Investigating progesterone and estrogen receptor crosstalk in breast cancer

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Declaration

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ABSTRACT

Progestins are synthetic compounds designed to mimic the natural hormone progesterone (Prog), and are widely used in hormone replacement therapy (HRT) and contraception. These compounds can be divided into four generations, with newer generations increasing in progesterone receptor (PR) specificity. Although progestins have many therapeutic benefits, a number of undesirable side-effects, such as increased risk of breast cancer, have been reported. As a result, many postmenopausal women have sought alternatives for HRT, such as compounded bio-identical hormones like bio-identical Prog (bProg), claimed not to increase breast cancer risk. Progestins, Prog and bProg (collectively referred to as progestogens) elicit their biological effects primarily by binding to the PR, which exists as two predominant isoforms, PR-A and PR-B, with PR-B being the more transcriptionally active and proliferative isoform in breast cancer. Emerging evidence suggest that the PR plays an important role in breast cancer development and progression, and that there is crosstalk between the PR and estrogen receptor (ER)-α, a major etiological factor in breast cancer biology. Moreover, it has been shown that ER-α is required for PR-B-mediated effects of medroxyprogesterone acetate (MPA) on activation of gene expression and breast cancer cell proliferation. The latter raised the questions of whether ER-α is needed for PR-B-mediated effects of other progestins, and whether the ERβ subtype would also be required. Given that PR-B has both transactivation and transrepression functions, this study used transactivation and transrepression transcriptional assays to investigate the PR-B-mediated agonist efficacies and potencies of Prog, bProg and select progestins from different generations (MPA, norethisterone acetate (NET-A), levonorgestrel (LNG), gestodene (GES) and drospirenone (DRSP)), and whether these were modulated by ERα and/or ERβ. Furthermore, the effects of the progestogens on breast cancer cell proliferation were evaluated in the absence and presence of ERα- and ERβ-specific antagonists. Results showed that progestins mostly displayed similar agonist efficacies and potencies for transactivation and transrepression via PR-B. The exception was first generation MPA that was less efficacious for transactivation and least potent for transrepression, and third generation GES that was more potent for transactivation. This study is the first to show that ERα and ERβ differentially decreased PR-B-mediated agonist efficacies of progestogens for transactivation and transrepression. However, the ERα-specific antagonist had no effect on progestogen-induced expression of the endogenous PR-B regulated c-myc gene or repression of the interleukin (IL)-8 gene in the T47D breast cancer cell line, while the ERβ-specific antagonist had no effect on progestogen-induced c-myc gene expression, and appeared to abolish repression of the IL-8 gene. Additionally, we showed that all progestogens, except NET-A and DRSP, displayed similar proliferative efficacies and potencies for cell proliferation. Interestingly, while the ERα-specific antagonist had no effect on progestogen-induced cell proliferation, increased cell proliferation by LNG- and GES was enhanced by the ERβ-specific antagonist. Taken together, the results from this study, although having limitations, emphasizes the complexity of crosstalk between the PR and ER subtypes in breast cancer. Although the physiological implications of these results have to be evaluated, our findings may assist us in our understanding of crosstalk between PR-B and the ER subtypes, and how it may be contributing to progestin-induced breast cancer cell growth.

OPSOMMING

Progestiene is sintetiese verbindings wat ontwerp is om die funksies van die natuurlike hormoon progesteroon (Prog) na te boots, en word wêreldwyd in hormoon vervagingsterapie (HVT) en voorbehoedmiddels gebruik. Hierdie verbindings kan in vier generasies verdeel word, met die nuwer generasie wat meer spesifiek is vir die progesteroon reseptor (PR). Alhoewel progestiene baie terapeutiese voordele het, is daar ook verskeie ongewenste newe-effekte, soos verhoogde risiko van borskanker, geassosieër met hul gebruik. As gevolg hiervan, het baie na-menopousale vrouens alternatiewe begin soek vir HVT, soos byvoorbeeld die saamgestelde bio-identiese hormone soos bio-identiese Prog (bProg), wat beweer word om nie die risiko van borskanker te verhoog nie. Progestiene, Prog and bProg (gesamentlik verwys daarna as progestogene) voer hul biologiese effekte uit deur hoofsaaklik te bind aan die PR, wat voorkom as twee hoof isoforme, PR-A en PR-B, met PR-B wat hoër transkripsionele aktiwiteit toon en die meer proliferatiewe isoform in borskanker is. Onlangse bewyse toon dat die PR ‘n belangrike rol in borskankerontwikkeling en -bevordering speel, en dat daar ‘n wisselwerking tussen die PR en die estrogeen reseptor (ER)-α, ‘n groot etiologiese faktor in borskankerbiologie, voorkom. Verder, is daar gevind dat ERα benodig word vir PR-B-bemiddelde effekte van medroksieprogesteroon asetaat (MPA) op die aktivering van geentranskripsie en borskanker proliferasie. Die laasgenoemde het gelei tot die vrae of ERα ook benodig word vir die PR-B-bemiddelde effekte van ander progestiene, en of die ERβ subtipe ook benodig sal word. Gegewe dat PR-B beide transaktivering en transonderdrukking funksies het, is daar in hierdie studie gebruik gemaak van transaktivering en transonderdrukking transkripsioneletoetse om die PR-B bemiddelde agonis effektiwiteit en potensie van Prog, bProg en geselekteerder progestiene van verskillende generasies (MPA, noretisteroon asetaat (NET-A), levonorgestrel (LNG), gestodeen (GES) en drospirenoon (DRSP)) te bepaal, asook om vas te stel of die effekte deur ERα en/of ERβ gemoduleer word. Verder, is die effekte van die progestogene op borskanker proliferasie in die afwesigheid en teenwordigheid van ERα- en ERβ-spesifieke antagoniste geëvalueer. Resultate het aangedui dat progestiene meestal soortgelyke agonis effektiwiteit en potensies vir transaktivering en transonderdrukking via PR-B getoon het. Die uitsonderings was die eerste generasie progestien MPA wat minder effektief vir transaktivering en minder potent vir transonderdrukking was, en die derde generasie progestien GES wat meer potent vir transaktivering was. Hierdie studie wys vir die eerste keer dat ERα en ERβ die PR-B-bemiddelde agonis effektiwiteit van die progestogene vir transaktivering en transoderdrukking differensieel verminder. Nietemin, het die ERα-spesifieke antagonis geen effek op progestogeen-geïnduseerde uitdrukking van die endogene PR-B-gereguleerde c-myc geen, of onderdrukking van die interleukin (IL)-8 geen in die T47D borskanker sellyn gehad nie, terwyl die ERβ-spesifieke antagonis geen effek op c-myc geen uidrukking gehad het nie, en wou dit voorkom asof dit die onderdrukking van die IL-8 geen verhoed. Verder het ons gewys dat alle progestogene, behalwe NET-A en DRSP, soortgelyke proliferatiewe effektiwiteit en potensies vir selproliferasie getoon het. Interessant genoeg, terwyl die ERα-spesifieke antagonis geen effek op progestogeen-geïnduseerde selproliferasie gehad het nie, is die LNG- en GES-geïnduseerde selproliferasie selfs verder verhoog deur die ERβ-spesifieke antagonis. Ten slotte, alhoewel daar sekere beperkinge is, beklemtoon die resultate van hierdie studie die kompleksiteit van die wisselwerking tussen PR-B en die ER subtipes in borskanker. Alhoewel die fisiologiese implikasies van ons resultate nog

geëvalueer moet word, mag ons bevindinge bydra tot die huidige begrip van die wisselwerking tussen PR-B en die ER subtipes, en hoe dit moontlik kan bydra tot progestien-geïnduseerde groei van borskankerselle.

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ALPHABETICAL LIST OF ABBREVIATIONS

AF-1	activation function-1
AF-2	activation function-2
AF-3	activation function-3
AP-1	activator protein-1
ANOVA	analysis of variance
AR	androgen receptor
ATCC	American Type Culture Collection
bHRT	bio-identical hormone replacement therapy
bp	base pair
bProg	bio-identical progesterone
CDK2	cyclin-dependent protein kinase 2
cDNA	complementary deoxyribonucleic acid
CEE	conjugated equine estrogen
CHD	coronary heart disease
ChIP	chromatin immunoprecipitation
COC	combined oral contraceptive
Co-IP	co-immunoprecipitation

C_q	quantification cycle
CS-FCS	charcoal-stripped fetal calf serum
DBD	DNA binding domain
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMPA	depot medroxyprogesterone acetate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DRSP	drosperinone
E	exponential amplification value
E_2	17β-estradiol
E	estriol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
ER	estrogen receptor
ERα	estrogen receptor alpha

ERβ	estrogen receptor beta
Erk	extracellular signal-regulated kinase
EtOH	ethanol
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GES	gestodene
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
HCL	hydrogen chloride
HIV	human immunodeficiency virus
hERα	human estrogen receptor alpha
hERβ	human estrogen receptor beta
hPR-B	human progrogesterone receptor B
HRP	horseradish peroxidase
HRT	hormone replacement therapy
Hsp	heat shock protein
HSV-2	herpes simplex virus type 2
ICI 182 780	fulvestrant

IF	inhibitory function
IgG	immunoglobulin G
IL-8	interleukin 8
LB	Luria Bertani
LBD	ligand binding domain
LNG	levonorgestrel
MAPK	mitogen-activated protein kinase
MOPS	morpholinopropanesulfonic acid
MPA	medroxyprogesterone acetate
MPP	methyl-piperidino-pyrazole dihydrochloride hydrate
mPR	membrane progesterone receptor
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWS	Million Women Study
NET	norethisterone
NET-A	norethisterone-acetate
NET-EN	norethisterone-enanthate

NFκB	nuclear factor kappa-B
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCOS	polycystic ovary syndrome
PELP1	proline-, glutamate- and leucine-rich protein 1
PHTPP	2-phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-alpha]pyrimidine
PI3K	phosphatidylinositol 3-kinase
PR	progesterone receptor
PR-A	progesterone receptor A
PR-B	progesterone receptor B
PR-C	progesterone receptor C
PRE(s)	progesterone response element(s)
Prog	progesterone
PAQR	progestin and adiponectin Q receptor
qPCR	quantitative polymerase chain reaction
R	relative expression
R5020	promegestone

