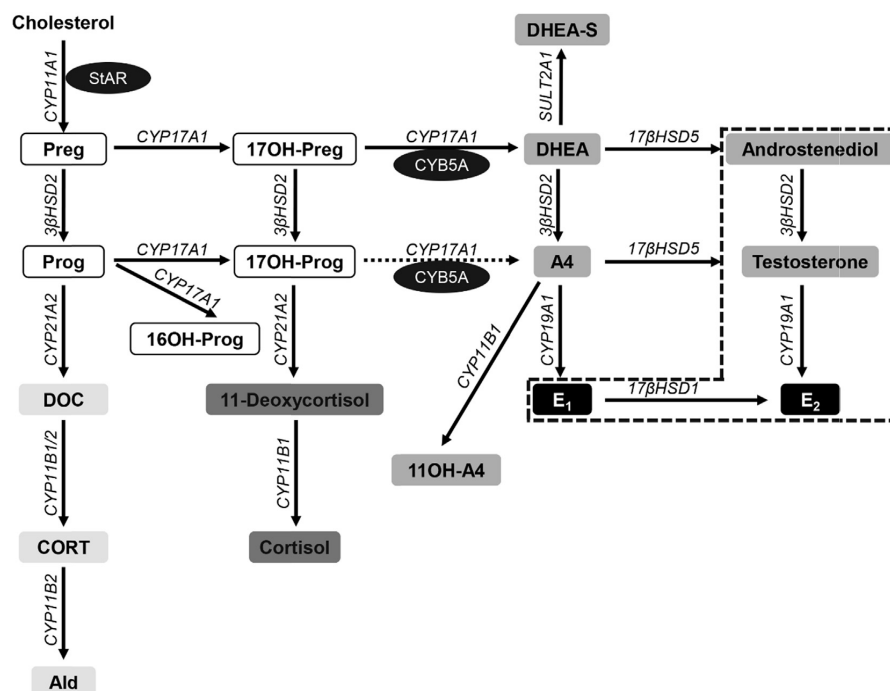
Steroid synthesis is initiated when cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane with the help of the steroidogenic acute regulatory protein (StAR) (Christenson and Strauss, 2000; Stocco, 2001; Strauss et al., 1999). The de novo biosynthesis of all steroid hormones then begins with cytochrome P450 side-chain cleavage (P450scc; CYP11A1) converting cholesterol to the Δ^5 C21-steroid Preg, which in turn serves as the initial precursor for the downstream biosynthesis of the mineralocorticoids, glucocorticoids and sex steroids as indicated in Fig. 1. The adrenal cortex is divided into three separate zones that each express a set of specific steroidogenic enzymes required for the synthesis of particular end products. The zona glomerulosa expresses CYP11A1, 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), cytochrome P450 21-hydroxylase (P450c21; CYP21A2), cytochrome P450 aldosterone synthase (CYP11B2), but not cytochrome P450 17α-hydroxylase/17,20-lyase

(P450c17; CYP17A1), thereby directing steroidogenesis towards the production of the mineralocorticoid aldosterone (Ald) (C21-steroid) (Miller and Auchus, 2011). The biosynthesis of the glucocorticoid cortisol, also a C21-steroid, occurs in the zona fasciculata due to the expression of CYP11A1, CYP17A1, 3βHSD2, CYP21A2 and cytochrome P450 11β-hydroxylase (P45011β; CYP11B1). Conversely, the expression of CYP11A1 and CYP17A1, and only low levels of 3βHSD2 in the zona reticularis, results in the synthesis of C19-steroids (androgen precursors). The expression of the accessory protein cytochrome b_5 (CYB5A) in this zone is also vital as it is required to augment the 17,20-lyase activity of CYP17A1, an activity which is not present in the zona fasciculata (Auchus et al., 1998; Geller et al., 1999; Katagiri et al., 1995; Lee-Robichaud et al., 1995). While mineralocorticoid production is mainly under the control of the renin–angiotensin–aldosterone system (Capper et al., 2016; Lalli et al., 2016), the synthesis of cortisol and androgen precursors is regulated by the HPA-axis (Capper et al., 2016; Catalano et al., 1986; Rege et al., 2013).

CYP17A1 catalyses the 17α-hydroxylation of Preg and Prog to produce 17OH-Preg and 17OH-Prog, respectively. It is noteworthy that CYP17A1 also catalyses the 16α-hydroxylation of Prog to produce 16OH-Prog (Swart et al., 1993), but unlike 17OH-Prog, 16OH-Prog is not further metabolised (Storbeck et al., 2011). The 17OH-Preg and 17OH-Prog intermediates are then cleaved at C17–C20 to produce the androgen precursors, Δ^5 C19-steroid DHEA and Δ^4 C19-steroid A4. It should, however, be noted that human CYP17A1 does not efficiently catalyse the conversion of 17OH-Prog to A4, but that the Δ^5 steroid 17OH-Preg is the preferred substrate for this enzyme (Auchus et al., 1998; Brock and Waterman, 1999; Lee-Robichaud et al., 1995). 3βHSD2 catalyses the conversion of the Δ^5 steroids, Preg, 17OH-Preg and DHEA, to their respective Δ^4 steroids, Prog, 17OH-Prog and A4. Moreover, DHEA is also converted by sulfotransferase family 2A member 1 (SULT2A1) to produce the abundant adrenal androgen precursor DHEA-S (Abraham, 1974; Nakamura et al., 2009; Turcu et al., 2014). CYP21A2 catalyses the hydroxylation of Prog and 17OH-Prog to produce the mineralocorticoid and glucocorticoid precursors, 11-deoxycorticosterone (DOC) and 11-deoxycortisol, respectively, while CYP11B1 catalyses a further hydroxylation of these precursors to produce corticosterone (CORT) and cortisol. Alternatively, DOC can be metabolised by CYP11B2 to Ald. The enzymatic actions of CYP11B1 are however not restricted to the mineralocorticoid and glucocorticoid pathways, as it can also hydroxylate A4 in the androgen pathway resulting in the production of 11β-hydroxyandrostenedione (11OH-A4) (Bloem et al., 2013; Shibusawa et al., 1980; Swart and Storbeck, 2015). Although DHEA-S, DHEA, A4 and 11OH-A4 are the primary C19-steroids produced by the adrenal, the low levels of 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) expressed in the adrenal results in the conversion of DHEA and A4 to androstenediol and testosterone, respectively (Fig. 1) (Labrie et al., 2003; Miller and Auchus, 2011). A4 and testosterone are also substrates for cytochrome P450 aromatase (CYP19A1), which catalyses the aromatization of A4 and testosterone (Δ^4 C19-steroids) to low levels of the estrogens (C18-steroids), estrone (E_1) and E_2, respectively. E_1 can subsequently be converted to E_2 by 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) (Labrie et al., 2003; Miller and Auchus, 2011; Payne and Hales, 2004). Although the adrenal produces low levels of estrogens, it is the main source of circulating estrogens in postmenopausal women (Simpson, 2002).

3.2. Gonadal steroidogenesis

The gonads are the primary site for the biosynthesis of sex steroids (Capper et al., 2016; Miller and Auchus, 2011), and use the same enzymes and pathways as in the adrenal to synthesise DHEA.

Fig. 1. The major pathways of *de novo* biosynthesis of steroids in the adrenal glands. Schematic representation of the biosynthesis pathways of progestogens (white), mineralocorticoids (light grey), glucocorticoids (dark grey), androgens and their precursors (grey), and estrogens (black) from cholesterol in the adrenal glands. 17OH-Preg and 17OH-Prog both serve as precursors for androgen biosynthesis, but the synthesis of A4 from 17OH-Prog is a minor reaction and is indicated by a dashed line. With the exception of androstenediol, testosterone and estrogens (boxed), minor metabolites are not presented. Enzymes: 3βHSD − 3β-hydroxysteroid dehydrogenase; 17βHSD − 17β-hydroxysteroid dehydrogenase; CYB5A − cytochrome b_5; CYP11A1 − cytochrome P450 side-chain cleavage; CYP11B1 − cytochrome P450 11β-hydroxylase; CYP11B2 − cytochrome P450 aldosterone synthase; CYP17A1 − cytochrome P450 17α-hydroxylase/17,20-lyase; CYP19A1 − cytochrome P450 aromatase; CYP21A2 − cytochrome P450 21-hydroxylase; StAR − steroidogenic acute regulatory protein; SULT2A1 − sulfotransferase family 2A member 1. Steroids: 11OH-A4 − 11β-hydroxyandrostenedione; 16OH-Prog − 16α-hydroxyprogesterone; 17OH-Preg − 17α-hydroxypregnenolone; 17OH-Prog − 17α-hydroxyprogesterone; Ald − aldosterone; A4 − androstenedione; CORT − corticosterone; DHEA − dehydroepiandrosterone; DHEA-S − dehydroepiandrosterone sulfate; DOC − deoxycorticosterone; E_1 − estrone; E_2 − estradiol; Preg − pregnenolone; Prog − progesterone.

Similar to the adrenals, the ovaries express specific steroidogenic enzymes required for the synthesis of androgens, estrogens and progestogens. The ovarian theca cells express CYP11A1, CYP17A1, CYB5A, 3βHSD2, 17βHSD2 and 17βHSD5 required for the synthesis of androgens and their precursors, while the granulosa cells express CYP11A1, 3βHSD2, 17βHSD1, CYP19A1, sulfatase (STS) and sulfotransferase family 1E member (SULT1E1), which are responsible for the production of estrogens. The synthesis of the progestogens, Preg and Prog, occurs in both the theca and granulosa cells due to the actions of CYP11A1 and 3βHSD2. However, as CYP17A1 is not expressed in the granulosa cells, 17OH-Preg and 17OH-Prog are only synthesised in the theca cells. Preg and Prog are therefore converted to the androgen precursor, A4, in the theca cells. The fate of A4 is determined by whether it remains in the theca cells or whether it diffuses into the adjacent granulosa cells. In case of the latter, estrogen will be synthesised via specific enzymatic reactions catalysed by CYP19A1 and 17βHSD1, while in the theca cells it will be converted to testosterone by 17βHSD5 (Capper et al., 2016; Miller and Auchus, 2011). Testosterone then diffuses into the granulosa cells where it will be converted to E_2 by CYP19A1. Notably, the ovary is the principal tissue contributing to the biosynthesis of estrogens and androgens in premenopausal women, while in postmenopausal women ovarian sex steroid production is terminated (Bulun et al., 2007; Simpson, 2002). In the testes, the Leydig cells express CYP11A1, CYP17A1, CYB5A, 3βHSD2

and 17βHSD3 required for the synthesis of androgens (Capper et al., 2016; Miller and Auchus, 2011), as well as CYP19A1, 17βHSD3 and SULT1E1 for the production of low levels of estrogens (Akingbemi, 2005; Miki et al., 2002; Simpson, 2002). It should be noted that in the adrenal and ovary, 17βHSD5 converts DHEA and A4 to androstenediol and testosterone, respectively, while 17βHSD3 catalyses these reactions in the Leydig cells (Capper et al., 2016; Lukacik et al., 2006; Peltoketo et al., 1999).

3.3. Metabolism of steroid hormones in peripheral tissues

The adrenals and gonads are not the only tissues that express steroid-metabolising enzymes. A number of these enzymes are also expressed in peripheral tissues and are responsible for either activating or inactivating steroid metabolites taken up from circulation. The importance of the metabolism of steroid hormones in peripheral tissues was first shown many years ago, when Grodin et al. (1973) showed that A4 was aromatised to E_1 in postmenopausal breast cancer patients, who no longer synthesised ovarian estrogens. This importance is further highlighted by more recent studies indicating that local synthesis of androgens and estrogens can cause increased prostate and breast cancer cell proliferation, respectively (Chetrite et al., 2000; Sharifi and Auchus, 2012; Suzuki et al., 2005). Conversely, in other tissues, steroidogenic enzymes play a protective role. For example, the expression of 11β-

hydroxysteroid dehydrogenase type 2 (11βHSD2) in the kidney converts the active glucocorticoid cortisol to its inactive form cortisone, thereby protecting the MR from activation by cortisol which has a similar affinity for the MR than the mineralocorticoid Ald (Arriza et al., 1987; Quinkler and Stewart, 2003), and which occurs at a significantly higher concentration in circulation than Ald (Martinerie et al., 2012; Stewart et al., 1988).

In the breast (Fig. 3), estrogens are produced from circulating DHEA, A4 and testosterone. The conversion of the inactive estrogen sulfates, E_1-sulfate (E_1-S) and E_2-S, to E_1 and E_2, respectively, is catalysed by STS, while the reverse reaction is catalysed by SULT1E1. Inactive E_1 is converted to active E_2 by the actions of both 17βHSD1 and 17βHSD7 (Labrie et al., 2003; Li et al., 2009), while 17βHSD5 catalyses the conversion of A4 to testosterone, and 17βHSD2 the reverse reaction (Labrie et al., 2003; Li et al., 2009). Steroid 5α-reductase (SRD5A) is also expressed in the breast (Suzuki et al., 2007; Yamana et al., 2010) and is the enzyme responsible for the synthesis of the potent androgen dihydrotestosterone (DHT). DHT can be produced either directly from testosterone or indirectly from A4 (Capper et al., 2016; Stanczyk et al., 1990), with A4 being converted to 5α-androstanedione (5α-dione) by SRD5A (Fouad Mansour et al., 2016; Stanczyk et al., 1990) followed by the conversion of 5α-dione to DHT by 17βHSD (Fouad Mansour et al., 2016; Labrie et al., 2005; Luu-The et al., 2008; Yamana et al., 2010). The conversion of 5α-dione to DHT in the breast has been suggested to occur via 17βHSD5 (Labrie et al., 2005; Vihko et al., 2005). Interestingly, low levels of DHT are synthesised in the breast (Suzuki et al., 2007).

A4 and testosterone are also precursors of local DHT production in other peripheral tissues such as the adipose tissue (Blouin et al., 2009; Fouad Mansour et al., 2016) and genital skin (Samson et al., 2010; Silva et al., 1987; Stanczyk et al., 1990). While the conversion of DHT from testosterone is preferred in male genital skin, A4 is the preferred precursor in the genital skin of normal and hirsute women (Silva et al., 1987). Notably, it has been shown that SRD5A1 has a higher affinity for A4 than testosterone in COS-M6 cells transiently transfected with human SRD5A1 (Andersson and Russell, 1990), while similar results were also obtained in HEK-293 cells stably transfected with either human SRD5A1, 2 or 3 (Yamana et al., 2010). The importance of peripheral androgen metabolism is highlighted in women with conditions associated with androgen excess, such as hirsutism and polycystic ovary syndrome (PCOS). For example, it has been suggested that women with PCOS have increased SRD5A activity as one study found increased 5α-reduced metabolites in the urine of patients with PCOS (Stewart et al., 1990), while another study showed increased levels of serum DHT (Fassnacht et al., 2003). These results are in agreement with earlier studies showing increased SRD5A activity in genital and pubic skin of hirsute women, resulting in increased production of DHT (Serafini and Lobo, 1985).

4. Progestins and the biosynthesis of endogenous steroid hormones

Understanding steroidogenesis and factors that may influence the process is vital to the understanding of numerous physiological responses including reproduction, hypertension, obesity, cancer and general homeostasis. An example displaying this importance is reflected in the use of synthetic glucocorticoids such as dexamethasone and prednisolone as anti-inflammatory drugs. These compounds were designed to exhibit anti-inflammatory properties via binding to the glucocorticoid receptor, and their longterm usage suppresses the HPA-axis resulting in the inhibition of endogenous glucocorticoid production. Upon discontinuation of use, the HPA-axis needs up to 18 months to recover, and during this time the

amount of glucocorticoids produced by the adrenal will not be sufficient for normal physiology. Hence, patients should be withdrawn from these drugs in a gradual manner so as to allow the recovery of adrenal function (Gupta and Bhatia, 2008). Surprisingly little research appears to have been carried out on the effects of progestins on endogenous steroid biosynthesis, and more so effects on specific steroidogenic enzymes. In the sections below, we provide an overview of the known effects of various progestins on the biosynthesis of endogenous steroid hormones, with emphasis on progestins regulating the expression and/or modulating the activity of steroid-producing enzymes.

4.1. Effects of progestins on the biosynthesis of

4.1.1. Progestogens

Few studies have investigated the effects of progestins on progestogen production and the results are often contradictory. Evidence from fish (Overturf et al., 2014; Petersen et al., 2015) and rat (Pridjian et al., 1987; Telleria et al., 1994) studies suggest that while some progestins influence the production of endogenous progestogens (Overturf et al., 2014; Pridjian et al., 1987), this is not true for all progestins (Petersen et al., 2015). For example, fish exposed to 0.32 nM LNG displayed reduced concentrations of Preg (~75%), Prog (~41%) and 17OH-Prog (~64%) (Overturf et al., 2014), while exposure to 0.001–10 μM NET had no effect on the production of these progestogens (Petersen et al., 2015). Interestingly, earlier studies in rats showed that FSH-induced production of Prog by ovarian granulosa cells was increased in response to 0.1 and 1 μM MPA, but decreased at concentrations higher than 1 μM (Pridjian et al., 1987). These results suggest that MPA may elicit effects in a concentration-dependent manner. In contrast to the results from this animal study, recent data from our group showed that 1 μM MPA (a dose similar to that used in cancer therapy (0.14–1.7 μM) (Focan et al., 2001; Thigpen et al., 1999)), has no effect on progestogen production in the human H295R adrenocortical carcinoma cell line (Louw-du Toit et al., In press). This highlights the fact that effects of progestins may differ depending on the model system used. Nonetheless, the results in the cell line model also showed that 1 μM NET-A, LNG and GES had no effect on the production of Prog or Preg metabolites, while NES and NoMAC increased the concentration of Preg, but decreased the concentrations of Prog, 17OH-Prog and 16OH-Prog. Treatment with DRSP, the unique progestin derived from the MR antagonist spironolactone, resulted in increased concentrations of all these progestogens. These results indicate that the influence of progestins on progestogen synthesis in the adrenal cell line is dependent on the type of progestin. To the best of our knowledge, only one study, more than 20 years ago, investigated the effects of a progestin on progestogen levels in humans. This particular study showed that plasma levels of Prog and 17OH-Prog were reduced at day 16 following the administration of the injectable contraceptive MPA to normal menstruating women (Aedo et al., 1981). Considering that the serum levels reported with the injectable contraceptive usage of MPA is 4.5–65 nM (Kirton and Cornette, 1974; Shrimanker et al., 1978) (Depo-Provera contraception injection, 2006, New York, Pharmacia and Upjohn Company, Pfizer Inc.), this suggests that MPA used as an injectable contraceptive would influence progestogen production.

The above *in vivo* animal experiments and *in vitro* cell culture experiments suggest that the modulation of progestogen biosynthesis by progestins may depend on the type and concentration of progestin used. However, results from these experiments should be cautiously interpreted as it is not feasible to use this information to extrapolate the effects in women using these progestins for hormone therapy. However, it is clear that there is a paucity in the research carried out in humans, and should be addressed by future

research.

4.1.2. Androgens

Studies have shown that LNG (Overturf et al., 2014), GES (Runnalls et al., 2013) and MA (Han et al., 2014) reduced testosterone production by fish, suggesting that progestins may inhibit androgen production. Similarly, MPA has been shown to reduce the serum concentrations of testosterone (Barbieri and Ryan, 1980; Satyaswaroop and Gurpide, 1978) and A4 (Satyaswaroop and Gurpide, 1978) in rats. Interestingly, our recent study in the human H295R adrenal cell line also showed decreased production of A4 and testosterone, this time by NES, NoMAC and DRSP (Louw-du Toit et al., In press).

Earlier studies investigating the effects of progestins on the biosynthesis of androgens in humans focused only on the effects of the first-generation progestin MPA (Dowsett et al., 1987; Van Veelen et al., 1984). The study by van Veelen and co-workers compared the adrenal function of postmenopausal breast cancer patients treated with MPA (300 mg daily) to that of a control group, and reported significantly lower A4 and DHEA-S levels. Similarly, Dowsett and co-workers also reported decreased serum levels of A4 and testosterone in postmenopausal breast cancer patients after the administration of MPA, at both low (100 mg three times a day) and high (250 mg four times a day) doses (Dowsett et al., 1987). Total testosterone serum concentrations were also reduced in men administered 100—1000 mg MPA weekly for the treatment of abnormal sexual behavior (Guay, 2008). More recent studies, however, have also investigated the effects of progestins from later generations, including LNG, norgestimate, GES, NoMAC and DRSP. For example, the use of LNG as a subcutaneous contraceptive implant has been shown to lower the serum concentrations of total testosterone, A4, DHT and DHEA-S (Kovalevsky et al., 2010). Although this study did not specify the initial concentration of LNG administered, the authors reported that the serum concentrations of LNG during a 3 month period were in the range of 1.04—3.52 nM. Interestingly, two other studies also showed lower serum concentrations of testosterone, A4, DHT and DHEA-S in women using progestins in combined oral contraceptives (COCs), and include NoMAC combined with E_2, or ethinyl estradiol (EE) combined with LNG, norgestimate or GES (Agren et al., 2011; Wiegratz et al., 1995). Similarly, De Leo and co-workers showed that the serum concentrations of total testosterone, free testosterone, A4 and DHEA-S were decreased in hyperandrogenic women diagnosed with PCOS using COCs containing EE combined with either DRSP, CMA, GES or DSG (De Leo et al., 2010; De Leo et al., 2007). Moreover, a study investigating the use of progestins to treat women with hirsutism showed that norgestimate, LNG, DNG, CPA and GES all decreased the production of the potent androgen DHT in genital skin, but that the degree of inhibition differed between progestins. Interestingly, an earlier study in non-hirsute premenopausal women showed that LNG and NET, but not MPA, inhibited the conversion of testosterone to DHT in genital and pubic skin (Cassidenti et al., 1991). Considering that A4 and testosterone are mainly produced by the ovaries in premenopausal woman, while DHEA-S is the main androgen precursor produced by the adrenals (De Leo et al., 2003; Simpson, 2002), results from the above-mentioned studies suggest that progestins have the potential to modulate both adrenal and ovarian androgen biosynthesis. Moreover, some progestins also inhibit peripheral androgen metabolism. In summary, the studies to date provide sufficient evidence to support a link between the use of progestins and decreased production of androgens. Indeed, progestins have been used in the treatment of women diagnosed with conditions associated with androgen excess such as PCOS, for many years (Falsetti and Galbignani, 1990; Frolich et al., 1980; Mathur et al., 2008; Mowszowicz et al., 1984).
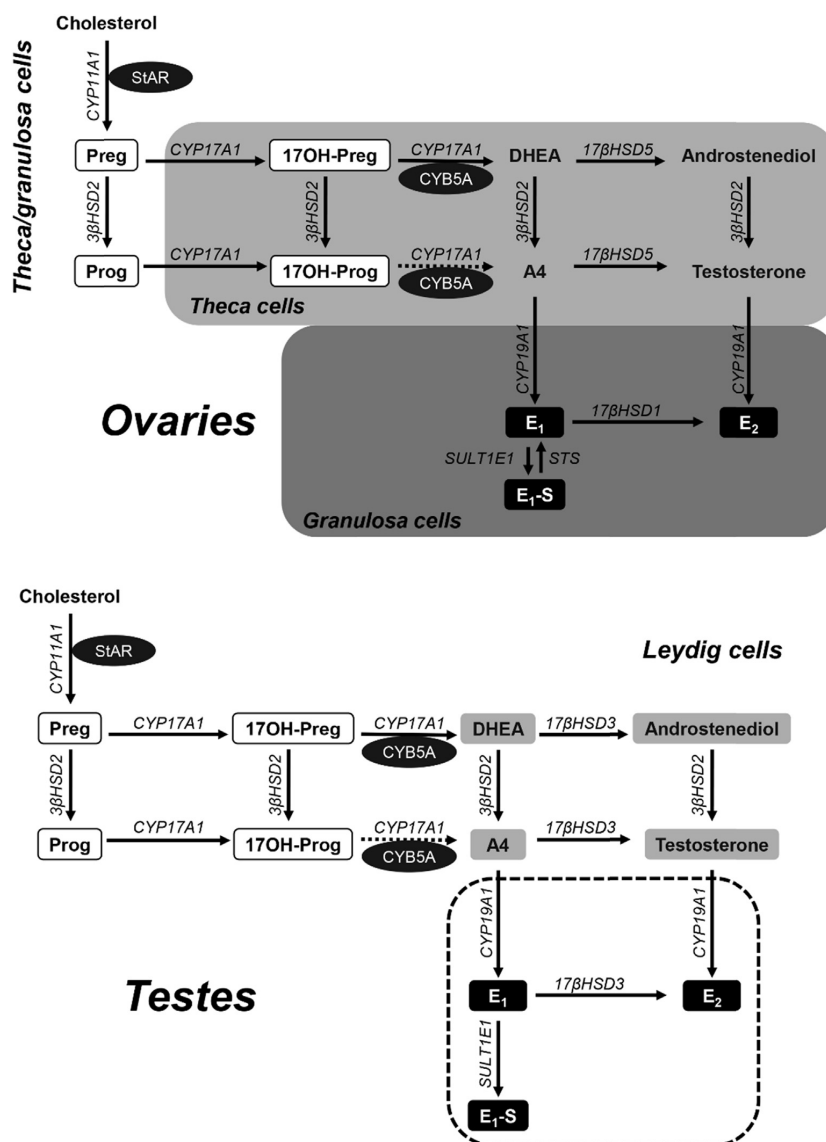
4.1.3. Estrogens

Studies have shown that progestins causing decreased concentrations of androgens, also decrease estrogen concentrations, which is not surprising given that androgens serve as the substrates for estrogen production (Figs. 1—3). For example, studies in fish showed that LNG, GES and MA all reduce the serum concentrations of both testosterone and E_2 (Han et al., 2014; Overturf et al., 2014; Runnalls et al., 2013). Dowsett et al. (1987) also reported decreased concentrations of A4, testosterone and E_2 in postmenopausal breast cancer patients receiving MPA. Interestingly, at least two studies have evaluated the effects of progestins on only estrogen production, and report a reduction in the serum concentration of E_2 in fish exposed to NET (Paulos et al., 2010), and decreased production of E_2 by cultured rat granulosa cells treated with NoMAC (Qian et al., 2001). From the limited number of studies reported above, it appears as though progestins that affect the biosynthesis of A4 and testosterone will also influence the biosynthesis of estrogens, though this may not always be the case. Further investigation is clearly needed to elucidate the effects of progestins on endogenous estrogen production.

4.1.4. Glucocorticoids and mineralocorticoids

Most studies investigating the effects of progestins on glucocorticoid and mineralocorticoid production have focused on the effects on serum cortisol and Ald levels in women, and the findings are often contradictory (Aedo et al., 1981; Amatayakul et al., 1988; Jones et al., 1974; Olsson et al., 1987; Toppozada et al., 1997). For example, some early studies showed that the intravenous administration of the contraceptive MPA (150 mg) to healthy menstruating women resulted in a significant decrease in the serum concentration of cortisol (Aedo et al., 1981; Jones et al., 1974), while no significant effect was reported by another study (Amatayakul et al., 1988). Interestingly, the serum concentration of cortisol was also reduced in postmenopausal breast cancer patients receiving 300—1500 mg MPA (intravenously or orally) (Blossey et al., 1984; Hellman et al., 1976; Lang et al., 1990; Papaleo et al., 1984; Van Veelen et al., 1984), as well as in men receiving 100—1000 mg MPA for the treatment of abnormal sexual behavior (Guay, 2008). Furthermore, studies evaluating the effects of progestins other than MPA mostly showed that serum cortisol levels were not modulated in women (Olsson et al., 1987; Amatayakul et al., 1988; Gaspard et al., 1983; De Leo et al., 2007), while some studies showed an increase (Wiegratz et al., 1995, 2003b) and at least one study showed a decrease in serum cortisol concentrations (Toppozada et al., 1997). For example, Toppozada et al. (1997) showed that a contraceptive implant containing 6 capsules of 36 mg LNG (Norplant®) decreased the serum levels of cortisol (Toppozada et al., 1997), while no effect was observed on the serum concentration of cortisol in women using the injectable contraceptive NET-EN (Amatayakul et al., 1988), a LNG contraceptive implant (Norplant® −2 − containing 2 rods of 70 mg LNG each) (Olsson et al., 1987) or COCs containing EE combined with LNG or DSG (Gaspard et al., 1983) or DRSP (De Leo et al., 2007). Conversely, data from the research group of Herbert Kuhl has shown an increase in serum cortisol levels in women using COCs containing EE and LNG, DSG, DNG, GES or norgestimate (Wiegratz et al., 1995, 2003b). These authors ascribe the increase in serum cortisol to the fact that EE can cause an increase in the serum levels of corticosteroid binding globulin (CBG) resulting in higher cortisol levels, or due to the progestins inhibiting the metabolism of corticosteroids in the liver. It should be noted that the extent to which these progestins increased the serum concentrations of cortisol were different, and has been suggested to be due to the progestin component counteracting the estrogen-dependent effects on CBG (Wiegratz et al., 2003a). The effects of progestins on the production of cortisol
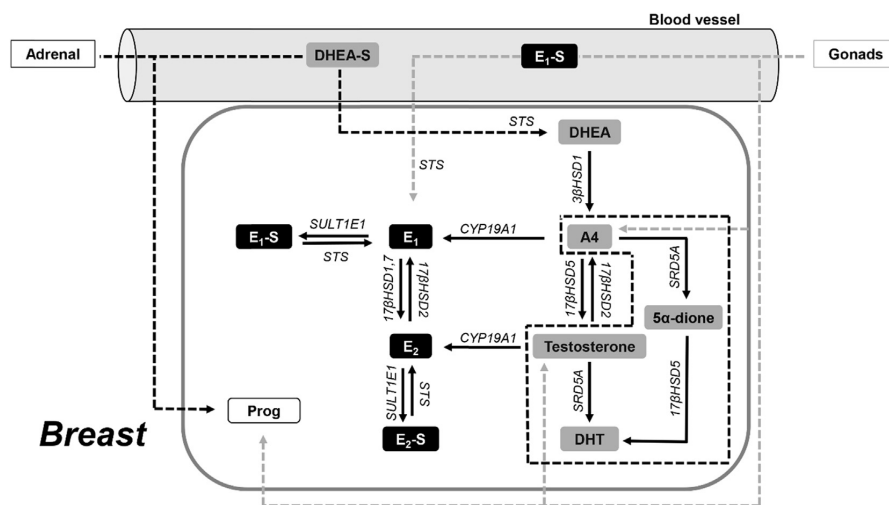
Fig. 2. Sex steroid synthesis in the gonads of females and males. The figure illustrates the *de novo* biosynthesis of progestogens (white), androgens (grey) and estrogens (black) from cholesterol in the ovaries and the testes. The enzymes and pathways used to synthesise DHEA are the same in the ovaries and testes, while the 17βHSD isozymes responsible for the biosynthesis of androgens and estrogens from DHEA differ. Estrogens are a minor product of testicular steroidogenesis and are indicated in the dashed box. The conversion of 17OH-Prog to A4 is indicated by a dashed line as it is a minor reaction. Enzymes: 3βHSD − 3β-hydroxysteroid dehydrogenase; 17βHSD − 17β-hydroxysteroid dehydrogenase; CYB5A − cytochrome b_5; CYP11A1 − cytochrome P450 side-chain cleavage; CYP17A1 − cytochrome P450 17α-hydroxylase/17,20-lyase; CYP19A1 − cytochrome P450 aromatase; StAR − steroidogenic acute regulatory protein; STS − sulfatase; SULT1E1 − sulfotransferase family 1E member 1. Steroids: 17OH-Preg − 17α-hydroxypregnenolone; 17OH-Prog − 17α-hydroxyprogesterone; A4 − androstenedione; DHEA − dehydroepiandrosterone; DHEA-S − dehydroepiandrosterone sulfate; E_1 − estrone; E_1-S − estrone sulfate; E_2 − estradiol; Preg − pregnenolone; Prog − progesterone.

have also been examined in at least one *in vivo* animal study (Baldwin et al., 1996), as well as our recent *in vitro* cell culture model (Louw-du Toit et al., In press). Baldwin and co-workers showed that LNG-containing contraceptive implants (releasing 0.06 mg per day) had no effect on the serum concentrations of cortisol in female cats (Baldwin et al., 1996), which is in agreement with our results in the human H295R adrenal cell line using cancer

therapy doses of LNG. Interestingly, we showed that MPA, NET-A, GES and NoMAC also had no effect, while NES and DRSP significantly decreased the concentration of cortisol (Louw-du Toit et al., In press).