RLU(s)	relative light unit(s)
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RU486	mifepristone
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SH3	Src-homology-3
siRNA	short interfering ribonucleic acid
TAT	tyrosine amino transferase
TBS	tris buffered saline
TBS-Tween	tris buffered saline tween
TNFα	tumor necrosis factor alpha
TPE	tris-phosphate-EDTA
VEGF	vascular endothelial growth factor
VTE	venous thromboembolism
WHI	Women's Health Initiative

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CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Progestogens refer to compounds that display progestational activity and include the natural hormone progesterone (Prog) as well as progestins (Sitruk-Ware 2004a; Stanczyk et al. 2013). Progestins are synthetic compounds that were designed to mimic the effects of Prog (Moore et al. 2012; Stanczyk et al. 2013), and are used clinically as contraceptives, in hormone replacement therapy (HRT) (Sitruk-Ware 2004a; Sitruk-Ware & Nath 2010; Africander, et al. 2011a), for the treatment of gynaecological disorders such as endometriosis (Harrison & Barry-Kinsella 2000; Irahara et al. 2001; Vercellini et al. 2003) and polycystic ovary syndrome (PCOS) (Archer & Chang 2004; Guido et al. 2004; Ehrmann 2005; Harwood et al. 2007; Badawy & Elnashar 2011). It should be noted that many different progestins have been designed and they are classified into four consecutive generations (Stanczyk 2003; Sitruk-Ware 2006). Although these progestins have been shown to have many beneficial effects, several side-effects have been associated with their clinical use. For example, results from the Women's Health Initiative (WHI) trial showed that the use of the first generation progestin medroxyprogesterone acetate (MPA) in HRT by postmenopausal women, increased the risk of developing coronary heart disease (CHD), stroke and breast cancer (Rossouw et al. 2002; Chlebowski et al. 2003; Chlebowski et al. 2013a; Chlebowski et al. 2013b), while the Million Women Study (MWS) showed an association between using progestins such as MPA, norethisterone acetate (NET-A) and levonorgestrel (LNG) in HRT, and increased risk of breast cancer (Beral 2003). Similarly, the French E3N cohort study showed that the use of MPA and NET-A in HRT increased breast cancer risk (Fournier et al. 2005; Fournier et al. 2008). As progestins differ in structure, and hence their biological activities (Sitruk-Ware 2006; Sitruk-Ware 2005; Sitruk-Ware & Nath 2010), it is possible that not all progestins will elicit the same side-effect profile in terms of breast cancer risk. Furthermore, the use of compounded bio-identical hormones such as bio-identical Prog (bProg) in HRT has gained popularity as it is claimed to be natural and safer in terms of breast cancer risk (Boothby & Doering 2008; Holtorf 2009; Files et al. 2011). Considering that breast cancer is the most common cause of cancer death amongst women

worldwide (Sommer & Fuqua 2001; Platet et al. 2004; Ferlay et al. 2014), it is of utmost importance to understand the mechanisms whereby hormones used in HRT may or may not contribute to the development and progression of this disease.

Progestins, like Prog, mainly elicit their biological effects by binding to the progesterone receptor (PR) (Moore et al. 2012; Stanczyk et al. 2013), which is a member of the steroid receptor family (Lu et al. 2006; Griekspoor 2007; Africander, et al. 2011a) that exists as two predominant isoforms, namely PR-A and PR-B (Kastner et al. 1990; Rękawiecki et al. 2011). Although recent evidence suggests that both the PR isoforms may have critical roles in the pathogenesis of hormone-responsive breast cancers (Daniel et al. 2011; Diep et al. 2015), the exact role of PR-A and PR-B in breast cancer development and progression still needs to be elucidated. Furthermore, emerging evidence suggest that crosstalk between different steroid receptors may be implicated in breast cancer biology. For example, a recent study by Giulianelli and co-workers (2012) showed that the estrogen receptor (ER)-α is essential for both PR-A and PR-B mediated effects of MPA on gene expression and breast cancer cell proliferation (Giulianelli et al. 2012). Considering that ERα is required for PR-mediated activity of MPA (Giulianelli et al. 2012), the question that arises is whether ERα is also required for the PR-mediated activity of other progestins. Moreover, as the ER exists as two subtypes, ERα and ERβ, another question that comes to mind is whether ERβ is also needed for PR-mediated effects of progestins.

1.2. Progestogens

1.2.1. Therapeutic applications

1.2.1.1. Prog and bProg

The natural progestogen, Prog, is a sex steroid hormone mainly synthesised in the ovaries of the female body. Interestingly, Prog is also synthesised *de novo* from cholesterol in the brain (Tsutsui et al. 2000; Hu et al. 2010) through a biosynthetic pathway that is similar to the pathway in the ovaries (Hanukoglu 1992; Wickenheisser et al. 2006). Prog plays a very important role in controlling brain

functions associated with sexual behaviour and receptivity, as well as in normal female development and maintenance of reproductive function in the uterus, ovaries and mammary glands (Graham & Clarke 1997; Conneely & Lydon 2000; Toh et al. 2013; Diep et al. 2015). In the uterus and ovaries for example, Prog is involved in reproductive processes such as ovulation as well as the establishment and maintenance of pregnancy (Conneely & Lydon 2000; Graham & Clarke 2002; Diep et al. 2015). It is noteworthy that Prog also elicits anti-proliferative effects in the uterus, so as to protect against possible hyperplasia induced by the rise in circulating estrogen levels at the time of ovulation during the menstrual cycle (Clarke & Sutherland 1990; Lydon et al. 1995; Conneely et al. 2000; Conneely et al. 2002). In contrast, Prog elicits proliferative effects in the normal mammary gland, thereby stimulating lobular-alveolar development and expansion to prepare for lactation (Lydon et al. 1995; Conneely et al. 2000; Graham & Clarke 2002; Diep et al. 2015). The various physiological effects of Prog in different target tissues are primarily mediated by the PR (Conneely et al. 2001; Conneely et al. 2003).

Clinically, Prog has been used either in the form of micronized Prog in conventional HRT (de Lignières 2002; Fournier et al. 2005; Fournier et al. 2008) or bProg in bio-identical HRT (bHRT) (Ruiz et al. 2011; White 2015). Micronized Prog refers to Prog which has undergone the process of micronization whereby the particle size is reduced in order to facilitate increased absorption and bioavailability (Maxson & Hargrove 1985; Chakmakjian & Zachariah 1987; Kimzey et al. 1991; Norman et al. 1991; Tavaniotou et al. 2000). It is administered either orally, via intramuscular injection or vaginally in the form of vaginal creams, suppositories, capsules, pessaries, gels and rings (Price et al. 1983; Kimzey et al. 1991; Cicinelli et al. 1996; Fanchin et al. 1997; Tavaniotou et al. 2000; Germond et al. 2002; Sitruk-Ware 2007). bProg is synthesized by chemically modifying the natural plant product diosgenin (figure 1.1), which can be extracted from plants such as the Mexican wild yam and soy (Boothby et al. 2004; Boothby & Doering 2008; Files et al. 2011; Bhavnani & Stanczyk 2012), and is reported to have the same chemical structure as natural Prog (Boothby et al. 2004; Boothby & Doering 2008; Holtorf 2009; Panay & Fenton 2010; Files et al.

2011; Bhavnani & Stanczyk 2012; Guidozzi et al. 2014). Thus, although compounding pharmacies claim that bio-identical hormones such as bProg are natural (Boothby et al. 2004), it is in fact semi-synthetic (Bhavnani & Stanczyk 2012). Compounded bProg can be administered to women either orally or vaginally in the form of gels and creams (White 2015). It is noteworthy that a position statement by the South African Menopause Society in 2014 indicated that the use of compounded bProg may not be as effective in counteracting the proliferative effects of estrogen on the endometrium, since bProg produced by compounding pharmacies are not regulated, and thus may vary in quality and potency (Guidozzi et al. 2014). Furthermore, claims by these pharmacies that bio-identical hormones are safer than the hormones traditionally used in conventional HRT have not been substantiated by scientific evidence. Thus, more research is needed to determine the possible benefits and risks associated with the use of bio-identical hormones such as bProg in HRT.

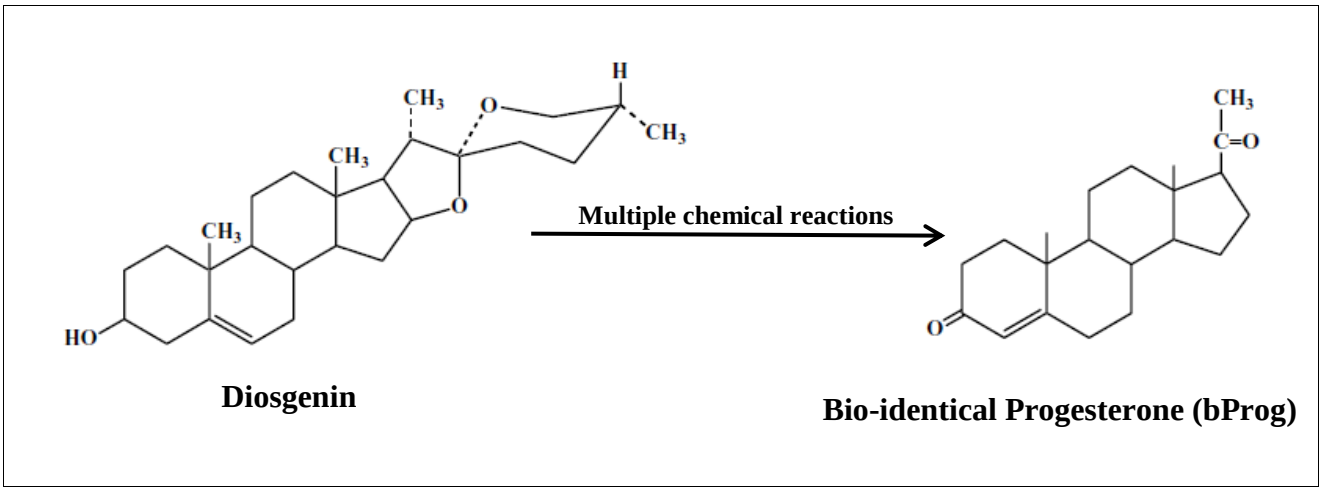


Figure 1.1. The plant product diosgenin is converted to bProg (chemical structure identical to that of natural Prog) by multiple chemical reactions. Adapted from Stanczyk et al. (2003).

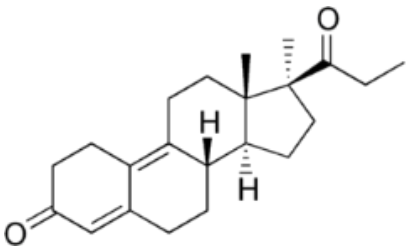
1.2.1.2. Progestins

The clinical use of Prog is however restricted due to its rapid metabolism and short half-life (Speroff & Darney 1996; Fotherby 1996). In contrast, progestins mostly display greater half-lives than Prog (Moore et al. 2012; Stanczyk et al. 2013) and are used in many therapeutic applications, most notably for contraception and HRT (Sitruk-Ware 2005a; Sitruk-Ware & Nath 2010; Africander, et al. 2011a). A number of different progestins have been designed and are classified according to successive generations. The first two generations are considered the older progestins,

while the third and fourth generations are regarded as the newer progestins (Sitruk-Ware 2004a; Sitruk-Ware & Plu-Bureau 2004; Sitruk-Ware 2005a). Although most progestins are structurally related to either Prog or testosterone, the chemical structures of these compounds differ greatly from each other and from Prog and testosterone (figure 1.2) (Stanczyk 2003; Sitruk-Ware 2005a; Sitruk-Ware 2005b; Sitruk-Ware & Nath 2010). Those progestins derived from Prog are referred to as either 17-hydroxyprogesterone or 19-norprogesterone derivatives, while those derived from testosterone are referred to as 19-nortestosterone derivatives (Stanczyk 2003; Sitruk-Ware & Nath 2010). The first generation progestin MPA is an example of a 17-hydroxyprogesterone derivative (Sitruk-Ware & Plu-Bureau 2004), while promegestone (R5020) is an example of a 19-norprogesterone derivative (Schindler et al. 2003; Stanczyk 2003; Sitruk-Ware 2006). Examples of 19-nortestosterone derivatives include the first generation progestin NET-A or norethisterone enanthate (NET-EN), the second generation progestin LNG, and the third generation progestin gestodene (GES). It is important to note that NET-A is used in HRT, while NET-EN is used in contraception (Schindler et al. 2003; Stanczyk 2003; Sitruk-Ware & Plu-Bureau 2004; Sitruk-Ware 2006), and that both NET-A and NET-EN are prodrugs which are metabolized to the active metabolite NET (Stanczyk & Roy 1990). The fourth and newest generation of progestins include drospirenone (DRSP) (Schindler et al. 2003; Stanczyk 2003; Sitruk-Ware & Plu-Bureau 2004; Sitruk-Ware 2006), which is unique in that it is derived from the anti-mineralocorticoid spironolactone (Krattenmacher 2000; Elger et al. 2003; Oelkers 2004; Sitruk-Ware 2004a; Sitruk-Ware 2006; Sitruk-Ware 2005). All of the above-mentioned progestins, except R5020, are used in both contraception (Lee et al. 1987; Althuis et al. 2003; Li et al. 2012; Wu et al. 2013; Dinger et al. 2014; Beaber et al. 2014; Stanczyk & Archer 2014; Vinogradova et al. 2015) and HRT (Rossouw et al. 2002; Beral 2003; Fournier et al. 2014; Schindler 2014). Notably, R5020 is used in only HRT and only in France (de Lignières et al. 2002; Fournier et al. 2005; Fournier et al. 2008). However, it is extensively used as an experimental tool to investigate PR-specific effects (Chwalisz et al. 2006).

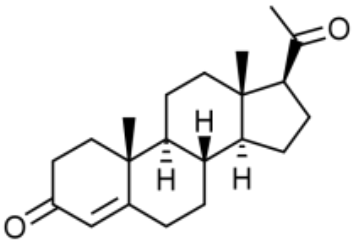
For contraception, progestins are administered to women either alone, or in combination with estrogens for enhanced cycle control (Sitruk-Ware 2005b; Sitruk-Ware & Nath 2010; Africander, et al. 2011a). Progestin-only contraceptives can be taken either orally or be administered as an injection, or in subcutaneous implants, vaginal rings and intrauterine devices, while estrogen-progestin combined contraceptives are administered either orally or in vaginal rings and contraceptive patches (Brache et al. 2000; Kahn et al. 2003; Erkkola & Landgren 2005; Sitruk-Ware 2006; Black & Kubba 2008; Nath & Sitruk-Ware 2009; Rakhi & Sumathi 2011; Brache et al. 2013; Sitruk-Ware et al. 2013; Jacobstein & Polis 2014). Interestingly, progestins are also being investigated for its possible use in male contraception (Gu et al. 2004; Ilani et al. 2012; Kanakis & Goulis 2015; Roth et al. 2015; Wang et al. 2016). Several studies have shown that combining progestins with testosterone suppresses spermatogenesis due to the progestin and testosterone synergistically suppressing gonadotropin hormone levels (Kamischke, et al. 2000a; Kamischke, et al. 2000b; Nieschlag et al. 2003; Gu et al. 2004; Amory 2008; Ilani et al. 2012; Costantino et al. 2014; Chao & Page 2016). In terms of HRT, estrogen combined with a progestin is commonly prescribed to menopausal women with an intact uterus. The estrogen is given to alleviate symptoms associated with decreasing levels of estrogen (Greendale et al. 1999; Hickey et al. 2005; Africander, et al. 2011a) such as hot flashes, night sweats and vaginal dryness (Greendale et al. 1999; Hickey et al. 2005), while a progestin is added to protect against endometrial cancer caused by the proliferative effects of estrogen on the endometrium (Whitehead et al. 1979; Greendale et al. 1999; Hickey et al. 2005). HRT regimens are administered either orally, via transdermal patches, gels or vaginal rings (Nath & Sitruk-Ware 2009; Hickey et al. 2012; Stanczyk et al. 2013).

A



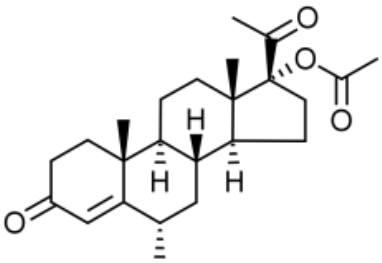
Promegestone (R5020)

B



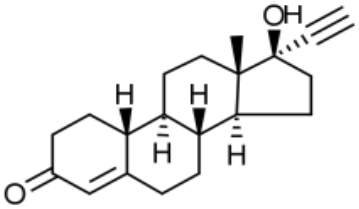
Progesterone (Prog)

C



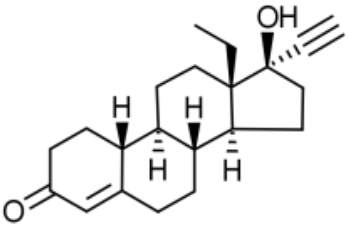
Medroxyprogesterone acetate (MPA)

D



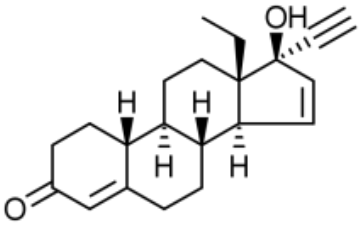
Norethisterone (NET)

E



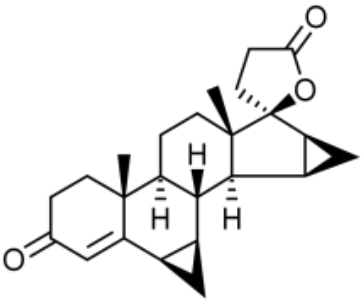
Levonorgestrel (LNG)

F



Gestodene (GES)

G



Drospirenone (DRSP)

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Figure 1.2. Chemical structures of select progestogens. The chemical structures for **(A)** promegestone (R5020) and **(B)** natural progesterone (Prog), as well as the progestins **(C)** medroxyprogesterone acetate (MPA), **(D)** norethisterone (NET), **(E)** levonorgestrel (LNG), **(F)** gestodene (GES) and **(G)** drospirenone (DRSP) are illustrated. Adapted from Louw-du Toit et al. (2016).

Other therapeutic applications of progestins include treatment of gynaecological disorders such as dysmenorrhea (painful menstruation), menorrhagia (heavy menstrual bleeding) (Williams & Creighton 2012), endometriosis (a disease that leads to pelvic pain and infertility) (Harrison & Barry-Kinsella 2000; Irahara et al. 2001; Vercellini et al. 2003) and PCOS (Archer & Chang 2004; Guido et al. 2004; Ehrmann 2005; Harwood et al. 2007; Setji & Brown 2007; Badawy & Elnashar 2011). PCOS is a endocrine disorder causing symptoms such as irregular ovulation (oligo-ovulation) or a complete lack of ovulation (anovulation), elevated levels of androgens and infertility, as well as complications such as insulin resistance and diabetes (Archer & Chang 2004; Guido et al. 2004; Ehrmann 2005; Harwood et al. 2007; Setji & Brown 2007; Badawy & Elnashar 2011). Interestingly, high dosages of progestins such as MPA (500 and 1500 mg/day [Blossey et al. 1984]) have also been used for the treatment of breast (Lundgren 1992; Yamashita et al. 1996; Cardoso et al. 2012; Cardoso et al. 2013) and endometrial cancer (Lentz et al. 1996; Thigpen & Brady 1999; Kim et al. 2013). Despite the number of beneficial effects associated with the therapeutic use of progestins, a number of side-effects have been reported for some progestins (Mostad et al. 2000; Kass-Wolff 2001; Rossouw et al. 2002; Anderson et al. 2003; Cromer et al. 2004; Morrison et al. 2004; Sitruk-Ware 2004b; Sitruk-Ware 2006; Ojule et al. 2010; Morrison et al. 2010).