Clinical and experimental studies investigating the effect of progestins on the serum concentrations of Ald, mostly indicate that progestins do not alter the production of this steroid hormone

Fig. 3. Peripheral steroid metabolism in breast tissue. The breast tissue obtains Prog (white), estrogens (black) and androgens (grey) from the adrenal (black dashed lines) and gonads (grey dashed lines). The dashed lines indicate steroids in circulation. Estrogens are synthesised from androgens obtained from both adrenal and gonadal steroidogenesis, as well as E_1-S produced by the ovaries. Low levels of DHT are produced from testosterone or A4 in minor reactions (enclosed in dashed box). Enzymes: 3βHSD – 3β-hydroxysteroid dehydrogenase; 17βHSD – 17β-hydroxysteroid dehydrogenase; CYP19A1 – cytochrome P450 aromatase; SRD5A – steroid 5α-reductase; STS – sulfatase; SULT1E1 – sulfotransferase family 1E member 1. Steroids: A4 – androstenedione; 5α-dione – 5α-androstanedione; DHEA – dehydroepiandrosterone; DHEA-S – dehydroepiandrosterone sulfate; DHT – dihydrotestosterone; E_1 – estrone; E_1-S – estrone sulfate; E_2 – estradiol; E_2-S – estradiol sulfate; Prog – progesterone.

(Blossey et al., 1984; Gaspard et al., 1983). For example, no effect was observed when MPA was used for the treatment of metastatic breast cancer in postmenopausal women (Blossey et al., 1984), or when LNG or DSG were used in COC (Gaspard et al., 1983). Similarly, we showed that none of the progestins investigated in our study (MPA, NET-A, LNG, GES, NES, NoMAC and DRSP) modulated the concentration of Ald produced by the H295R adrenal cell line (Louw-du Toit et al., In press). However, one human study reported an increase in serum concentrations of Ald in normal menstruating women using the progestin-only contraceptive DRSP (Oelkers et al., 1991).

It is evident from the above human studies that there is no definitive answer regarding the effects of progestins on cortisol levels, and effects seem to depend on the type and dosage of the progestin used, as well as whether it is used in combination with EE. Results from the limited number of studies investigating the effects on Ald levels seem to suggest that progestins do not affect the production of this steroid.

4.2. Effects of progestins on key steroid-producing enzymes

It is clear from the above discussion that clinical studies investigating the effects of progestins on steroid hormone levels are lacking and the available studies do not explain the mechanism by which steroid levels are modulated. Evidence from animal studies and *in vitro* cell culture systems do however indicate that progestins can directly inhibit the expression and/or activity of key steroidogenic enzymes. These studies will be discussed in the sections below.

4.2.1. 3βHSD2 and CYP17A1

3βHSD2 and CYP17A1 are key enzymes involved in the biosynthesis of progestogens and androgens in both the adrenals and gonads, and thus changes in the production of these steroid hormones following treatment with progestins may be due to the modulation of the activity and/or expression of either or both of these enzymes. Indeed, the observed decrease in mRNA expression of both ovarian 3βHSD and CYP17A1 by LNG, may account for the decrease in the production of Preg, Prog, 17OH-Prog and testosterone by fish (Overturf et al., 2014). Furthermore, an earlier study in rats indicated that LNG inhibits the activity of ovarian 3βHSD and the 17,20-lyase activity of CYP17A1 (Arakawa et al., 1989). Interestingly, this study showed that the first-generation progestin NET, which is structurally related to LNG (Stanczyk and Roy, 1990), also inhibited the activity of these two enzymes (Arakawa et al., 1989). Although the results from a recent study in fish showed that NET also appeared to decrease 3βHSD mRNA expression, this decrease was not significant and correlated with their findings showing no effect on the production of progestogens and androgens (Petersen et al., 2015).

Other progestins have also been shown to inhibit the 17,20-lyase activity of CYP17A1 in fish, this time in the testes, and include DRSP and CPA (Fernandes et al., 2014). However, inhibition of the 17α-hydroxylase activity cannot be excluded as it was not measured in this study. Interestingly, results from a previous study suggest that DRSP may inhibit both the 17α-hydroxylation and 17,20-lyase activities of human CYP17A1, as a decrease in the ratio of 17OH-Prog/Prog and A4/17OH-Prog was observed in women using a DRSP-containing COC (De Leo et al., 2007). However, these authors did not investigate the direct effect of DRSP on the activity of this enzyme. In contrast, we recently showed that DRSP inhibits the activity of both CYP17A1 and 3βHSD2 in COS-1 cells transfected with either human CYP17A1 or 3βHSD2 (Louw-du Toit et al., In press). These results correlated to our findings in the human H295R adrenal cell line showing an increase in the production of progestogens and decrease in the production of androgens in the presence of DRSP (Louw-du Toit et al., In press). Furthermore, we showed that NES and NoMAC inhibited the activity of 3βHSD2, but not CYP17A1, which is in agreement with our observation of reduced concentrations of progestogens and androgens in the H295R cells. Notably, the inhibition constant (K_i) values determined for the inhibition of 3βHSD2 by NES (9.5 ± 0.96 nM), NoMAC

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(29 ± 7.1 nM) and DRSP (232 ± 38 nM) are within the reported serum concentration ranges for the contraceptive use of NES (0.086–27.3 nM), NoMAC (3–33 nM) and DRSP (26.7–253 nM) (Bahamondes and Bahamondes, 2014; Blode et al., 2001, 2012; Brache et al., 2001; Gerrits et al., 2013; Ito et al., 2016; Massai et al., 2001; Robbins and Bardin, 1997) (Louw-du Toit et al., In press), therefore strongly suggesting that these progestins have the potential to alter endogenous steroid production *in vivo*.

Whether or not MPA inhibits 3βHSD2 and/or CYP17A1 is contradictory and appears to be dependent on the species and/or tissue and/or expression levels of these enzymes. Earlier findings indicated decreased serum concentrations of androgens by MPA and attributed the decrease to inhibition of 3βHSD activity in rat testes (Barbieri and Ryan, 1980; Satyaswaroop and Gurpide, 1978). In contrast, MPA had no effect on 3βHSD activity in the rat ovary (Mizutani et al., 1992), suggesting that MPA elicits tissue-specific effects. Interestingly, recent data from our group also showed no effect on the activity of human 3βHSD2 transiently transfected into the non-steroidogenic COS-1 cells (Louw-du Toit et al., In press), while an earlier study indicated that MPA binds to and inhibits the activity of human 3βHSD2 expressed in a yeast system (Lee et al., 1999). It should however be noted that these investigators determined a Kᵢ of 3 μM for 3βHSD2 and showed low levels of inhibition (26%) with 1 μM MPA. In terms of CYP17A1, the results from our group as well as that of the Auchus research group show that MPA has no effect on the activity of human CYP17A1 (Lee et al., 1999) (Louw-du Toit et al., In press), whereas findings from one animal study indicates that MPA inhibits the 17α-hydroxylase activity, but not the 17,20-lyase activity, of rat CYP17A1 (Mizutani et al., 1992).

Taken together, the majority of the above-mentioned studies suggest that while some progestins affect adrenal and gonadal steroid biosynthesis due to the inhibition of 3βHSD and CYP17A1 activity, this is not true for all progestins. However, as most of the reported findings are from animal studies and considering that different steroidogenic enzyme isoforms, with distinct functions and substrate preferences, are expressed in different species (Auchus et al., 1998; Bird and Conley, 2002; Gilep et al., 2011; Labrie et al., 1992; Miller and Auchus, 2011; Payne and Hales, 2004), it is likely that the effects observed with progestins in animals may not always be reflected in humans. For example, humans only have two 3βHSD isoforms, while rodents have 6 isoforms (Miller and Auchus, 2011; Payne and Hales, 2004). Furthermore, human CYP17A1 has a preference for Δ⁵ steroids, while the rodent enzyme favors Δ⁴ steroids as substrates (Brock and Waterman, 1999). Nevertheless, it is possible that progestins that do affect the activity of 3βHSD2 and CYP17A1 may have physiological implications when used clinically, especially considering that the Kᵢ values determined for 3βHSD2 are in the nanomolar range.

4.2.2. SRD5A

The biosynthesis of androgens also requires additional steroidogenic enzymes (Figs. 1–3), such as SRD5A which converts testosterone to the more potent androgen DHT in peripheral tissues. LNG, NET, DNG, CPA and GES have all been shown to inhibit the activity of SRD5A, resulting in decreased DHT production in the genital skin of women (Cassidenti et al., 1991; Rabe et al., 2000). In contrast, Cassidenti et al. showed that MPA does not modify the production of DHT in human genital skin, suggesting that the effects of progestins on the activity of SRD5A may be progestin-dependent (Cassidenti et al., 1991). To our knowledge, effects of progestins on other enzymes involved in the biosynthesis of androgens remain to be elucidated.

4.2.3. CYP19A1, 17βHSD and STS

Few studies have investigated the effects of progestins on enzymes involved in the biosynthesis of estrogens, and the available studies in the literature mostly focus on effects on CYP19A1, 17βHSD and STS in breast carcinoma cell lines. Increased expression and/or activity of these enzymes are associated with increased production of E₂, which in turn may lead to increased development of breast cancer. CYP19A1 mRNA expression and activity was increased when human MCF-7 breast cancer cells, stably transfected to express CYP19A1, were treated with equimolar concentrations of MPA and E₂ (Xu et al., 2007). These authors also showed that the endogenous mRNA expression and activity of 17βHSD1 was increased in T47D breast cancer cells, as well as that of endogenous STS in MCF-7 cells. These increases were not observed with E₂ alone, suggesting that MPA was responsible for the observed effects on these enzymes. Similarly, Sivik et al. showed that MPA upregulated the mRNA expression of 17βHSD1 and 17βHSD5 in MCF-7 breast cancer cells, as well as 17βHSD5 in T47D cells, while downregulating 17βHSD2 mRNA expression (Sivik et al., 2012), suggesting increased biosynthesis of E₂. Collectively, these results suggest that MPA promotes the local production of E₂ in breast cancer cell lines. In contrast, progestins such as NET, LNG and DNG in combination with E₂ had no effect on the mRNA expression or activity of CYP19A1, STS and 17βHSD1 (Xu et al., 2007). It is important to note that the concentrations of the progestins used in these studies correlate to those used in contraception and HRT. Other studies investigating the effects of MPA and NET, at doses used in cancer therapy, showed that these progestins decrease the activity of STS in both the MCF-7 and T47D breast cancer cell lines (Chetrite et al., 1997; Pasqualini et al., 2003). Similarly, studies by the same group have shown that the cancer therapy doses of NoMAC inhibit the activity of both STS and 17βHSD1 in the above-mentioned cell lines (Chetrite et al., 1996; Pasqualini et al., 1995; Shields-Botella et al., 2005). Considering that progestins are used in HRT to prevent estrogen-induced hyperplasia of the endometrium (Campagnoli et al., 2005; Pike and Ross, 2000) or as medical treatment for endometriosis, an estrogen-dependent disease (Kitawaki et al., 2002; Rizner, 2009), some studies have also investigated the effects of MPA and DNG on local estrogen production in endometrial cells. These studies report a decrease in the mRNA expression of CYP19A1 and 17βHSD1 in the Z-12 human endometrial epithelial cell line (Beranic and Rizner, 2012), and CYP19A1 in a different human endometrial epithelial cell line, EM-PR (Shimizu et al., 2011). Interestingly, MPA and DNG also decreased the mRNA expression of CYP19A1 in mice implanted with human endometrial fragments (Fechner et al., 2007).

4.2.4. CYP21A1, CYP11B1/2 and 11βHSD1/2

To our knowledge, no studies have investigated the direct effects of the progestins on the activity and/or mRNA expression of steroidogenic enzymes involved in the synthesis of glucocorticoids and mineralocorticoids. Our recent study is thus the first to show that all the progestins used in our study (MPA, NET-A, LNG, GES, NES, NoMAC and DRSP) had no effect on the activity CYP21A1 transfected into COS-1 cells (Louw-du Toit et al., In press). Interestingly, although we do not show direct effects on the activity of 11βHSD2, we showed that NES decreased the concentration of cortisol, while increasing the concentration of cortisone in human H295R adrenal cells, alluding to an increase in the activity of this enzyme.

5. Conclusions

Despite the beneficial effects of progestins in endocrine therapy, concerns have been raised about the side-effects associated with their clinical use. Many side-effects are believed to be due to off-target effects elicited via steroid receptors other than the PR.

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However, it is known that the biological activities of these structurally diverse progestins are not only determined by their actions via steroid receptors, but may also be influenced by factors such as metabolism, bioavailability and the regulation of endogenous steroid hormone biosynthesis. Studies on the latter are few and far between, as indicated by the small number of studies reviewed here, and to our knowledge we have reviewed most, if not all, of the studies that have previously investigated the effects of progestins on the biosynthesis of endogenous steroid hormones. One of the most striking observations is the fact that effects are mostly examined in animal and cell culture models, while human studies are severely lacking. The limited number of human studies that are however available, measured the serum levels of specific endogenous steroid hormones and did not investigate the enzymes involved. The few studies that do in fact investigate effects on steroidogenic enzymes are limited to effects in the gonads and the breast, while effects in the adrenal are mostly overlooked.

From the available data summarised in this review, it is clear that the effects for the same progestin often differs between animal, cell culture and clinical studies. This highlights the fact that animal and cell culture experiments, although informative, may not reflect effects in humans and are thus not sufficient to infer effects in women using progestins in hormone therapy. Moreover, the majority of clinical and molecular studies investigate effects of the first-generation progestin MPA. This is probably due to the fact that MPA has been available for many years and is used by women worldwide, as well as the fact that its structure closely resembles that of 17OH-Prog, a metabolite in steroid biosynthesis. As we know that all other progestins differ in their structures and pharmacological properties, it is impossible to draw conclusions about the effects of all progestins from effects exhibited by MPA. Results from the limited studies that have in fact investigated effects of progestins other than MPA, together with the studies evaluating MPA, are inconsistent. However, it appears that the modulation of steroid hormone biosynthesis is dependent on the type and dosage of the progestin used in the different types of therapies, and whether it is a progestin-only therapy versus a therapy where the progestin is combined with an estrogen component. It would thus be useful for these various factors to be comparatively evaluated in future clinical studies to improve our understanding of whether, and to what extent, progestins modulate endogenous steroid hormone production. In addition to clinical data, molecular data is also needed to understand the mechanisms of progestin effects on steroid biosynthesis. For example, whether progestin metabolites, rather than the parent compound, or even both, are responsible for some effects needs to be elucidated. Moreover, given that some human studies do allude to inhibitory effects of progestins on steroidogenic enzymes, it would be of interest to elucidate which enzymes are involved, what type of inhibition is involved and if the inhibition constants are within the expected serum concentrations found in women using progestins in different applications. Our recent *in vitro* study has shown that the fourth-generation progestins NES, NoMAC and DRSP inhibit human 3βHSD2, with the K_i values determined found to be within the serum concentrations reported in contraceptive users of these progestins.

Data from the suggested clinical and molecular studies will improve our understanding of the relative benefit to risk ratio of progestins as well as their mechanisms of action, particularly in terms of effects of progestins on endogenous steroid biosynthesis. Considering that progestins are widely used by women as contraception or treatment of menopausal symptoms such studies will provide answers that may aid in the type and concentration of progestins used in hormone therapy.

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A4: Conference Outputs

A4.1 National Conferences

1. **Cartwright, M**., Louw-du Toit, R., Hapgood, J., Africander, D. Progestin-Induced Breast Cancer: Investigating the Role of the PR isoforms. 25th SASBMB Congress, East London Convention Centre, South Africa. 10 - 14 July 2016.

A4.2 International Conferences

1. **Cartwright, M**., Louw-du Toit, R., Africander, D. Progestin-Induced Breast Cancer: Identifying the Role of Progesterone Receptor Isoforms. Society for Endocrinology/British Endocrine Society International Conference, Scottish Events Campus, Glasgow, Scotland, UK. 19-21 November 2018. (Presented by Africander, D) (Published Abstract – see below) (*Endocrine Abstracts,* Volume 59, pages 121)

2. **Cartwright, M**., Louw-du Toit, R., Africander, D. Progestins and the Progesterone Receptor Isoforms: Friend or Foe for Breast Cancer? 17th International ICHSHC Congress, STIAS, Stellenbosch, South Africa. 26 - 29 November 2018.

3. Skosana, S., Woodland, J., **Cartwright, M**., Enfield, K., Komane, M., Louw-du Toit, R., Avenant, C., Storbeck, K., Africander, D., Hapgood, J. Differential *in vitro* metabolism of clinically relevant progestins used in contraception. 17th International ICHSHC Congress, STIAS, Stellenbosch, South Africa. 26 - 29 November 2018.

4. **Cartwright, M**., Louw-du Toit, R., Africander, D. Progesterone Receptor Isoforms: Defining a Role in Progestin-Induced Breast Cancer. 18th International Congress of Endocrinology/53rd SEMDSA Congress, CTICC, Cape Town, South Africa. 1 - 4 December 2018.

Published Abstract

Here, we delineate the oncogenic mechanisms of PBF, along with its binding partner PTTG, in head and neck cancer (HNSCC), in which TP53 mutations (mutTP53) are common ($>50\%$). HNSCC tissue revealed significant upregulation of PBF and PTTG mRNA (>1.6-fold), which was consistent with a TCGA cohort ($n=520$). Importantly, a panel of 129 p53-target genes showed a more significant correlation with PBF ($P=0.0006$) and PTTG ($P=5.9\times10^{-9}$) expression in TCGA than the background transcriptome ($n=19,764$ genes), supporting a functional relationship. In agreement, there were significant mRNA changes in PBF- and PTTG-depleted HNSCC cells for key p53-responsive genes such as BCL2. Co-immunoprecipitation studies confirmed that PBF and PTTG are specific interactors of p53 in HNSCC. PTTG retained the ability to bind p53 in the absence of PBF, but the degree of interaction was significantly attenuated (4-fold) suggesting that PBF facilitates binding of PTTG to p53. Half-life studies showed that PBF and PTTG inhibit p53 stability, with joint over-expression giving the most pronounced decrease (~13-fold). HNSCC TCGA patients with mutTP53 and high PBF/PTTG showed poorer overall survival (median$=28.98$ months) than those with low PBF/PTTG (median$=71.16$ months). A significant increase in the incidence of metastatic disease was further evident for wtTP53 HNSCC with high PBF/PTTG expression. In summary, our results indicate that PBF and PTTG functional interaction is not confined to endocrine cancers. HNSCC patients with high tumoural PBF/PTTG have worse outcomes due in part to greater aberration of p53-dependent signalling. These findings may be of relevance to poorly differentiated or anaplastic thyroid cancers which have a higher incidence of TP53 alterations than WDTC.

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P120

Progestins used in menopausal hormone therapy is not a 'one-size-fits-all' for breast cancer risk

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Women worldwide are using progestins in combination with an estrogen to relieve menopausal symptoms. Although the progestin component of menopausal hormone therapies is effective in terms of preventing estrogen-induced endometrial cancer, it has been associated with an increased risk of developing invasive breast cancer. Notably, most studies investigating an association between progestins and breast cancer, have examined older progestins such as medroxyprogesterone acetate, norethisterone and levonorgestrel. Considering that a variety of progestins with distinct structures and functions are available, it is possible that not all progestins increase breast cancer risk. Our study directly compared the effects of selected progestins on the mRNA expression of genes that are markers for specific tumour cell behaviours such as proliferation and apoptosis, and showed that progestins differentially regulate the expression of these genes. Moreover, we investigated the role of signal transduction pathways in progestin-induced regulation of the above-mentioned processes. Specifically, we examined pathways known to play a crucial role in growth, survival and metastasis, such ERK1/2 and JNK. All progestins, except the newer progestin drospirenone (DRSP), increased proliferation and migration of the human T47D breast cancer cell line to the same extent. DRSP was also the only progestin that did not stimulate phosphorylation of the ERK1/2 and JNK pathways in the T47D cells. Moreover, blocking activation of these kinase pathways by highly selective inhibitors prevented the effects all the progestins, except DRSP, on proliferation, apoptosis and migration. These results suggest that activation of the ERK1/2 and JNK pathways may be a mechanism by which the older progestins increase breast cancer risk. Finally, the results also suggest that DRSP may promote breast cancer pathogenesis to a lesser extent that the older progestins used in this study.

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P121

Progestin-induced breast cancer: Identifying the role of progesterone receptor isoforms

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Breast cancer is the most common oncology-related cause of death in women worldwide. The use of progestins in combined hormone replacement therapy (HRT) has been implicated in increasing the risk of developing breast cancer in postmenopausal women. Since various progestins are available for clinical use, all differentiated by structure, it is possible that not all progestins would lead to increased breast cancer risk. Progestins are synthetic ligands of the progesterone receptor (PR), designed to mimic the actions of natural progesterone. Although the PR exists as two isoforms, PR-A and PR-B, studies investigating the role of the PR in breast cancer seldom distinguish between the two isoforms. This is important, as the isoforms are functionally distinct and present in equimolar concentrations in the normal breast, while PR-A is overexpressed in breast cancer. The current study investigated the role of PR-A and PR-B in mediating progestin-induced regulation of genes involved in breast cancer biology, as well as their respective roles in physiological processes involved in breast cancer development and progression. In addition, effects of overexpression of PR-A relative to PR-B on the above-mentioned responses, was also investigated. Results showed differential regulation of genes by progestins via the individual PR isoforms. Moreover, effects on physiological processes such as cell proliferation, apoptosis, migration and invasion were progestin- and isoform-specific. These results not only highlight the importance of studying effects of individual progestins, but also effects via the individual PR isoforms. Moreover, in the presence of most progestins, overexpression of PR-A relative to PR-B inhibited physiological processes involved in breast cancer development and progression, suggesting that enhanced PR-A expression may be a positive prognostic marker for breast cancer.

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P122

The human oestrogen receptor beta variant 5 (ERß5) can alter the oestrogen sensitivity of oestrogen receptor alpha positive endometrial cancer cells

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Endometrial cancer is the most common gynaecological malignancy in the developed world: lifetime exposure to oestrogen is a key risk factor. Oestrogen action is mediated by ligand activated receptors encoded by the *ESR1* (ERα) and *ESR2* (ERß) genes: ERα plays a key role in regulating endometrial cell proliferation. ERß5, is a truncated variant isoform of ERß formed by alternative splicing of *ESR2* that contains a DNA binding domain but lacks the ability to bind E2. ERß5 is expressed in endometrial cancer tissue but its functional impact is unknown. Double fluorescent immunostaining for ERα and ERß5 was performed on sections of endometrial adenocarcinomas recovered from post-menopausal women ($n=271$) undergoing total abdominal hysterectomy. Reproductive cell lines where infected with lentivirus expressing an ERß5 construct to generate cells with altered ratios of ERß5/ERα to examine the functional impact in an ERE reporter assay. A lentivirus YFP-ERß5 construct was used to investigate intra-nuclear mobility (FRAP) in the cell lines. Fluorescent immunohistochemistry detected cells co-expressing ERß5 and ERα in stage I cancers. Co-expression of ERß5 in an ERα^{pos} endometrial cancer cell line (Ishikawa) increased ligand-dependent activation of an ERE-luciferase reporter by the ERα-selective ligand PPT. FRAP analysis of YFP-ERß5 in Ishikawa cells revealed incubation with E2 resulted in a transient reduction in intra-nuclear mobility. In ERα^{neg} MDA breast cancer cells, there was no E2-dependent change in mobility of YFP-ERß5 or activation of the reporter gene. Our results show ERß5 can act as heterodimeric partner to ERα in cells of endometrial stage 1 cancers that may increase their sensitivity to E2. These data suggest immunostaining for ERß5 should be considered in risk assessment of women with stage I endometrial cancers as they could benefit from treatment with drugs that block receptor dimerisation.

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P123

ELL2 and EAF2 co-regulation of AKT in prostate cancer cells

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Elongation factor, RNA polymerase II, 2 (ELL2) is an RNA Pol II elongation factor with functional properties similar to ELL that can interact with the prostate

Addendum B
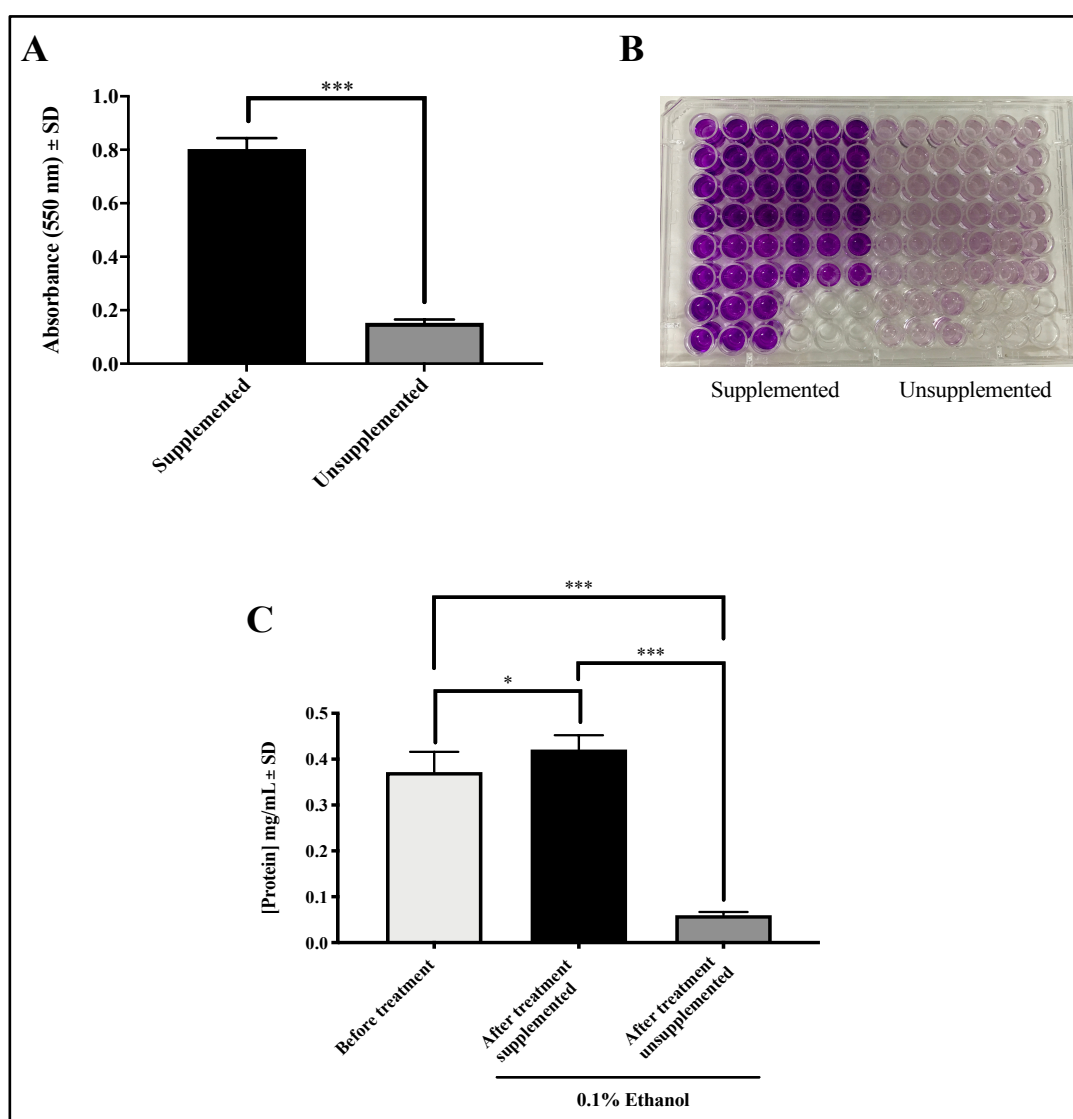
Additional experimental data supporting

Chapter 2

Table B1. The molecular ion species, multiple reaction monitoring (MRM) mass transitions and retention times of the steroids used in Chapter 2.