1.2.2. Side-effects

To the best of our knowledge, no major side-effects have been reported with the clinical use of Prog and bProg, while a number of side-effects have been associated with the clinical use of some progestins. Some of the less severe side-effects include bloating, weight gain, headaches, nausea, fatigue, depression, insomnia, abdominal pain, reduced libido, vaginal itchiness, breast tenderness, mood changes, amenorrhea and irregular bleeding (Li et al. 2000; Greydanus et al. 2001; Sitruk-

Ware 2004b; Erkkola & Landgren 2005; Sitruk-Ware 2006; Ojule et al. 2010; Moore et al. 2012; Williams & Creighton 2012). More severe side-effects include changes in lipid and lipoprotein levels in postmenopausal women using progestins like MPA and NET-A in HRT, which in turn could increase cardiovascular risk (Sitruk-Ware 2000). In addition, progestins such as MPA, NET, LNG, GES and DRSP used in both contraception and HRT have been shown to increase the risk of venous thromboembolism (VTE) and stroke in a number of independent studies (Rossouw et al. 2002; Warren 2004; Lidegaard et al. 2012; Manzoli et al. 2012; Sidney et al. 2013; Wu et al. 2013; Dinger et al. 2014; Vinogradova et al. 2015). Furthermore, several studies have shown that the use of the injectable contraceptive depot-MPA (DMPA) by adolescent females is associated with decreased bone mineral density (Kass-Wolff 2001; Cromer et al. 2004; Lara-Torre et al. 2004; Williams & Creighton 2012), a condition which is reversed when the use of this contraceptive is discontinued (Cundy et al. 1994). Interestingly, postmenopausal women using combined HRT formulations containing MPA have been shown to be at an increased risk of dementia (Rossouw et al. 2002; Warren 2004). Alarmingly, the contraceptive use of MPA has also been shown to modulate the local immune response in the female genital tract, thereby increasing susceptibility to genital tract infection such as herpes simplex virus type (HSV)-2 (Mostad et al. 2000), chlamydia (Morrison et al. 2004), gonorrhoea (Morrison et al. 2004) and human immunodeficiency virus (HIV)-1 (Morrison et al. 2010). Although MPA can be used for the treatment of breast and endometrial cancer, evidence in the literature suggests that MPA may in fact be associated with increased risk of developing breast (Lee et al. 1987; Riis et al. 2002; Rossouw et al. 2002; Beral 2003; Althuis et al. 2003; Stahlberg et al. 2003; Li et al. 2012; Beaber et al. 2014), as well as ovarian cancer (Anderson et al. 2003). For example, the use of MPA, NET and LNG in contraception (Lee et al. 1987; Althuis et al. 2003; Li et al. 2012; Beaber et al. 2014) and HRT (Riis et al. 2002; Rossouw et al. 2002; Beral 2003; Stahlberg et al. 2003) have all been associated with increased risk of breast cancer.

Many of the undesirable side-effects observed with the clinical use of progestins are thought to be due to the cross-reactivity of progestins with steroid receptors other than the PR. For example, bloating, weight gain, as well as salt and water retention is associated with progestins lacking anti-mineralocorticoid activity (Li et al. 2000; Greydanus et al. 2001; Elger et al. 2003; Sitruk-Ware 2006; Ojule et al. 2010; Moore et al. 2012; Stanczyk et al. 2013), whereas interference with the local immune response in the female genital tract and decreased bone mineral density, may be attributed to the glucocorticoid-like properties of progestins such as MPA (Ishida et al. 2002; Ishida et al. 2008; Tomasicchio et al. 2013; Louw-du Toit et al. 2014). However, despite the ability of some progestins to exert off-target biological effects via other steroid receptors, the actions of progestins via the PR itself have also been implicated in side-effects such as increased risk of breast cancer. For example, results from a study by Wargon et al. (2014) showed that the stimulatory effects of MPA on breast tumour growth were mediated by the PR. Details of the PR structure and general mechanism, as well as the cellular mechanism of action of progestins via the PR, will be discussed in Section 1.3.

1.3. Structure and general mechanism of the progesterone receptor (PR)

1.3.1. Structure

The PR is a steroid receptor which belongs to the nuclear receptor superfamily, comprising the PR, androgen receptor (AR), mineralocorticoid receptor (MR), glucocorticoid receptor (GR) and ER (Lu et al. 2006; Griekspoor 2007). These receptors are ligand-activated transcription factors that share similar structures and mechanisms of action (Griekspoor 2007; Africander, et al. 2011a). In females, the PR is expressed in various target tissues including the uterus, ovary, mammary gland, brain, pituitary gland and the pancreas (Graham & Clarke 1997; Africander, et al. 2011a). The PR, like other steroid receptors, consists of the following functional domains: a highly variable amino-terminal domain, a central highly conserved DNA binding domain (DBD), a flexible hinge region and a carboxy-terminal domain containing a moderately conserved ligand binding domain (LBD) (figure 1.3) (Kastner et al. 1990; Giangrande et al. 1997; Scarpin et al. 2009; Rękawiecki et al.

2011). The amino-terminal domain contains a ligand-independent activation function (AF)-1 domain which is important for optimal transcriptional activity and is responsible for protein-protein interactions with transcription factors and co-factors (Giangrande et al. 1997; Rękawiecki et al. 2011). The DBD allows binding of the receptor to target DNA sequences, dimerization of the receptor and interactions with certain co-factors involved in transcription, while the LBD is responsible for ligand-binding (Giangrande et al. 1997; Rękawiecki et al. 2011). The LBD contains a ligand-dependent AF-2 domain, and the LXXLL motif found within this domain is involved in protein-protein interactions with transcription factors and chaperone proteins (Scarpin et al. 2009; Rękawiecki et al. 2011; Jacobsen & Horwitz 2012). Moreover, both the DBD and LBD are essential for nuclear translocation of the steroid receptor-hormone complex (Griekspoor 2007).

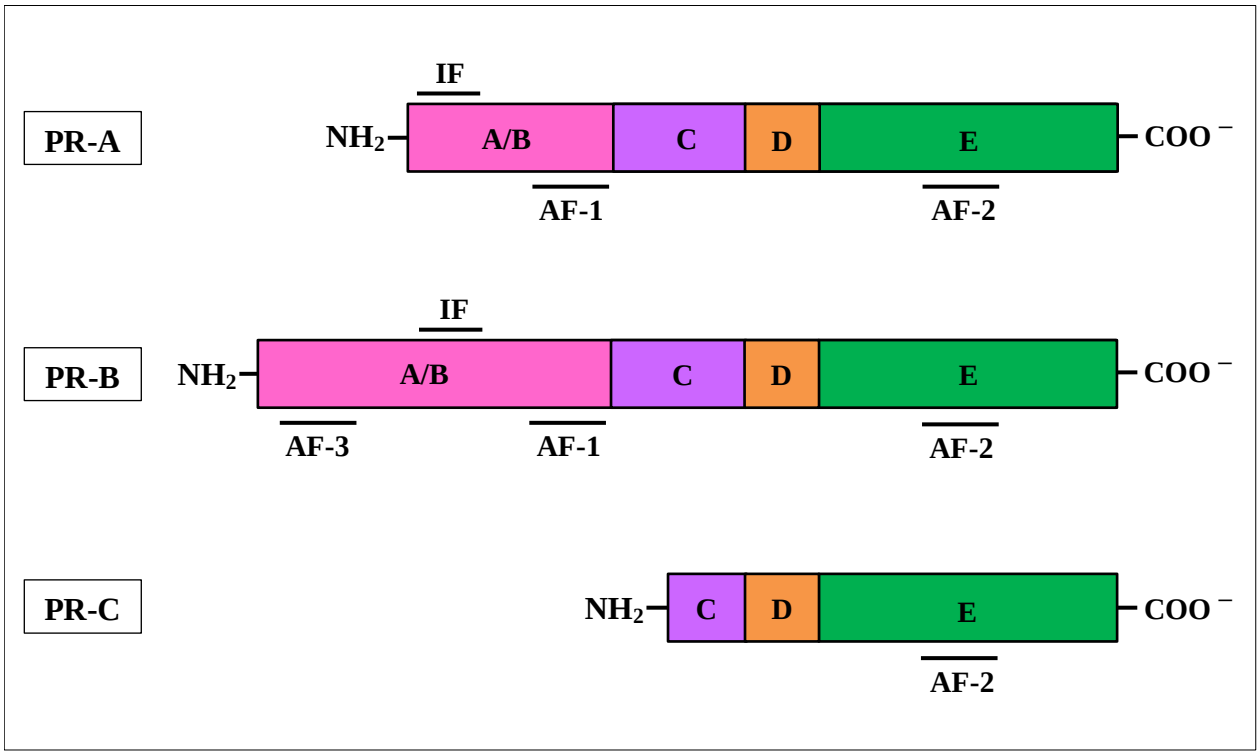


Figure 1.3. A schematic illustration of the structural and functional domains of the PR isoforms. The PR consists of the following domains: an amino-terminal domain (A/B), the DNA binding domain (DBD; C), the flexible hinge region (D) and the carboxy-terminal domain containing the ligand binding domain (LBD; E). PR-A has a truncated version of the A/B domain (lacking AF-3), whilst PR-C has a truncated C domain and lacks the A/B domain. AF - activation function; IF - inhibitory function. Adapted from Rękawiecki et al. (2011) and Africander et al. (2011a).

Three distinct PR isoforms exist namely PR-A, PR-B and PR-C (figure 1.3) (Kastner et al. 1990; Wei & Gonzalez-Aller 1990; Daniel et al. 2011; Rękawiecki et al. 2011), which are transcribed

from three different promoters of a single gene (Kastner et al. 1990; Wei & Gonzalez-Aller 1990; Rękawiecki et al. 2011). PR-A is a 94 kDa protein, while PR-B (~110 kDa) is larger as it contains an additional 164 amino acids at the amino-terminal (Kastner et al. 1990; Giangrande et al. 1997; Giangrande et al. 2000). An AF-3 domain is found in this amino-terminal region (Kastner et al. 1990; Giangrande et al. 1997; Rękawiecki et al. 2011), which leads to the binding of certain co-activators to PR-B, but not PR-A (Giangrande et al. 2000; Graham & Clarke 2002; Tung et al. 2006). Furthermore, both PR-A and PR-B contain an inhibitory function (IF) domain in their amino-terminal domains which have been shown to interact with co-repressors (Giangrande et al. 1997; Rękawiecki et al. 2011; Jacobsen & Horwitz 2012). In contrast to PR-A and PR-B, PR-C is a small 60 kDa protein that lacks the entire amino-terminal domain as well as a large part of the DBD (Wei & Gonzalez-Aller 1990; Daniel et al. 2011). PR-C thus cannot bind DNA and is transcriptionally inactive (Daniel et al. 2011; Rękawiecki et al. 2011; Abdel-Hafiz & Horwitz 2014).