Steroid (Abbreviation)	Mass Transition Quantifier Ion	Mass Transition Qualifier Ion	Retention Time (minutes)
Promegestone (R5020)	327.0 > 161.0	327.0 > 135.0	0.98
Progesterone (P_4)	315.2 > 97.1	315.2 > 109.1	0.89
Gestodene (GES)	311.2 > 109.4	311.2 > 135.0	1.36
Nomegestrol acetate (NOMAC)	371.3 > 175.1	371.3 > 209.2	1.13
Drospirenone (DRSP)	367.2 > 97.3	367.2 > 159.3	1.75
Testosterone (T)	289.2 > 97.2	289.2 > 109.0	1.58

Figure B1. Loss of MDA-MB-231 cell viability in unsupplemented DMEM. MDA-MB-231 cells were transiently transfected with 900 ng pSG5-hPR-B cDNA expression vector. The cells were treated with 0.1 % (v/v) ethanol (vehicle control) in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS and 1% penicillin-streptomycin or in unsupplemented phenol red-free DMEM, for 24 hours. The colorimetric MTT assay was used to assess cell viability **(A and B),** and the Bradford protein assay to measure protein concentration **(C). (A)** Shows the absorbance values at 550 nm for the supplemented and unsupplemented treatments, while **(B)** is a photograph of the 96-well tissue culture plate following the colorimetric assay. **(C)** The protein concentrations (mg/mL) of the cells before treatment, as well as after treatment with 0.1% ethanol in either supplemented DMEM or serum-free DMEM. The results shown are the averages (±SD) of two independent experiments. **(A)** An unpaired *t*-test was used to determine statistical difference between the supplemented and unsupplemented samples, while **(C)** one-way ANOVA with Bonferroni (compares all pairs of columns) post-test was used compare cell viability between the before treatment and after treatment samples. Statistically significant differences are represented by * and ***, indicating $p < 0.05$ and $p < 0.001$, respectively.

Addendum C

Additional experimental data supporting

Chapter 3

C1: Table comparing the relative efficacy values of all progestogens at all densities of PR-A (1x, 2x and 5x), PR-B (1x), as well as the different PR-A:PR-B ratios.
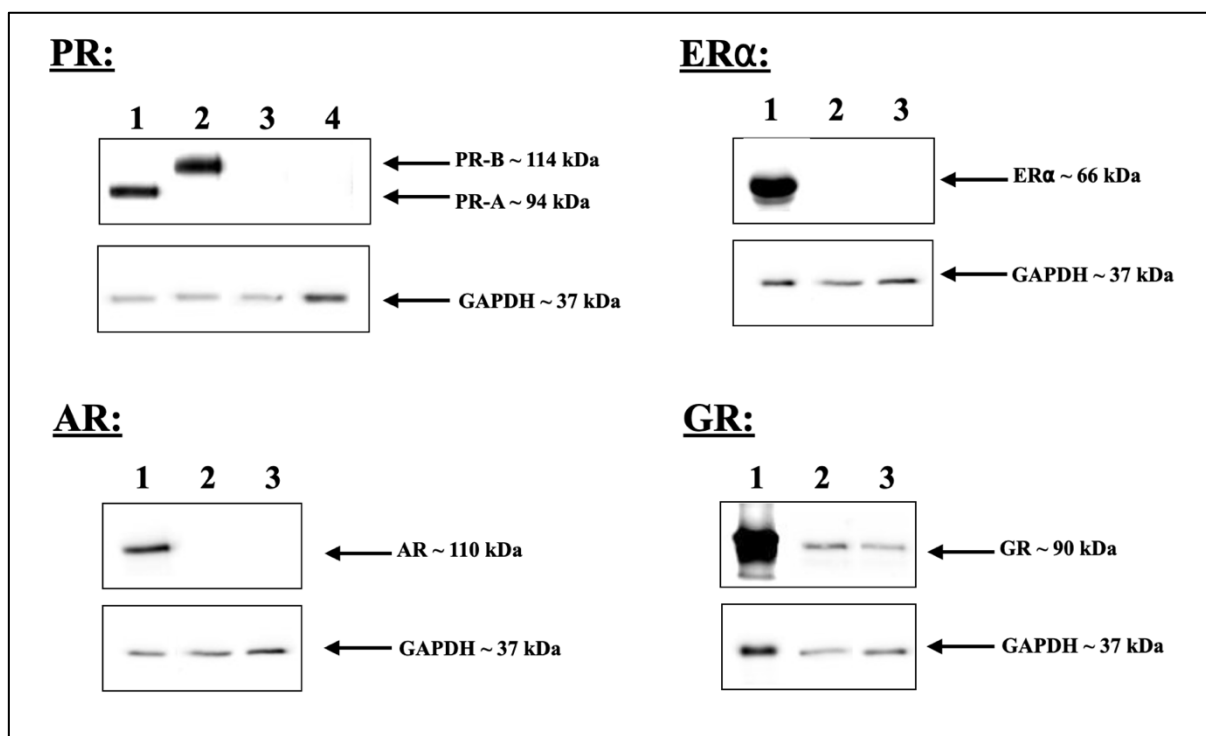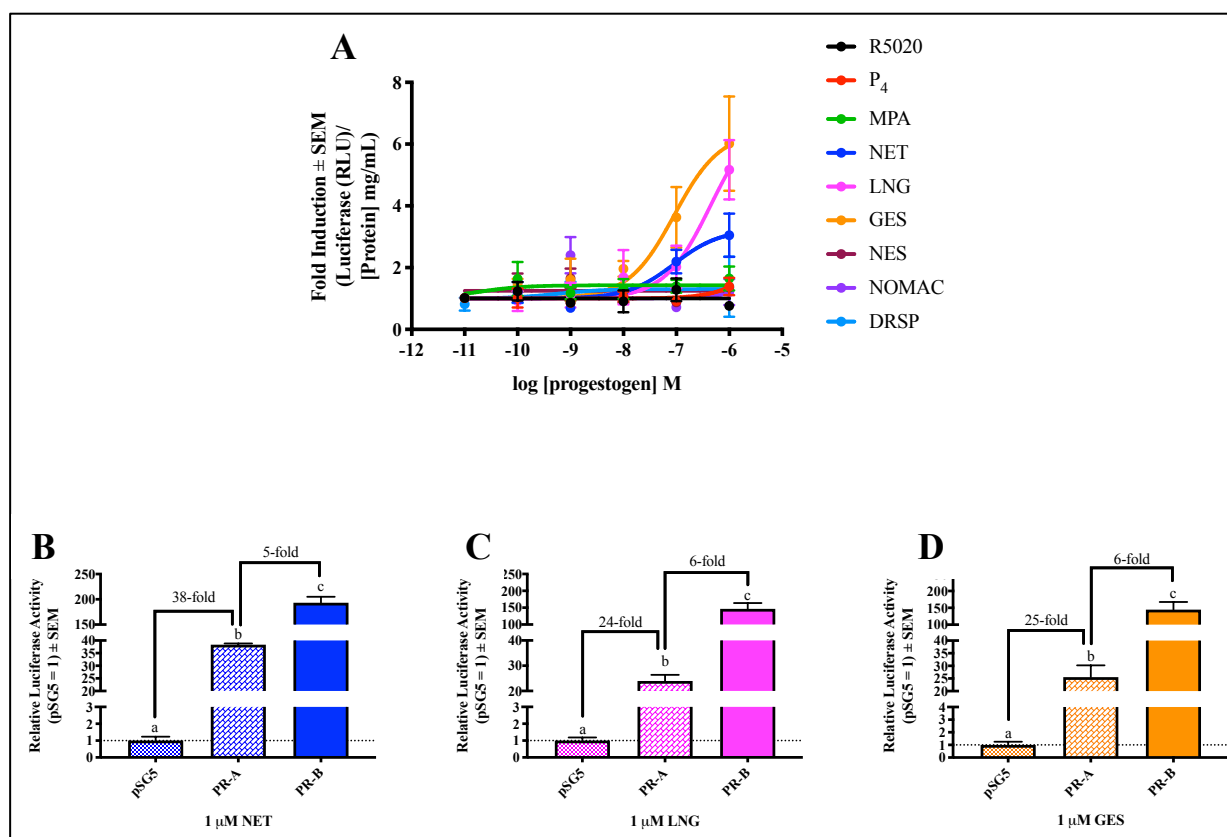
	Maximal response (R5020 Max = 100%) ± SEM[*]						
Progestogen	*PR-A (1x)*	*PR-B (1x)*	*1:1*	*2:1*	*5:1*	*PR-A (2x)*	*PR-A (5x)*
R5020	100.0 ± 5.2	100.0 ± 3.5	100.0 ± 6.1	100.0 ± 5.0	100.0 ± 3.7	100 ± 4.3	100 ± 3.1
P₄	47.1 ± 3.3	100.4 ± 2.9	61.8 ± 5.8	63.1 ± 2.6	60.7 ± 5.3	47.1 ± 3.8	57.9 ± 6.0
MPA	43.4 ± 2.5	55.9 ± 3.6	57.6 ± 3.5	54.5 ± 4.0	37.8 ± 2.8	37.3 ± 3.3	44.2 ± 3.8
NET	74.1 ± 6.	80.9 ± 4.6	75.9 ± 6.0	54.7 ± 3.7	50.5 ± 3.8	47.6 ± 3.6	56.0 ± 4.7
LNG	81.8 ± 5.5	83.8 ± 3.1	80.8 ± 6.0	70.2 ± 4.2	68.2 ± 4.8	52.9 ± 4.0	88.6 ± 5.4
GES	74.1 ± 3.7	99.1 ± 4.3	104.3 ± 5.2	98.2 ± 5.7	86.9 ± 5.7	69.0 ± 5.4	95.4 ± 6.4
NES	57.0 ± 3.7	84.5 ± 4.8	85.0 ± 4.2	54.8 ± 4.1	46.2 ± 3.6	39.7 ± 3.2	54.3 ± 2.3
NOMAC	43.0 ± 2.7	60.6 ± 3.9	53.7 ± 3.6	40.1 ± 3.5	42.9 ± 2.8	26.4 ± 2.3	56.4 ± 5.6
DRSP	64.0 ± 4.2	55.6 ± 4.3	73.5 ± 6.7	49.8 ± 3.4	37.8 ± 3.9	60.7 ± 3.8	47.6 ± 5.5

[*]Dose-response curves were used to determine the efficacy values for P_4 and the selected progestins from the four generations relative to the maximal response of R5020 for each transfection condition set as 100%.

C2: Table comparing the relative potency values of all progestogens at all densities of PR-A (1x, 2x and 5x), PR-B (1x), as well as the different PR-A:PR-B ratios.

	EC_{50} (pM) ± SEM[*]						
Progestogen	*PR-A (1x)*	*PR-B (1x)*	*1:1*	*2:1*	*5:1*	*PR-A (2x)*	*PR-A (5x)*
R5020	81.3 ± 28.4	18.03 ± 6.2	36.8 ± 17.6	154 ± 65.5	9.7 ± 2.9	976 ± 216	1437 ± 280
P₄	2.0 ± 1.3	791 ± 265	0.62 ± 0.34	0.06 ± 0.04	0.45 ± 0.44	0.01 ± 0.008	87.06 ± 71.6
MPA	4.9 ± 2.9	0.71 ± 0.57	0.04 ± 0.02	0.15 ± 0.12	0.02 ± 0.01	0.01 ± 0.007	0.03 ± 0.02
NET	8.5 ± 7.1	399.7 ± 180	272 ± 175	0.76 ± 0.52	0.50 ± 0.37	0.012 ± 0.01	0.07 ± 0.05
LNG	0.7 ± 0.4	8.22 ± 3.82	23.7 ± 15.1	0.27 ± 0.12	0.38 ± 0.27	0.13 ± 0.12	6.6 ± 3.7
GES	0.08 ± 0.05	3.3 ± 1.3	0.8 ± 0.4	8.3 ± 4.1	5.5 ± 3.1	40.9 ± 25.8	36.0 ± 18.5
NES	0.04 ± 0.03	7.9 ± 3.5	0.26 ± 0.14	0.05 ± 0.04	0.24 ± 0.19	0.21 ± 0.13	0.08 ± 0.03
NOMAC	2.3 ± 1.6	0.4 ± 0.2	0.07 ± 0.05	0.06 ± 0.05	0.05 ± 0.04	0.062 ± 0.04	0.6 ± 0.4
DRSP	0.1 ± 0.09	1707 ± 775	0.11 ± 0.09	0.53 ± 0.39	254 ± 127	0.13 ± 0.08	0.04 ± 0.02

[*]Dose-response curves were used to determine the potency values for P_4 and the selected progestins from the four generations relative to the maximal response of R5020 for each transfection condition set as 100%.
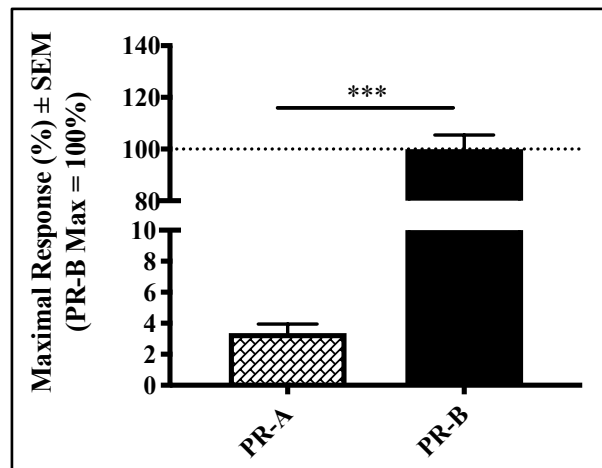
Figure C1. Endogenous steroid receptor expression characterization of MDA-MB-231 breast cancer cells. Total protein of MDA-MB-231 cells was harvested to perform western blot analysis using antibodies specific for the PR (PR-A and PR-B), ERα, AR, GR as well as GAPDH (loading control). Representative western blots are shown. **PR blot:** Lane 1, PR-A positive control (PR-A transfected COS-1 cells); Lane 2, PR-B positive control (PR-B transfected COS-1 cells; Lane 3, untransfected MDA-MB-231 cells; Lane 4, negative control (pSG5-empty transfected COS-1 cells). **ERα/AR/GR blots:** Lane 1, positive control (receptor transfected COS-1 cells); Lane 2, untransfected MDA-MB-231 cells; Lane 3, negative control (pSG5-empty transfected COS-1 cells). Low levels of the GR was detected in the untransfected COS-1 cell line (negative control) as the GR is ubiquitously expressed in these cells.