The evidence in the literature suggests that PR-A and PR-B may display differential physiological functions in different target tissues. For example, PR-B is more proliferative in the breast as it is mainly involved in mammary gland branching and alveologenesis (Conneely et al. 2003), whereas PR-A is more proliferative in the uterus as it plays an important role in the development of the uterus and implantation of the fertilized ovum (Conneely et al. 2001; Mulac-Jericevic & Conneely 2004; Diep et al. 2015). It is well-known that PR-A is a repressor of PR-B activity (Vegeto & Shahbaz 1993), as well as that of the ER, AR, MR and GR (McDonnell & Goldman 1994; McDonnell et al. 1994; Kraus et al. 1995; Kraus et al. 1997; Conneely & Lydon 2000). As PR-B has been reported to be more transcriptionally active than PR-A in the presence of ligand (Kastner et al. 1990; Edwards et al. 1995; Rękawiecki et al. 2011; Jacobsen & Horwitz 2012), we mainly focussed on the activity of PR-B in this thesis.

1.3.2. Mechanisms of action

Unliganded PR-B is evenly distributed between the cytoplasm and the nucleus (Lim et al. 1999; Li 2005; Griekspoor 2007), and is associated with chaperone proteins such as heat shock protein (hsp)90 and hsp70, p23 and immunophillins (Griekspoor 2007; Rękawiecki et al. 2011). In the presence of ligand, PR-B undergoes a conformational change which ultimately leads to the dissociation of the multiprotein complex, leading to the activation of signalling pathways through either non-genomic (Boonyaratanakornkit et al. 2001; Boonyaratanakornkit et al. 2007; Carnevale et al. 2007; Boonyaratanakornkit et al. 2008; Kariagina et al. 2008) or genomic mechanisms (Rękawiecki et al. 2011). Non-genomic mechanisms involves rapid signalling pathways and include a direct interaction of cytoplasmic ligand-bound PR-B with the membrane-associated c-Src tyrosine kinase (Boonyaratanakornkit et al. 2001; Boonyaratanakornkit et al. 2007; Carnevale et al. 2007; Boonyaratanakornkit et al. 2008; Kariagina et al. 2008), while genomic mechanisms can take hours and involves the translocation of ligand-bound PR-B to the nucleus where it regulates gene expression (Griekspoor 2007; Africander, et al. 2011a; Rękawiecki et al. 2011). It is important to note that ligand-induced non-genomic mechanisms can also occur via a membrane PR (mPR) (Thomas et al. 2007; Thomas 2008; Stanczyk et al. 2013)

1.3.2.1. Non-genomic mechanisms

PR-B contains a proline-rich PXXPXR motif in its amino-terminal domain enabling the cytoplasmic ligand-bound PR-B to directly bind to the Src-homology (SH-3) domain of c-Src (Boonyaratanakornkit et al. 2001; Carnevale et al. 2007). This results in activation of either the c-Src/Ras/mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathways (Boonyaratanakornkit et al. 2007). Activation of these signalling pathways ultimately leads to the activation of other transcription factors via phosphorylation events (Saitoh et al. 2005; Carnevale et al. 2007; Boonyaratanakornkit et al. 2008). For example, the transcription factors activator protein-1 (AP-1) and nuclear factor kappa B (NFκB) have been shown to be activated by phosphorylation when the MAPK and PI3K/Akt signalling pathways are activated

(Faivre et al. 2005; Saitoh et al. 2005). It has been suggested that this mechanism allows PR-B to activate transcription of genes that do not contain progesterone response elements (PREs) in their promoter regions, such as the PR-B-mediated upregulation of cyclin D1 by MPA (Saitoh et al. 2005).

Non-genomic mechanisms can also be mediated via binding of a progestogen to an mPR, which has been shown to be a part of the progestin and adiponectin Q receptor family (PAQR) (Tang et al. 2005; Thomas et al. 2007; Thomas 2008; Dressing et al. 2011). Similarly to a G protein-coupled receptor (GPCR), the PAQR contains a seven transmembrane domain and can couple and activate G-proteins (Tang et al. 2005; Thomas et al. 2007; Thomas 2008; Gellersen et al. 2009; Dressing et al. 2011). Interestingly, phylogenetic analysis has revealed that PAQRs have a different ancestral origin than GPCRs (Tang et al. 2005; Thomas et al. 2007; Thomas 2008; Dressing et al. 2011). Following the binding of a PR ligand to mPR and the activation of G-proteins, downstream MAPK and/or PI3K/Akt signalling pathways are activated, leading to the subsequent phosphorylation and activation of nuclear proteins, which include other transcription factors (Edwards 2005; Stanczyk et al. 2013).

1.3.2.2. Genomic mechanisms

Genomic mechanisms of PR-B refers to the transcriptional regulation of target genes either positively (transactivation) or negatively (transrepression) (Griekspoor 2007; Africander, et al. 2011a; Rękawiecki et al. 2011). The ligand-bound PR-B binds as a dimer to PREs located in the promoter regions of PR regulated genes (figure 1.5) (Bagchi et al. 1988; Giangrande et al. 1997; Rękawiecki et al. 2011). Components of the basal transcription machinery and other necessary co-regulatory proteins such as co-activators as well as chromatin remodelling proteins are subsequently recruited to the promoters of the target genes (Beato & Klug 2000; Griekspoor 2007; Africander, et al. 2011a; Rękawiecki et al. 2011). Histones are then acetylated which leads to chromatin decondensation (McKenna et al. 1999), and the subsequent activation of transcription in a process

called transactivation (Beato & Klug 2000; Griekspoor 2007; Africander, et al. 2011a; Rękawiecki et al. 2011).

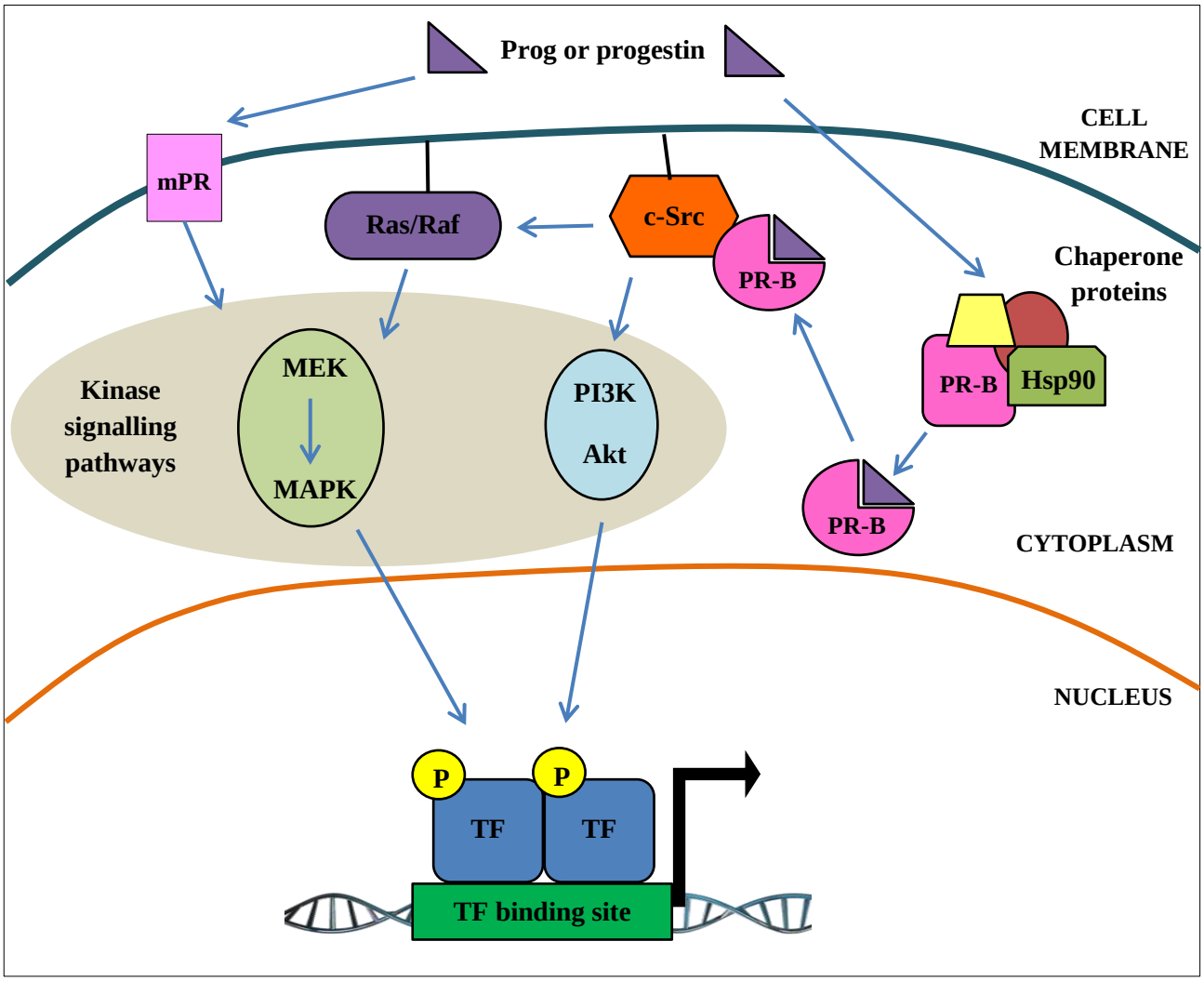


Figure 1.4. An illustration of the non-genomic mechanisms of action of progestogens via intracellular PR-B and the membrane-bound PR (mPR). PR ligands such as Prog or progestins can elicit biological effects through non-genomic signalling mechanisms by either binding to intracellular PR-B or the mPR. For the rapid activation of intracellular PR-B, the ligand diffuses across the cell membrane and binds to cytoplasmic PR-B, followed by the dissociation of heat-shock proteins, immunophillins and other chaperone proteins from PR-B. The ligand-bound PR-B binds and subsequently activates c-Src, which leads to the activation of the MAPK and/or PI3K/Akt signalling pathways. For activation of mPR, the ligand binds to mPR which in turn activates kinases such as MAPK and/or PI3K/Akt. Finally, these signalling cascades activate other transcription factors through phosphorylation, and lead to the regulation of genes without a progesterone response element (PRE) sequence. Adapted from Giulianelli et al. (2012) and Stanczyk et al. (2013).

PR-B can negatively regulate transcription (Rękawiecki et al. 2011; Abdel-Hafiz & Horwitz 2014) when the ligand-activated PR-B represses the activity of the transcription factor NFκB (Kalkhoven et al. 1996; Kobayashi et al. 2010) via an interaction between the PR and the p65-subunit of NFκB.

Co-repressors and chromatin remodelling proteins are subsequently recruited to the promoter regions of the target genes (Daniel et al. 2009; Africander et al. 2011a), and transcription is inhibited as a result of condensed chromatin due to histone deacetylation (McKenna et al. 1999; Gronemeyer et al. 2004; Rękawiecki et al. 2011). This negative regulation of target gene expression is referred to as transrepression (figure 1.5) (Kalkhoven et al. 1996).

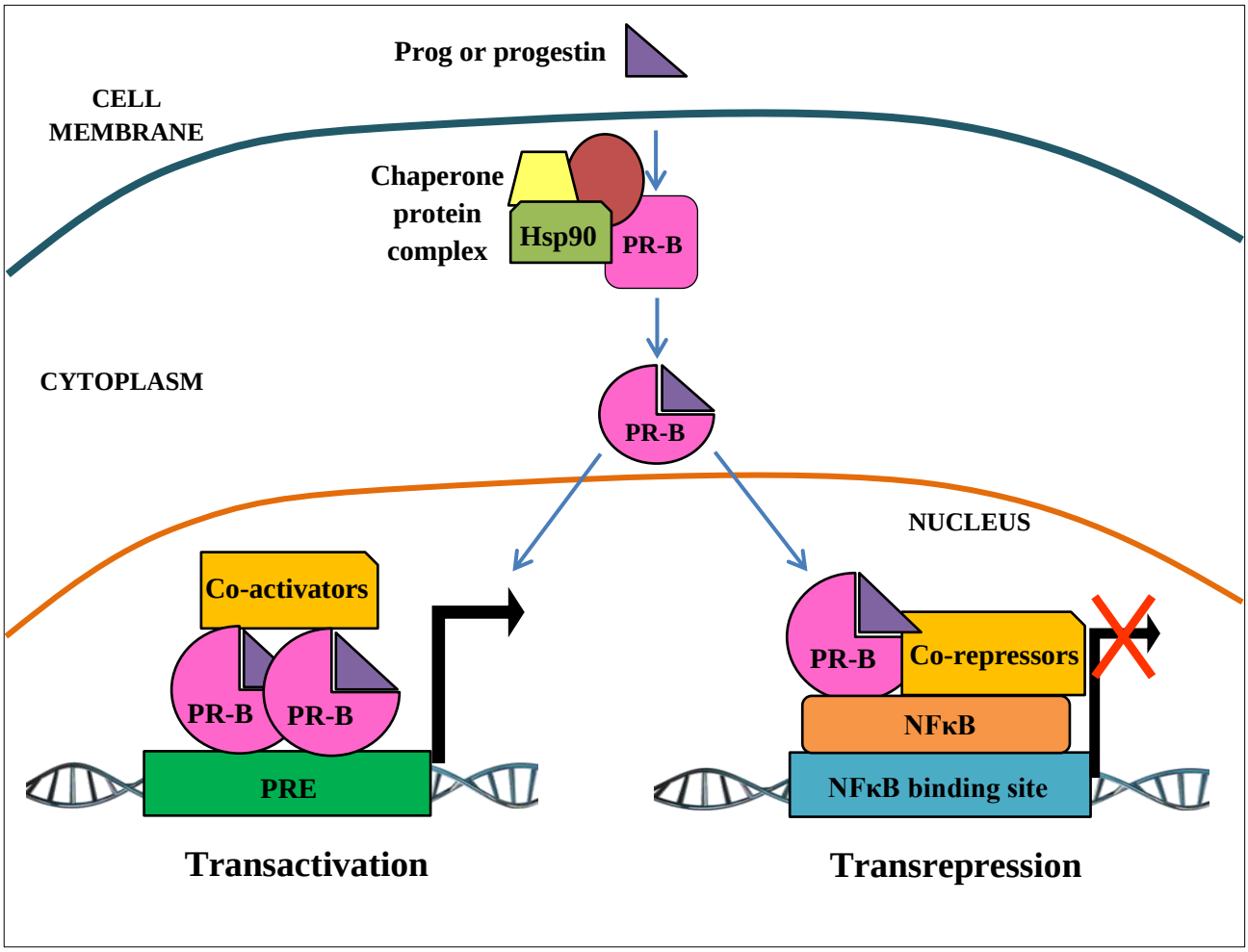


Figure 1.5. An illustration of the genomic mechanisms of PR-B. The ligand (e.g. Prog or progestin) diffuses across the cell membrane and binds to the intracellular PR-B. The receptor undergoes a conformational change allowing the dissociation of heat-shock proteins, immunophillins and other chaperone proteins. The activated PR-B then translocates to the nucleus where it either activates transcription of target genes by binding as a dimer to PREs (transactivation) or represses transcription of target genes due to the PR monomer tethering to a DNA-bound transcription factor such as NFκB (transrepression). Adapted from Africander et al. (2011a).

1.4. The PR, progestins and breast cancer

For many years, the role of the PR in breast cancer was thought to be limited to only a prognostic marker of ER functionality, while the ER was considered the main etiological factor in the

development and progression of breast cancer (Horwitz & McGuire 1978; Hefti et al. 2013). However, recent evidence suggest that the PR itself can directly contribute to the development of breast cancer by upregulating the expression of genes known to be involved in the development and progression of breast cancer (Giulianelli et al. 2012; Wargon et al. 2014). Whilst PR-A and PR-B are present at equimolar concentrations in the normal breast (Mote et al. 2002), PR-A is often overexpressed in breast cancer (Graham et al. 1995; Bamberger et al. 2000; Ariga et al. 2001; Hopp et al. 2004). In fact, studies have shown that the ratio of the PR isoforms, PR-A:PR-B, is an important determinant of breast cancer development and progression (Graham et al. 1995; Mote et al. 2002; Hopp et al. 2004; Cui 2005). For example, breast tumours expressing high PR-A:PR-B ratios have been shown to be more aggressive and pose a higher risk of relapse (Hopp et al. 2004). Although the exact cause for the altered PR-A:PR-B ratio in breast cancer has not been fully elucidated, it has been suggested that it may be due to an increase in the activity of kinases such as MAPK in breast cancers (Daniel et al. 2007; Diep et al. 2015). This increased kinase activity leads to increased phosphorylation of PR-B, which in turn leads to hyperactivation and an increase in the rate of PR-B protein turnover (Daniel et al. 2011; Diep et al. 2015).

Breast cancer is one of the most commonly diagnosed cancers (Platet et al. 2004), and the most common cause of cancer death amongst women worldwide (Sommer & Fuqua 2001; Ferlay et al. 2014). Understanding factors which may contribute to the development of this disease, such as the PR and the activity of ligands binding to the receptor, is therefore essential. Although a number of studies have started investigating the role of the PR in breast cancer (Hyder et al. 1998; Hyder et al. 2001; Moore et al. 2006; Jacobsen et al. 2003; Mueller et al. 2003; Sartorius et al. 2003; Wu et al. 2004; Liang et al. 2007; Giulianelli et al. 2012; Bellance et al. 2013; Kariagina et al. 2013; Wargon et al. 2014; Diep et al. 2015), and examined whether PR ligands (progestogens) increase the risk of developing breast cancer (Rossouw et al. 2002; Beral 2003; Anderson et al. 2004; Li et al. 2012), results are often contradictory and many questions remain unanswered. In the next section, the

existing knowledge on the PR and progestogens in breast cancer development and progression will be reviewed.

1.4.1. Clinical and epidemiological studies

Concerns that progestins increase breast cancer risk were raised by the results of the Women's Health Initiative (WHI), a large-scale randomised clinical trial (Rossouw et al. 2002). This study examined the health benefits and risks associated with the use of HRT, either administered as conjugated equine estrogen (CEE) alone or as an estrogen-progestin combination (CEE–MPA) (Rossouw et al. 2002; Anderson et al. 2004). The estrogen-progestin combined HRT treatment component of the trial was stopped earlier than planned due to an increase in the risk of developing several adverse conditions such as CHD, stroke, pulmonary embolism and most relevant to this thesis, invasive breast cancer (Rossouw et al. 2002; Chlebowski et al. 2003; Chlebowski et al. 2013a; Chlebowski et al. 2013b). Notably, the estrogen only trial was stopped two years later due to an increased risk of stroke, but no significant effect on CHD or breast cancer risk was observed (Anderson et al. 2004). These results suggest that the MPA component was responsible for the increased CHD and breast cancer risk in the estrogen-progestin arm of the trial. Evidence from observational studies also indicate an association between HRT and increased risk of breast cancer (Ross et al. 2000; Newcomb et al. 2002). For example, results from an observational study two years prior to the publication of the WHI results showed that the use of CEE alone, as well as the use of estrogen-progestin HRT (CEE-MPA) was associated with increased risk of breast cancer in postmenopausal women, with a higher risk associated with the CEE-MPA HRT (Ross et al. 2000). Similarly, a population-based case-control study by Newcomb et al. (2002) also showed that CEE-MPA therapy was associated with a greater breast cancer risk in postmenopausal women (Newcomb et al. 2002). In agreement with the above-mentioned studies, results from the Million Women Study found that both estrogen only HRT and combined HRT preparations containing MPA increased the risk of developing breast cancer in long-term HRT users (Beral 2003). However, this study examined the effects of different types of progestins and estrogens on breast

cancer incidence and mortality in over one million HRT users, and found that even though combined estrogen-progestin formulations were associated with a greater breast cancer risk than estrogen only formulations, there were no significant differences in terms of risk between the different estrogens or progestins (Beral 2003). The estrogens examined were CEE and ethinyl estradiol (EE), while the progestins examined were the first generation progestins MPA and NET-A, as well as the second generation progestin LNG. This higher breast cancer risk associated with different estrogen-progestin formulations was also observed in the French E3N cohort study evaluating the risk of breast cancer associated with the use of MPA- and NET-A-containing combined HRT formulations and estrogen-only HRT formulations in post-menopausal women (Fournier et al. 2005; Fournier et al. 2008). Interestingly, this same study also showed that estrogen-progestin combined formulations containing the synthetic progestin R5020, considered to be PR-specific, significantly increased breast cancer risk (Fournier et al. 2005; Fournier et al. 2008).