Figure C2. NET, LNG and GES display negligible activity in the PR negative MDA-MB-231 cells. Human MDA-MB-231 cells were transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase reporter construct and 900 ng of either the pSG5-empty vector, pSG5-PR-A or pSG5-PR-B expression vectors. **(A)** Cells transfected with the pSG5-empty vector were treated with either 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 24 hours. Luciferase activity was measured as relative light units (RLU) and normalised to protein concentration (mg/mL). The results in **(A)** are shown as fold induction where all responses are set relative to the vehicle, set as 1. **(B-D)** The relative luciferase activity is shown for 1 µM **(B)** NET, **(C)** LNG and **(D)** GES in cells transfected with the pSG5-empty vector**,** pSG5-PR-A or pSG5-PR-B, with the response of the pSG5-empty vector set as 1, and all other responses calculated relative to this. The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by letters a, b and c, where the values that differ significantly from other values are assigned a different letter.
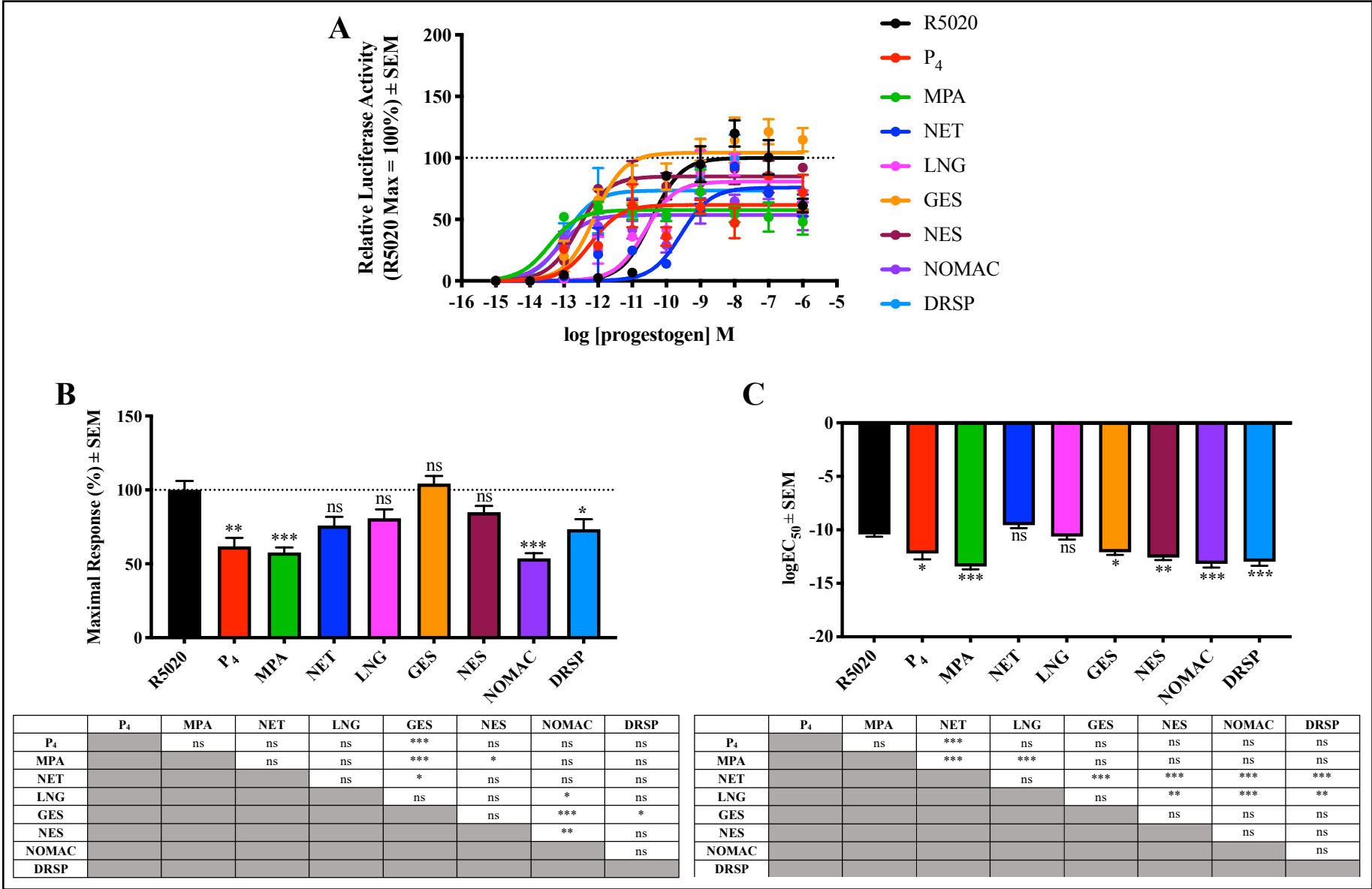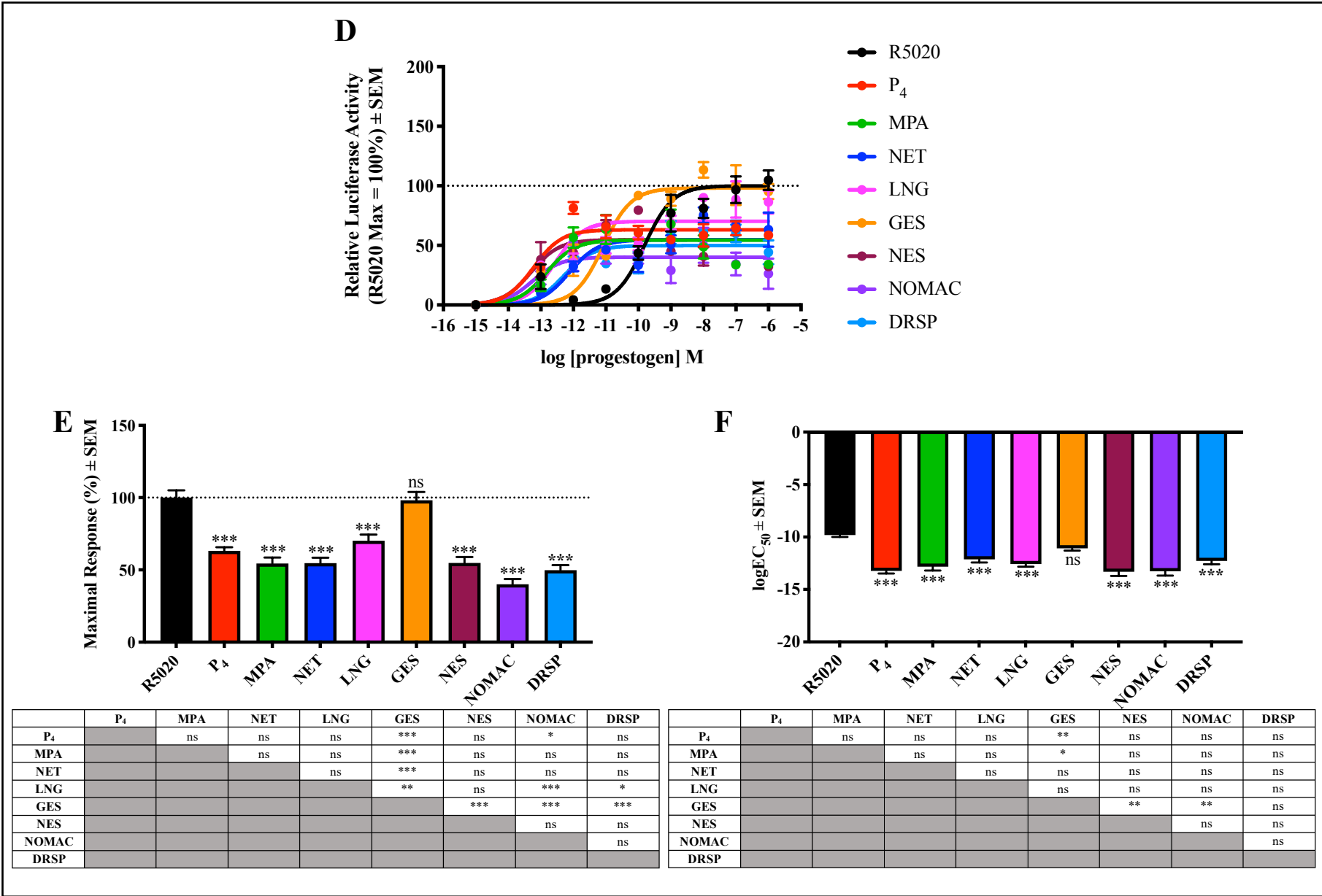
Figure C3. In the presence of R5020, PR-B is more transcriptionally active than PR-A. The MDA-MB-231 cells were transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase reporter construct and 900 ng pSG5-PR-A or pSG5-PR-B. Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of R5020 for 24 hours. Luciferase activity was normalised to protein concentration and the maximal response of R5020 via PR-A was set relative to the maximal response of R5020 via PR-B, set to 100%. Results shown are representative of at least three independent experiments with each condition performed in triplicate (±SEM). An unpaired *t*-test was used to determine the statistically significant difference between PR-A and PR-B with *** representing $p < 0.001$.

Figure C4. Increasing the concentration of PR-A decreases ligand independent effects on transcription. MDA-MB-231 cells transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase reporter construct and either 900 ng (1x), 1 800 ng (2x) or 4 500 ng (5x) pSG5-PR-A, were treated with 0.1% (v/v) ethanol (vehicle control) for 24 hours. Luciferase activity was normalised to protein concentration. Relative luciferase activity is shown, with the vehicle response in cells transfected with 2x and 5x PR-A, set relative to the vehicle response at 1x PR-A (100%). The result shown is the average (± SEM) of at least two independent experiments with each condition performed in triplicate. Unpaired *t*-tests were used for statistical analysis and statistically significant differences are represented by a and b, where the values that differ significantly from other values are assigned a different letter.

Figure C5. continues on the following page.

D

E

	P₄	MPA	NET	LNG	GES	NES	NOMAC	DRSP
P₄		ns	ns	ns	***	ns	*	ns
MPA			ns	ns	***	ns	ns	ns
NET				ns	***	ns	ns	ns
LNG					**	ns	***	*
GES						***	***	***
NES							ns	ns
NOMAC								ns
DRSP								

F

	P₄	MPA	NET	LNG	GES	NES	NOMAC	DRSP
P₄		ns	ns	ns	**	ns	ns	ns
MPA			ns	ns	*	ns	ns	ns
NET				ns	ns	ns	ns	ns
LNG					ns	ns	ns	ns
GES						**	**	ns
NES							ns	ns
NOMAC								ns
DRSP								

Figure C5. continues on the following page.

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Figure C5. Figure legend on the following page.

Figure C5. The relative agonist efficacy and potency of the progestogens are dependent on the PR isoform ratio. The MDA-MB-231 cells were transiently transfected with 9 000 ng pTAT-2xPRE-E1b-luciferase reporter construct together with 900 ng pSG5-PR-B in combination with **(A)** 900 ng (1:1), **(D)** 1 800 ng (2:1) or **(G)** 4 500 ng (5:1) pSG5-PR-A. The cells were treated with either the 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 24 hours. Luciferase activity was normalised to protein concentration and results are shown as relative luciferase activity for which the maximal response with R5020 was set to 100% with the other responses calculated relative to this. Maximal response and logEC$_{50}$ values of the progestogens for PR-A and PR-B in the following ratios, **(B and C)** 1:1, **(E and F)** 2:1, **(H and I)** 5:1 are plotted. The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented by *, ** and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns. Statistically significant differences relative to R5020 (reference agonist) are shown on the graph, while significant differences between progestogens are indicated in the tables below the graph.

Figure C6. Figure legend on the following page.

Figure C6. The efficacies of all progestogens decrease when excess PR-A is co-expressed with PR-B. MDA-MB-231 cells transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase reporter construct and either 900 ng pSG5-PR-B only or in combination with 900 ng (1:1), 1 800 ng (2:1) or 4 500 ng (5:1) pSG5-PR-A, were treated with either the 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 24 hours. Luciferase activity was normalised to protein concentration. Responses of the progestogens in the presence of different PR-A:PR-B ratios were set relative to the maximal response of R5020 via PR-B set as 100%. Results shown are representative of at least three biological repeats with each condition performed in triplicate ($\pm$SEM).

Figure C7. The ratio of PR-A and PR-B expression influences ligand independent effects. MDA-MB-231 cells transiently transfected with 9 000 ng of the pTAT-2xPRE-E1b-luciferase reporter construct and either 900 ng pSG5-PR-B only, or in combination with 900 ng (1:1), 1 800 ng (2:1) or 4 500 ng (5:1) pSG5-PR-A, were treated with 0.1% (v/v) ethanol (vehicle control) for 24 hours. Luciferase activity was normalised to protein concentration. Relative luciferase activity is shown with the vehicle response in cells transfected with PR-A and PR-B at a 1:1, 2:1 and 5:1 ratio, set relative to the vehicle response with PR-B only (100%). Results shown are representative of at least three independent experiments with each condition performed in triplicate (±SEM). One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis, and statistically significant differences are represented by different letters a, b and c, where the values that differ significantly from other values are assigned a different letter.

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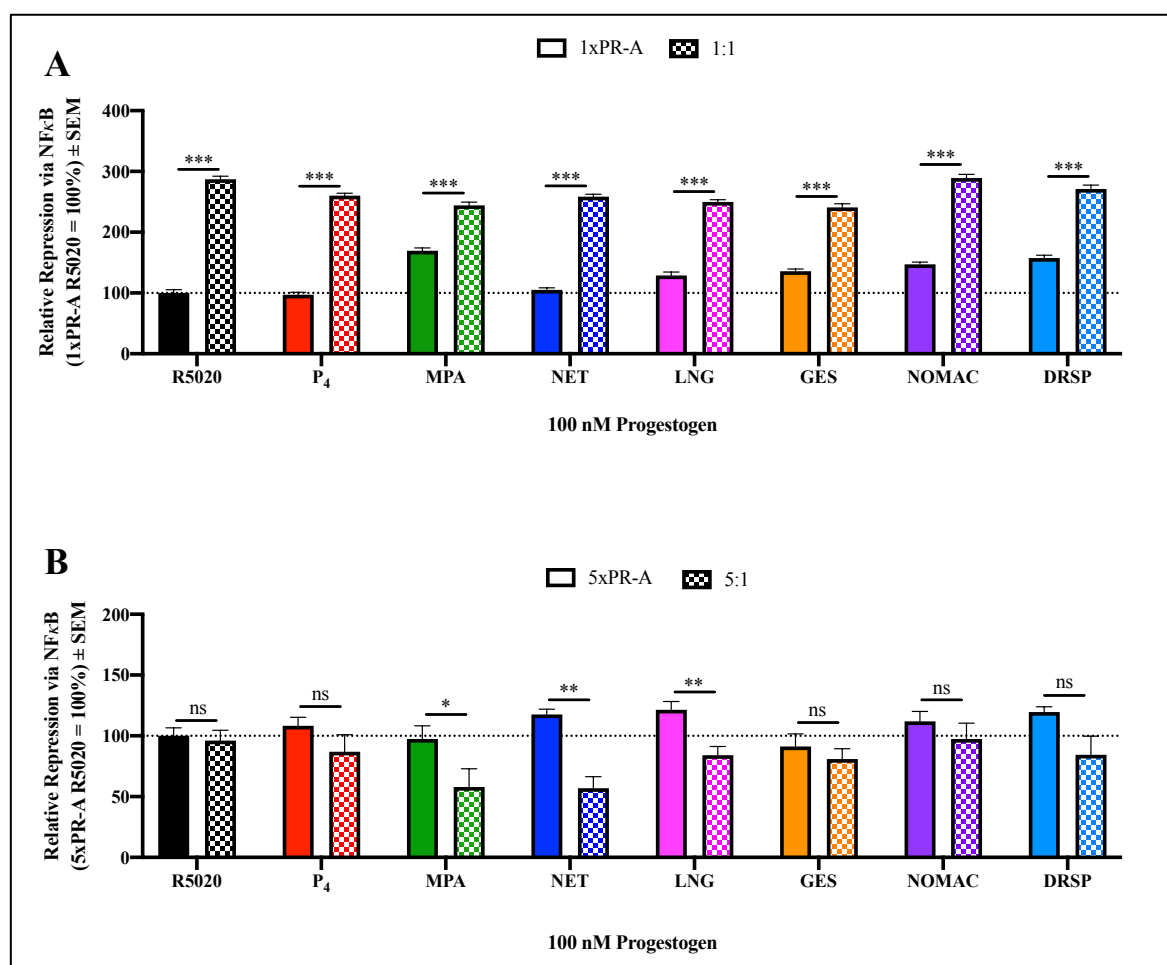
Figure C8. continues on the following page.

Figure C8. The presence of PR-B decreases the efficacy of all progestogens when co-expressed with excess PR-A. MDA-MB-231 cells transiently transfected with 9 000 ng pTAT-2xPRE-E1b-luciferase reporter construct and either **(A)** 1 800 ng (2x) or **(B)** 4 500 ng pSG5-PR-A (5x), in the absence or presence of 900 ng pSG5-PR-B, were treated with either the 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogens for 24 hours. Luciferase activity was normalised to protein concentration. Results are shown as relative luciferase activity for which the dose-response curves of the progestogens in the presence of PR-A:PR-B at 2:1 and 5:1 was set relative to the maximal response of R5020 set as 100% via **(A)** 2x PR-A or **(B)** 5x PR-A, respectively. The results shown are the averages (± SEM) of at least two biological repeats with each condition performed in triplicate. The efficacies **(C and D)** and potencies **(E and F)** of the progestogens via 2x **(C and E)** and 5x PR-A **(D and F)** in the absence and presence of PR-B is compared. Unpaired *t*-tests were used and statistical differences are represented by *, **, *** indicating p < 0.05, p < 0.01 and p < 0.001, respectively. Absence of stars indicate no significant differences (p > 0.05).
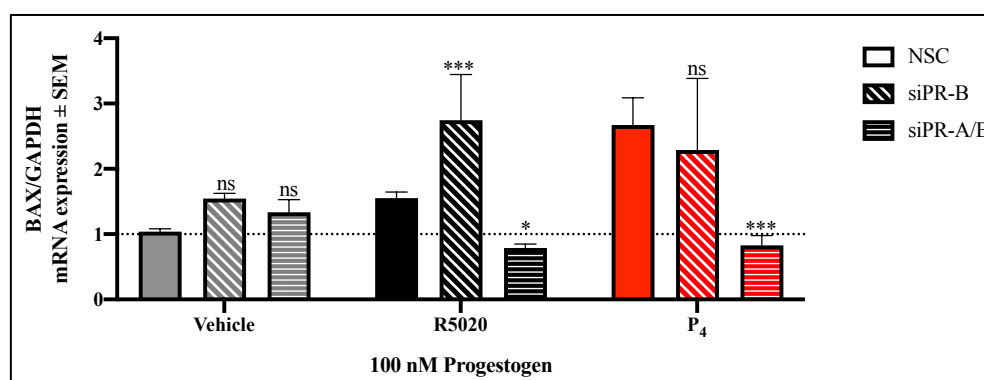
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Figure C9. Unlike the other progestins, NES displays agonist activity for transrepression via a synthetic NFκB-containing promoter in the MDA-MB-231 breast cancer cell line lacking PR-A and PR-B. (A) MDA-MB-231 cells transiently transfected with 3 000 ng of the 5xNFκB-luciferase reporter plasmid and 1 500 ng pSG5-empty vector, were treated with 0.2% (v/v) ethanol (vehicle control) or 10 ng/mL PMA in the absence or presence of 100 nM progestogens for 24 hours. Luciferase activity was measured and normalised to protein concentration. The PMA response was set as 100% and all other responses were calculated as a percentage of this. **(B) Repression by NES is similar in the absence and presence of PR-A or PR-B.** MDA-MB-231 cells transfected with the 5xNFκB-luciferase reporter plasmid and either 1500 ng pSG5-empty vector, pSG5-PR-A or pSG5-PR-B, were treated with 10 ng/mL PMA in the absence or presence of 100 nM NES for 24 hours. The % repression was set relative to the response of R5020 via PR-B, which was set as 100%. The results shown are averages (±SEM) of at least two biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences between PMA and the progestins are represented by * indicating $p < 0.05$, while statistically significant differences between the vehicle and PMA are represented by ### indicating $p < 0.001$. No statistical significance ($p > 0.05$) is indicated by ns.

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Figure C10. Repression by NES increases when MDA-MB-231 cells are transfected with 5x excess PR-A and when co-transfected with PR-A and PR-B (1:1 and 5:1). MDA-MB-231 cells transiently transfected with 3 000 ng of the 5xNFκB-luciferase reporter plasmid and 1 500 ng pSG5-empty vector, or 1 500 ng of both pSG5-PR-A and pSG5-PR-B, or 7 500 ng pSG5-PR-A only or in combination with 1 500 ng pSG5-PR-B, were treated with 0.2% (v/v) ethanol (vehicle control) or 10 ng/mL PMA in the absence or presence of 100 nM NES for 24 hours. Luciferase activity was measured and normalised to protein concentration. The % repression by NES was set relative to the response of NES in the pSG5-empty transfected cells, set as 100%. The results shown are averages (±SEM) of at least two biological repeats with each condition performed in triplicate. Unpaired *t*-tests were used for statistical analysis and statistically significant differences are represented by different letters a, b and c, where the values that differ significantly from other values are assigned a different letter.

Figure C11. Repression by all the progestogens increases when PR-A is co-expressed at equivalent levels to PR-B, while the presence of 5x excess PR-A causes a decrease in repression by MPA, NET and LNG. MDA-MB-231 cells transiently transfected with 3 000 ng of the 5xNFκB-luciferase reporter plasmid and 1 500 ng (1x) or 7 500 ng (5x) pSG5-PR-A in the absence and presence of 1x pSG5-PR-B were treated with 100 nM progestogen for 24 hours. The percentage repression by R5020 via **(A)** 1x PR-A or **(B)** 5x PR-A only was set as 100% and the percentage repression for the progestogens when PR-A and PR-B are co-expressed was set relative to this. The results shown are the averages (±SEM) of at least two biological repeats with each condition performed in triplicate. Unpaired *t*-tests were used for statistical analysis to assess differences between 1x PR-A and 5x PR-A in the absence and presence of PR-B, with statistically significant differences represented by *, ** or ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. No statistical significance ($p > 0.05$) is indicated by ns.
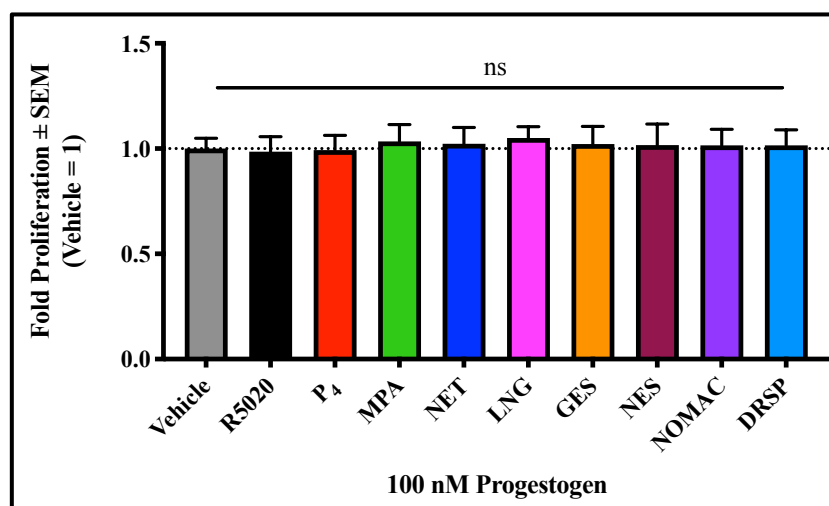
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Figure C12. BAX upregulation is abrogated when both PR isoforms are silenced. T47D cells were transiently transfected with either 10 nM non-silencing scrambled sequence control (NSC) siRNA or siRNA directed against only PR-B or both PR-A and PR-B. Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM R5020 and P_4 for 2 hours. Total RNA was isolated, cDNA synthesized and realtime qPCR performed to determine the relative mRNA expression levels of BAX. GAPDH was used as the reference gene. The responses of all progestogens were set relative to the vehicle control of the T47D cells transfected with the NSC siRNA set as 1. The results shown are averages (±SEM) of at least three biological repeats. Two-way ANOVA with Bonferroni's post-test was used for statistical analysis to compare the responses within both treatment groups after PR-B or PR-A/B siRNA knockdown to the NSC siRNA (first bar of every group). Statistically significant differences are represented with either *, or ***, indicating $p < 0.05$ and $p < 0.001$, respectively. No statistical differences ($p > 0.05$) are indicated by ns.

Addendum D

Additional experimental data supporting

Chapter 4

Figure D1. Progestogens have no effect on the proliferation of MDA-MB-231 cells in the absence of transfected PR. The MDA-MB-231 cells were transiently transfected with 900 ng pSG5-empty vector and subsequently treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM progestogen for 48 hours, then retreated for a further 48 hours. Proliferation was quantified using the colorimetric MTT cell viability assay. Results are shown as fold proliferation where the vehicle is set as 1, while all other responses are calculated relative to this. The result shown is the average (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis. No statistical significance ($p > 0.05$) is indicated by ns.
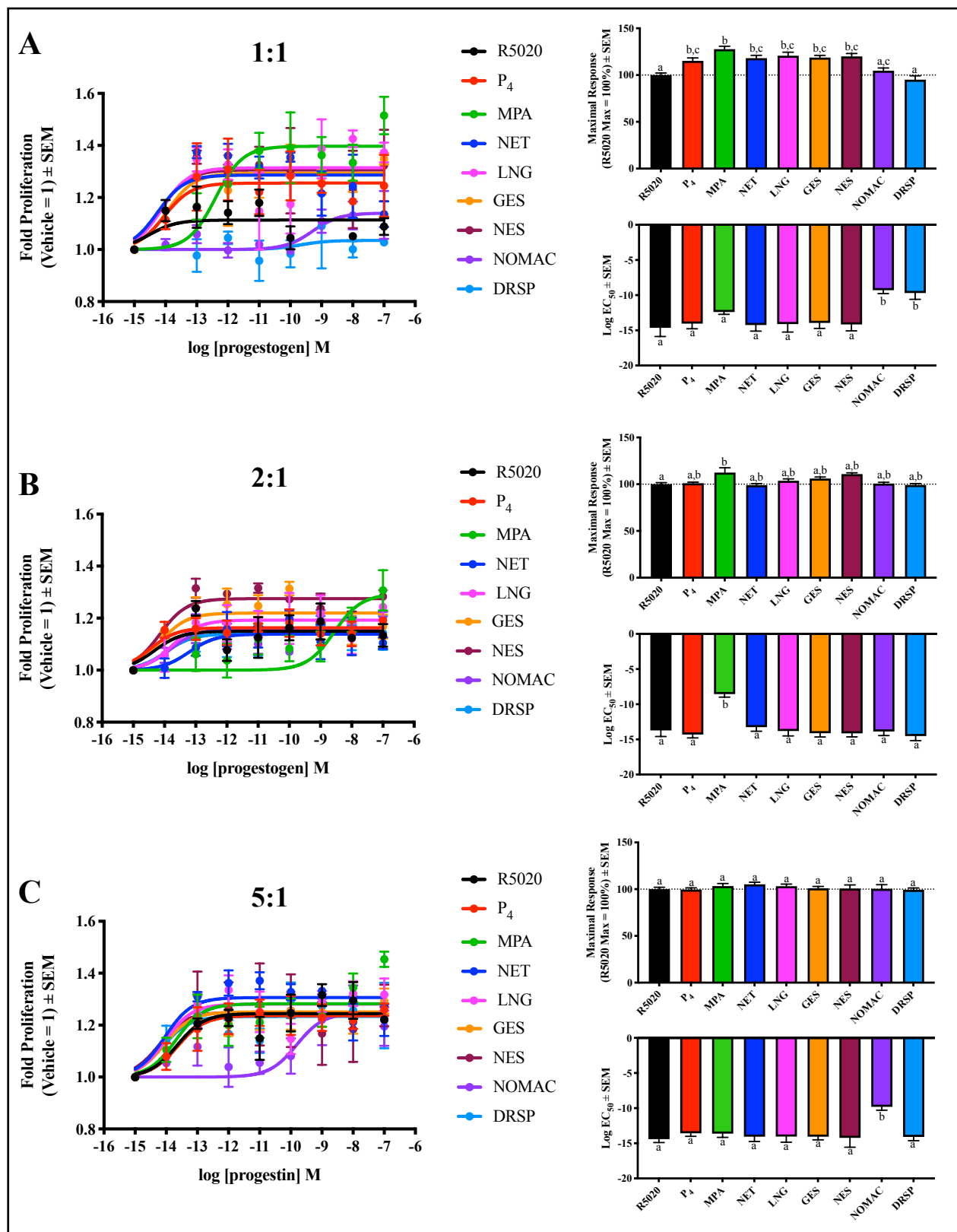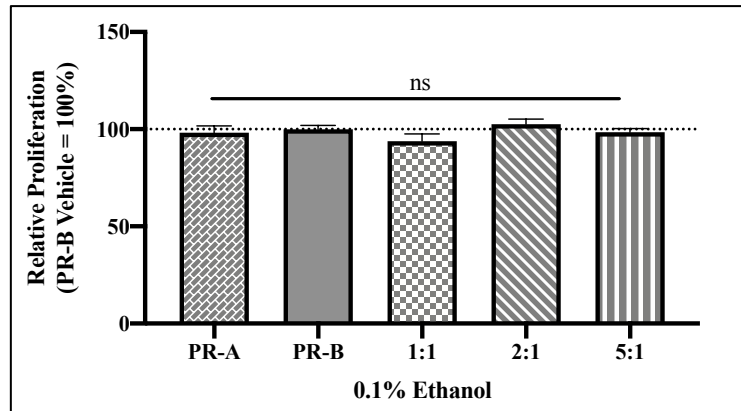
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Figure D2. Figure legend on the following page.

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Figure D2. The effects of the progestogens on proliferation is PR-A:PR-B ratio-dependent. The MDA-MB-231 cells were transiently transfected with 900 ng pSG5-PR-B and **(A)** 900 ng (1:1), **(B)** 1 800 ng (2:1) or **(C)** 4 500 ng **(**5:1) pSG5-PR-A. The cells were treated with either 0.1% (v/v) ethanol (vehicle control) or increasing concentrations of the progestogen for 48 hours, then retreated for a further 48 hours. Proliferation was quantified using the colorimetric MTT cell viability assay. Results are shown as fold proliferation where the vehicle is set as 1, while all other responses were calculated relative to this. Plots are shown for the maximal responses and $logEC_{50}$ values of the progestogens via PR-A and PR-B co-expressed at **(A)** 1:1, **(B)** 2:1 and **(C)** 5:1. The results shown are averages (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis and statistically significant differences are represented with letters a, b and c, where the values that differ significantly from other values are assigned a different letter.