Studies have shown that, in contrast to HRT formulations containing the above-mentioned estrogens and progestins, formulations containing estrogenic compounds such as estradiol (E_2) and CEE in combination with micronized Prog, did not affect breast cancer risk (de Lignières et al. 2002; Fournier et al. 2005; Fournier et al. 2008). Similarly, no incidence of breast cancer was reported with the use of compounded bProg either alone or in combination with E_2 as part of bio-identical HRT preparations, (Ruiz et al. 2011). In agreement with this, a recent study on breast cancer risk in 101 women administered a combined HRT regimen of compounded bProg in combination with biest (combination of E_2 and estriol (E_3), reported no incidence of breast cancer in any of the women tested over a four year period (White 2015). Although these results may be promising, much larger randomised control studies on the use of bProg over longer periods are, however, needed to definitively prove that the use of bProg in HRT is “safer” than the use of progestins in terms of breast cancer risk.

Most studies investigating effects of progestins on breast cancer, focus on the association of breast cancer risk in postmenopausal women using HRT. However, some studies have also investigated

the association between the contraceptive use of progestins and increased breast cancer risk (Lee et al. 1987; Althuis et al. 2003; Hunter et al. 2010; Li et al. 2012; Beaber et al. 2014), with the results often contradictory. For example, two studies have shown that the injectable progestin-only contraceptive DMPA enhanced breast cancer risk (~2.2 to 2.6-fold) in women aged 20 to 58 (Lee et al. 1987; Li et al. 2012), while two other studies showed no effect on breast cancer risk (Paul et al. 1989; Shapiro et al. 2000). Interestingly, the use of combined oral contraceptive (COC) formulations containing either NET or LNG has also been shown to increase breast cancer risk (Althuis et al. 2003). Similarly, Hunter et al. (2010) reported an increase in breast cancer risk with the use of triphasic LNG-containing COCs when compared to women not using oral contraception (Hunter et al. 2010). Triphasic COCs refer to contraceptive formulations that are given in three stages and each stage contains different concentrations of estrogens and progestins. In contrast to Althuis et al. (2003), Hunter and co-workers (2010) reported no increase in breast cancer risk with the use of NET-containing COCs (Hunter et al. 2010). It is noteworthy that a recent population-based case-control study by Beaber et al. (2014) showed that COC formulations containing either NET, LNG or DRSP, increased the risk of breast cancer to similar extents (Beaber et al. 2014). Collectively, these results suggest that the first- (MPA and NET-A), second- (LNG) and fourth (DRSP) generation progestins all increase the risk of breast cancer. However, considering that a large number of progestins are available for clinical use and that progestins are structurally different (Stanczyk 2003; Sitruk-Ware 2004a), it may be possible that not all progestins would cause an increase in breast cancer risk.

1.4.2. Experimental studies

Various processes are implicated in the development and progression of breast cancer, including continual proliferation, evasion of apoptosis, sustained angiogenesis as well as migration and invasion of breast cancer cells (Hanahan & Weinberg 2000; Sledge & Miller 2003; Hanahan & Weinberg 2011). In the next sections, the effects of progestogens and/or the PR on these processes will be reviewed.

1.4.2.1. Proliferation

Proliferation is an important process for cell growth and renewal, maintaining tissue homeostasis and normal cellular function (Hall & Levison 1990; Sears & Nevins 2002; DeBerardinis et al. 2008; Hanahan & Weinberg 2011). In normal cells, this process is under tight regulation of the cell cycle (Vermeulen et al. 2003; DeBerardinis et al. 2008), while this process becomes dysregulated in cancer allowing the uncontrolled proliferation of cells to continue (Vermeulen et al. 2003; Hanahan & Weinberg 2011). A number of studies have investigated the effects of progestogens (Horwitz & Freidenberg 1985; van der Burg et al. 1992; Catherino et al. 1993; Botella et al. 1994; Kalkhoven et al. 1994; Krämer et al. 2006; Ruan et al. 2012) and the role of the ligand-bound PR (Giulianelli et al. 2012; Wargon et al. 2014) on breast cancer cell proliferation. Findings from several *in vitro* studies investigating the effects of progestins on proliferation of normal and cancerous breast epithelial cell lines are contradictory, with the effects appearing to be cell line dependent (Horwitz & Freidenberg 1985; van der Burg et al. 1992; Catherino et al. 1993; Botella et al. 1994; Kalkhoven et al. 1994; Krämer et al. 2006; Ruan et al. 2012). For example, NET, LNG and GES were shown to cause proliferation of the human HCC1500 and MCF-7 breast cancer cell lines (van der Burg et al. 1992; Catherino et al. 1993; Kalkhoven et al. 1994; Krämer et al. 2006; Ruan et al. 2012), while having no effect on the normal human breast epithelial cell line MCF10A (Krämer et al. 2006). Interestingly, the newer generation progestin, DRSP, also displayed a proliferative effect on the MCF-7 breast cancer cell line (Ruan et al. 2012). GES has also been shown to have proliferative effects in the T47D breast cancer cell line, while NET and R5020 have been shown to inhibit proliferation of the T47D cells (Horwitz & Freidenberg 1985; Botella et al. 1994; Kalkhoven et al. 1994). In contrast to NET, LNG and GES, MPA has been shown to stimulate proliferation of the normal MCF-10A breast cell line (Krämer et al. 2006), while inhibiting proliferation of the HCC1500 and T47D breast cancer cell lines (Botella et al. 1994; Krämer et al. 2006), and having no significant effect on the proliferation of the MCF-7 breast cancer cells (Catherino et al. 1993; Ruan et al. 2012). Two other studies, however, have shown that MPA stimulates T47D breast cancer cell

proliferation (Liang et al. 2006), and that the PR antagonist mifepristone (RU486) was able to inhibit this effect (Giulianelli et al. 2012; Wargon et al. 2014). These authors thus suggested that the PR mediates the proliferative effect of MPA. However, this result should be carefully interpreted as RU486 is not only a PR antagonist, but also an AR and a GR antagonist, both of which are expressed in T47D cells (Spitz & Bardin 1993; Song et al. 2004). Wargon and co-workers (2014) provided more convincing data for a role of the PR when showing that MPA-induced T47D breast cancer cell proliferation is abolished once PR-B expression is silenced (Wargon et al. 2014).

Prog has been shown to have no effect on the growth of normal MCF-10A breast cells, or the HCC1500 and MCF-7 breast cancer cells (Krämer et al. 2006; Ruan et al. 2012). In contrast, Wiebe and co-workers (2000) showed that Prog has an anti-proliferative effect on both the normal MCF-10A breast cells and the MCF-7 breast cancer cells (Wiebe et al. 2000). Similarly, Prog has also been shown to inhibit proliferation of MDA-MB-231 breast cancer cells co-transfected with PR-A and PR-B (Lin et al. 1999), as well as T47D breast cancer cells endogenously expressing both PR-A and PR-B (Formby & Wiley 1998). Conversely, Liang and co-workers (2006) showed that Prog has pro-proliferative effects on T47D cells, as well as BT-474 breast cancer cells (Liang et al. 2006). To the best of our knowledge, the effect of bProg on breast cancer cell proliferation has not been investigated.

Progestins have been shown to regulate the expression of genes which play important roles in breast cancer cell proliferation (Wong & Murphy 1991; Moore et al. 1997; Thuneke et al. 2000; Giulianelli et al. 2012; Wargon et al. 2014). For example, R5020 (Moore et al. 1997) and MPA (Wong & Murphy 1991; Giulianelli et al. 2012; Wargon et al. 2014) have been shown to upregulate c-myc mRNA expression in T47D breast cancer cells. The c-myc gene is a marker for proliferation which has been shown to be overexpressed in breast cancer (Moore et al. 1997). Considering that the c-myc proto-oncogene contains a PRE sequence in its promoter, it has been suggested that the upregulation of this gene, at least for R5020, is mediated by the PR binding to PRE (Moore et al. 1997). MPA has also been shown to upregulate the mRNA (Giulianelli et al. 2012; Wargon et al.

2014) and protein expression of another well-known marker of proliferation, Cyclin D1 in T47D cells (Thuneke et al. 2000). Interestingly, chromatin immunoprecipitation (ChIP) assays showed that the PR is recruited to both the c-myc and Cyclin D1 gene promoters upon treatment with MPA, suggesting that the PR is involved in the MPA-induced upregulation of these genes (Giulianelli et al. 2012; Wargon et al. 2014). Surprisingly, information on the effects of Prog on c-myc and Cyclin D1 mRNA expression is not readily available.

1.4.2.2. Apoptosis

Apoptosis, also known as programmed cell death, is a naturally occurring process which plays an important role in normal cell turnover and the elimination of improperly developed and damaged cells (Thompson 1995; Elmore 2007). In cancer, however, apoptosis is often evaded (Thompson 1995; Hanahan & Weinberg 2000; Hanahan & Weinberg 2011) To date, a number of studies have investigated the effects of progestins and the role of the PR on apoptosis. For example, R5020 (Moore et al. 2006) and MPA (Ory et al. 2001; Franke & Vermes 2003) have been shown to display anti-apoptotic effects on the T47D and MCF-7 cell lines, while MPA has also been shown to inhibit apoptosis of the H466B breast cancer cell line (Ory et al. 2001). Similarly, NET-A (Franke & Vermes 2003) and R5020 (Moore et al. 2006) inhibited apoptosis of the MCF-7 and MDA-MB-231 breast cancer cells, respectively. Moreover, Moore et al. (2006) showed that the anti-apoptotic effect of R5020 in the T47D cell line is at least partly mediated via the PR, as the PR, GR and AR antagonist, RU486, was able to partially abrogate the R5020-induced effect (Moore et al. 2006). In contrast to the progestins that all appear to inhibit apoptosis, evidence from the literature regarding the effects of Prog on cell death is contradictory. For example, Moore and co-workers showed that 100 nM Prog inhibits apoptosis of T47D breast cancer cells (Moore et al. 2006), while other studies have shown that 10 µM Prog exhibits pro-apoptotic effects in both the T47D (Formby & Wiley 1998; Formby & Wiley 1999) and MCF-7 breast cancer cells (Franke & Vermes 2003), suggesting concentration-specific effects.