Figure D3. Proliferation of MDA-MB-231 cells co-expressing PR-A and PR-B at equivalent levels or excess PR-A relative to PR-B, does not occur in the absence of the progestogens. The MDA-MB-231 cells were transiently transfected with 900 ng of either pSG5-PR-A or pSG5-PR-B in the absence and presence of 900 ng (1:1), 1 800 ng (2:1) or 4 500 ng (5:1) pSG5-PR-A. Cells were subsequently treated with 0.1% (v/v) ethanol for 48 hours, then retreated for a further 48 hours. Proliferation was quantified using the colorimetric MTT cell viability assay. Results are shown as relative proliferation where the vehicle of PR-B is set as 100%. The result shown is the average (±SEM) of at least three biological repeats with each condition performed in triplicate. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test was used for statistical analysis. No statistical significance ($p > 0.05$) is indicated by ns.
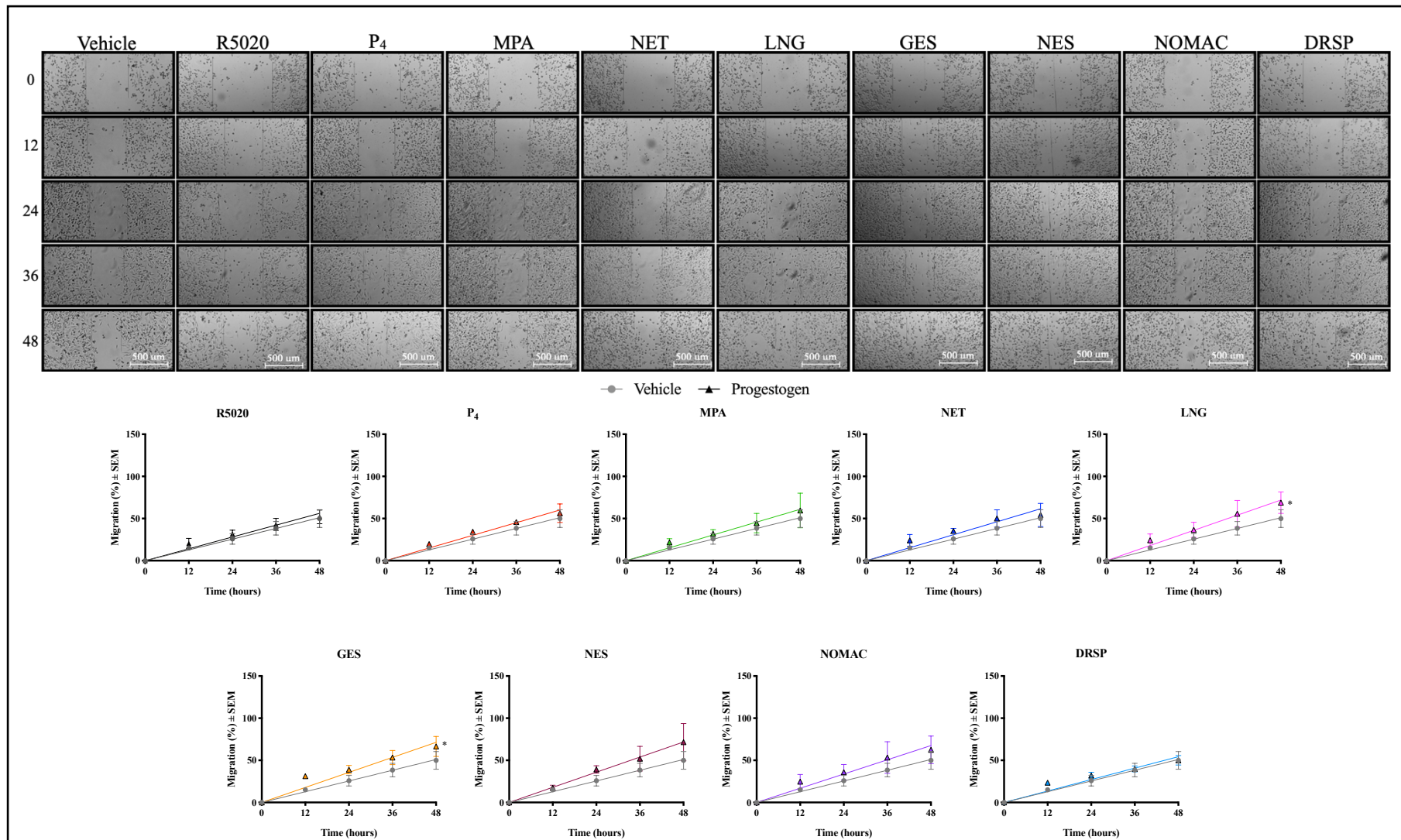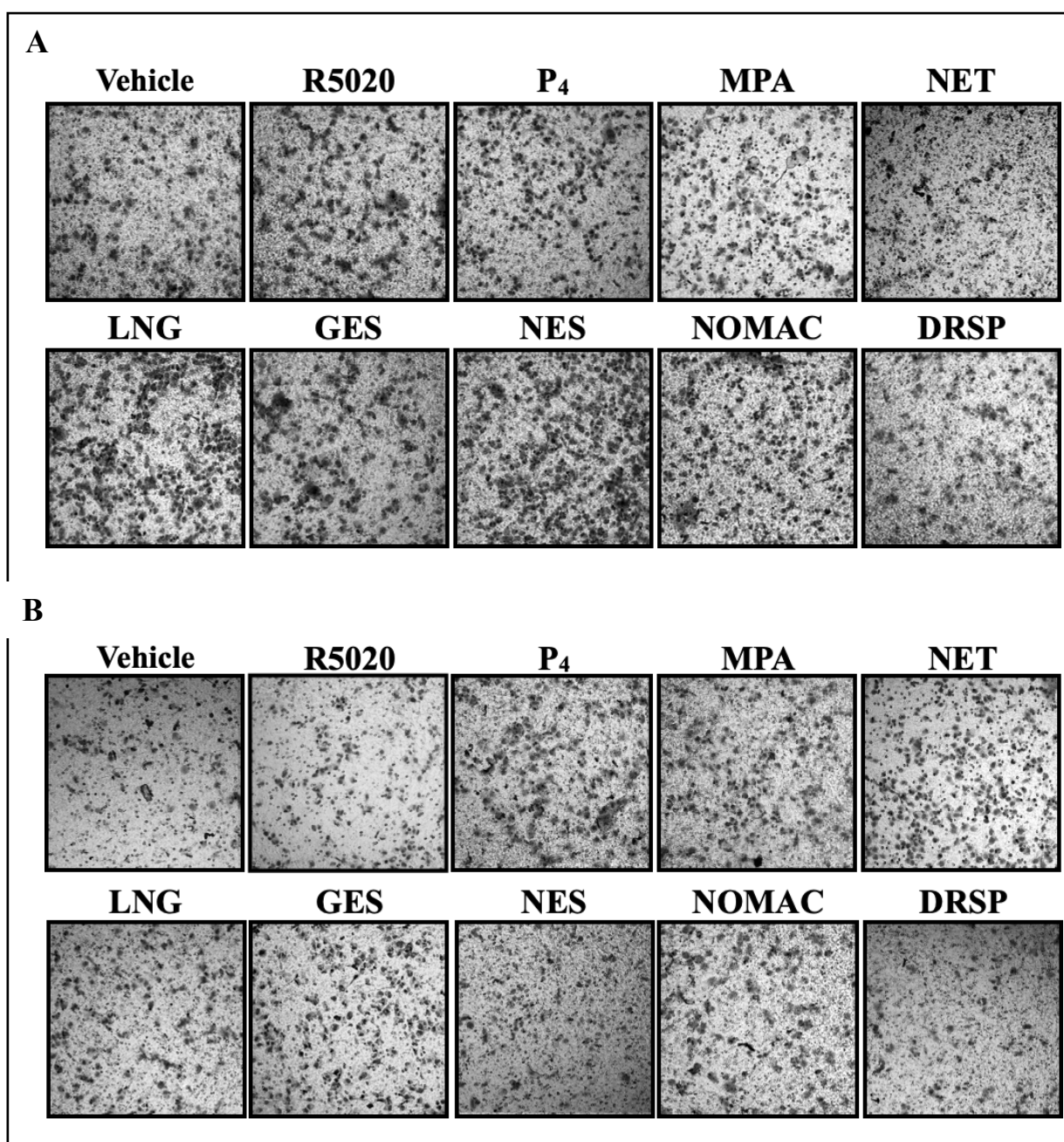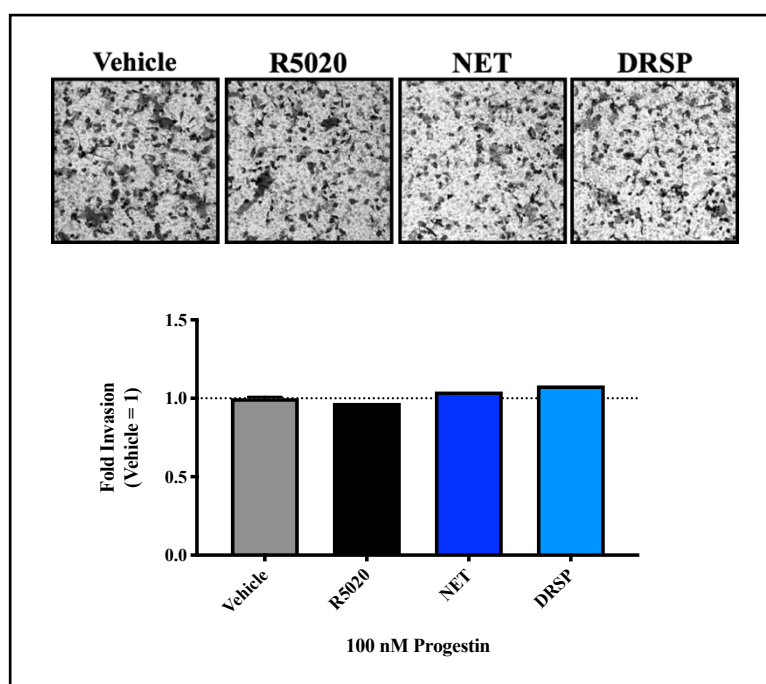
Figure D4. Figure legend on the following page.

Figure D4. LNG and GES increase cell migration in the absence of PR-A and PR-B. The MDA-MB-231 cells were transiently transfected with 900 ng pSG5-empty vector. The cells were pre-treated for 2 hours with mitomycin C to inhibit proliferation, then treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for a total of 48 hours. Images were captured every 12 hours and migration quantified over time using ImageJ analysis software. Results shown are averages (±SEM) of at least three biological repeats and the percent migration is plotted against time. Simple linear regression was used to fit the line and a two-way ANOVA, with Dunnett's post-test was performed to determine statistical differences between the curves of the vehicle and treatment, with statistically significant differences shown using *, indicating $p < 0.05$. Absence of stars indicate no significant differences ($p > 0.05$).
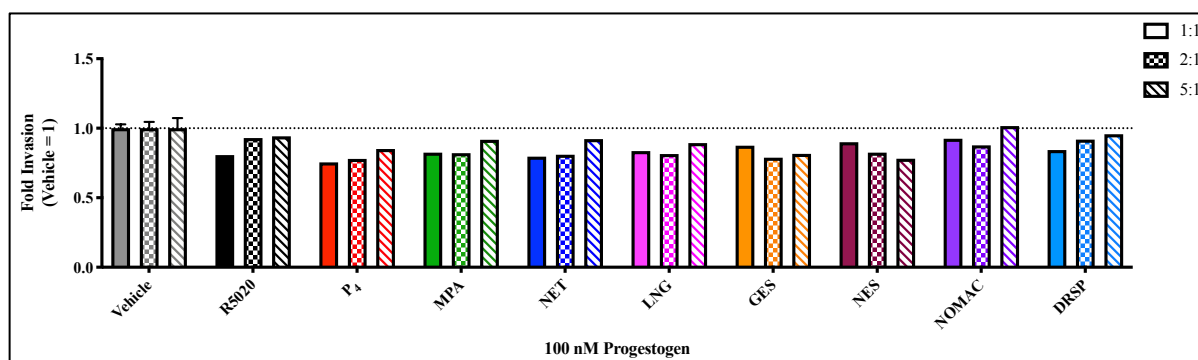
Figure D5. While NET decreases cell invasion via PR-A, most progestogens increase invasion via PR-A or PR-B. The MDA-MB-231 cells were transiently transfected with 900 ng of either **(A)** pSG5-PR-A or **(B)** pSG5-PR-B. Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for 24 hours and invasion was assessed using Matrigel pre-coated Boyden chambers. Images were captured and cells counted using a script in Mathematica (as described in the methods).
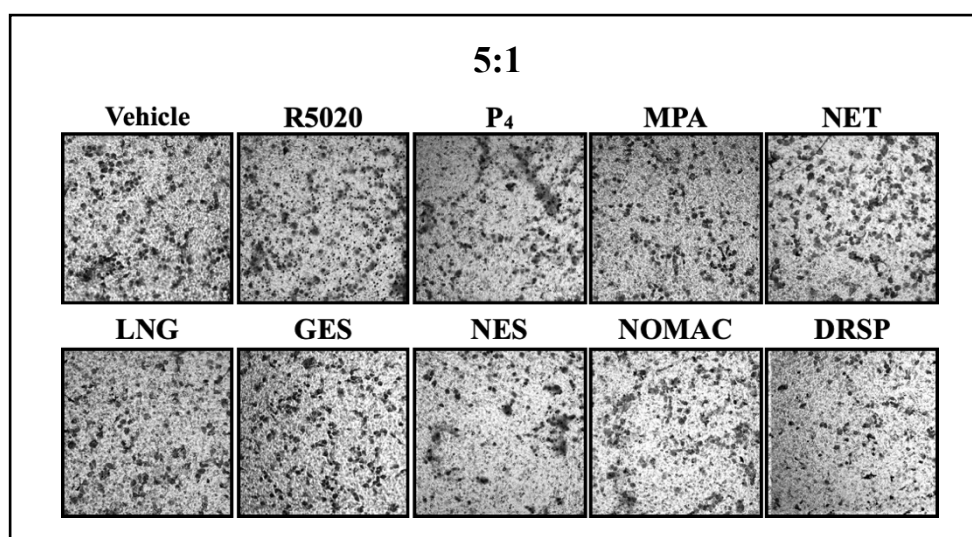
Figure D6. R5020, NET and DRSP have no effect on cell invasion in the absence of PR-A and PR-B. The MDA-MB-231 cells were transiently transfected with 900 ng pSG5-empty expression vector. Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM R5020, NET or DRSP for 24 hours and invasion was assessed using Matrigel pre-coated Boyden chambers. Images were captured and cells counted using a script in Mathematica (as described in the methods). Fold invasion was determined relative to the vehicle control. Shown are the results of 1 experiment with the vehicle conducted in duplicate.

Figure D7. There are no differences in progestogen effects on invasion of MDA-MB-231 cells expressing PR-A and PR-B at various ratios. The MDA-MB-231 cells were transiently transfected with either 900 ng pSG5-PR-B and 900 ng (1:1), 1 800 ng (2:1) or 4 500 ng (5:1) pSG5-PR-A. Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for 24 hours, and invasion was assessed using Matrigel pre-coated Boyden chambers. Images were captured and cells counted using a script in Mathematica (as described in the methods). Fold invasion was determined relative to the vehicle control set as 1. Results shown are from one experiment with the vehicle conducted in duplicate (±SD).

Figure D8. When PR-A is co-expressed in excess relative to PR-B (5:1), P₄, NES and DRSP decrease cell invasion. The MDA-MB-231 cells were transiently transfected with 4 500 ng pSG5-PR-A and 900 ng pSG5-PR-B (5:1). Cells were treated with either 0.1% (v/v) ethanol (vehicle control) or 100 nM of the progestogens for 24 hours and invasion was assessed using Matrigel pre-coated Boyden chambers. Images were captured and cells counted using a script in Mathematica (as described in the methods).