The process of apoptosis is dependent on a balance between the expression of pro-apoptotic and anti-apoptotic genes (Ory et al. 2001). Studies investigating the effects of progestins on the expression of pro-apoptotic and anti-apoptotic genes are limited. Findings from one study showed that R5020 downregulated the mRNA expression of the pro-apoptotic tumor-suppressor p53 gene in T47D cells, suggesting that R5020 is anti-apoptotic (Hurd et al. 1995). In contrast, Moore et al. (2000) showed that R5020 downregulated the expression of the anti-apoptotic bcl-2 gene, suggesting that R5020 promotes apoptosis (Moore et al. 2000). The first generation progestin, MPA has been shown to upregulate the mRNA expression of the pro-apoptotic bcl-xS gene and downregulate bcl-2 mRNA expression in T47D cells, suggesting that MPA is pro-apoptotic (Ory et al. 2001). However, this same study also reported that MPA upregulated the mRNA expression of the anti-apoptotic bcl-xL gene in T47D cells, while having no effect on the mRNA expression of the pro-apoptotic Bax gene, which suggests that MPA is anti-apoptotic (Ory et al. 2001). Considering the above results, it is clear that no definitive conclusions can be drawn from the results of individual apoptosis-related genes, and that future studies should directly compare the effects of progestins on several both pro-apoptotic and anti-apoptotic genes.

The effects of Prog on apoptosis-related genes have also previously been studied, with most studies indicating that Prog may be pro-apoptotic as it downregulates bcl-2 mRNA and protein expression (Formby & Wiley 1998; Moore et al. 2000), downregulates the mRNA expression levels of the apoptosis inhibitor, survivin (Formby & Wiley 1999), and upregulates p53 mRNA expression (Formby & Wiley 1998). However, effects may also be anti-apoptotic as at least one study has shown that Prog upregulates bcl-xL protein expression, while having no effect on Bax protein expression (Moore et al. 2000). Taken together, the above-mentioned evidence suggests that progestins promote breast cancer by inhibiting cell death (anti-apoptotic), while Prog can either promote (pro-apoptotic) or inhibit (anti-apoptotic) programmed cell death.

1.4.2.3. Migration and invasion

Migration and invasion refers to the process of metastasis whereby cancerous cells from the primary tumour site spread to other organs in the body via the circulatory blood- and lymphatic systems (Chambers et al. 2002; Polyak 2007). To date, studies investigating the effects of progestins on migration and invasion are scarce. The research group of Simoncini has however investigated effects of MPA and DRSP, and showed that both these progestins increase migration and invasion of the T47D breast cancer cell line, with the increase by MPA being more pronounced (Fu et al. 2008a). Interestingly, Prog also increased T47D breast cancer cell migration and invasion (Fu et al. 2010; Diaz et al. 2012) to a greater extent than DRSP, but lesser extent than MPA (Fu, et al. 2008a; Fu, et al. 2008b). In a different study, Kariagina and co-workers showed that R5020 downregulates the protein expression of the epithelial adhesion protein E-cadherin, a protein, often downregulated in more invasive breast tumours (Kariagina et al. 2013). This result suggests that R5020 promotes invasion of T47D breast cancer cells (Kariagina et al. 2013). Interestingly, using T47D breast cancer cells that have been engineered to silence PR expression (T47D-Y), or express only PR-A (T47D-YA) or only PR-B (T47D-YB), Kariagina and co-workers (2013) showed that the R5020-induced downregulation of E-cadherin is mediated via PR-B, suggesting that PR-B, but not PR-A, promotes R5020-induced breast cancer cell invasion (Kariagina et al. 2013). In contrast, a study by Bellance et al. (2013) using bi-inducible MDA-MB-231 breast cancer cells showed that PR-A and PR-B differentially contributed to the R5020-induced increase in breast cancer cell migration and invasion (Bellance et al. 2013).

1.4.2.4. Angiogenesis

Angiogenesis promotes tumour growth and metastasis by providing tumours with nourishment through the formation of new blood vessels (Hanahan & Weinberg 2000; Sledge & Miller 2003; Hsieh et al. 2005; Hanahan & Weinberg 2011). In terms of investigating the effects of progestins on angiogenesis, most studies have investigated the effects on the mRNA and protein expression levels of vascular endothelial growth factor (VEGF) (Hyder et al. 1998; Hyder et al. 2001; Wu et al. 2004;

Mirkin et al. 2005; Liang et al. 2007). VEGF is a very potent angiogenic growth factor (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011) commonly overexpressed in metastatic breast cancer (Sledge & Miller 2003; Hsieh et al. 2005). However, these studies have been limited to the first generation progestins, MPA and NET, and the second generation progestin LNG. For example, MPA, NET and LNG have previously been shown to increase VEGF mRNA expression in T47D cells (Hyder et al. 1998; Mirkin et al. 2005), while MPA and NET increased VEGF protein expression in T47D cells (Hyder et al. 2001) and BT464 breast cancer xenografts (Liang et al. 2010). In contrast to the above results, MPA and NET had no effect on VEGF protein expression in the MCF-7, MDA-MB-231 or ZR-75 breast cancer cell lines (Hyder et al. 1998). This could possibly be due to the fact that MCF-7, MDA-MB-231 and ZR-75 cells express little to no endogenous PR (Hyder et al. 1998).

The effects of Prog on VEGF expression have been extensively studied, and like progestins, Prog has been shown to increase VEGF mRNA (Hyder et al. 1998; Wu et al. 2004; Mirkin et al. 2005) and protein (Hyder et al. 2001) expression in T47D cells and BT-474 breast cancer xenografts (Liang et al. 2010), while having no effect on VEGF protein expression in the MCF-7, MDA-MB-231 and ZR-75 breast cancer cell lines (Hyder et al. 1998). Two separate studies by Hyder and co-workers showed that the Prog-, MPA- and NET-induced upregulation of VEGF protein expression in T47D cells was mediated by the PR (Hyder et al. 1998; Hyder et al. 2001). In another study, Wu and co-workers (2004) attempted to delineate the role of the individual PR isoforms in the progestogen-induced upregulation of VEGF protein expression in T47D cells (Wu et al. 2004). Using T47D-Y, T47D-YA and T47D-YB cells, these authors showed that MPA, NET and Prog upregulates VEGF protein expression to a greater extent in the T47D-YB cells than in the T47D-YA cells, while having no significant effect on the T47D-Y cells, suggesting that progestogen-induced upregulation of VEGF is mainly mediated via PR-B (Wu et al. 2004). Furthermore, it has been shown that the VEGF gene is under the direct transcriptional regulation of the PR as the gene promoter contains three functional PRE sequences (Mueller et al. 2003). Taken together, these

results suggest that progestogens may be acting via PR-B to promote angiogenesis and ultimately breast cancer growth and metastasis.

1.4.3. Steroid receptor crosstalk in breast cancer

Multiple steroid receptors, in addition to the PR and ERα, have been implicated in the etiology and pathogenesis of breast cancer. For example, increased expression of ERβ and the AR in breast tumours correlate with good prognosis (Peters et al. 2009; Leygue & Murphy 2013). It is known that ERβ inhibits ERα-mediated breast cancer cell proliferation (Williams et al. 2008), while it has been shown that the AR inhibits ERα-mediated transcriptional activation (Peters et al. 2009). In addition, glucocorticoids acting via the GR are known to have anti-proliferative and anti-apoptotic effects on breast cancer (Abduljabbar et al. 2015; reviewed in McNamara & Sasano 2015), and have also been shown to inhibit ERα-mediated breast cancer cell proliferation (Karmakar et al. 2013). Moreover, Hopp et al. (2004) have shown that overexpression of PR-A in ER- and PR-positive breast cancer negatively influences breast cancer treatment with the selective ER modulator tamoxifen, by causing tamoxifen resistance (Hopp et al. 2004). PR-A has in fact been shown to repress the activity of PR-B (Conzen 2008), as well as that of the ER, AR, MR and GR (Vegeto & Shahbaz 1993; Wen et al. 1994; McDonnell & Goldman 1994; McDonnell et al. 1994).

Daniel and co-workers showed that in the absence of ligand, PR-B, ERα and the scaffolding protein proline-, glutamate- and leucine-rich protein 1 (PELP1) form transcriptional complexes at the promoter sequences of certain ER target genes, enhancing the responsiveness of MCF-7 breast cancer cells to E_2, thus resulting in a more aggressive breast cancer phenotype (Daniel et al. 2015). In another study it was shown that in the presence of Prog, the PR inhibits breast tumour growth by associating with ERα and thereby altering the recruitment of ERα to the chromatin, which ultimately changes the transcriptional activity of ERα and activates a gene expression programme that favours a good prognosis (Mohammed et al. 2015). All of the above clearly indicates the complexity of crosstalk between steroid receptors in breast cancer. Interestingly, multiple steroid receptors have been implicated in the mechanism whereby MPA increases breast cancer. For

example, MPA has been shown to mediate its breast cancer promoting effects via the PR (Hyder et al. 1998; Hyder et al. 2001; Fu, et al. 2008a; Wargon et al. 2014) and the AR (Ochnik 2012). An example of steroid receptor crosstalk which is of particular importance for the present study is the reported crosstalk between the PR and ERα. Giulianelli et al. (2012) showed that ERα is required for MPA-induced gene regulation and breast cancer cell proliferation mediated by PR-B (Giulianelli et al. 2012).

1.5. Conclusion

The available clinical, epidemiological and experimental studies on progestins and breast cancer indicate an association between the use of progestins in HRT and increased risk of developing breast cancer, with the majority of research focussing on the first generation progestin MPA. A few studies have, however, examined the effects of the first generation progestin NET, and second generation progestin LNG, while only one or two have investigated newer generation progestins like GES and DRSP. Considering that a number of different progestins are available for clinical use, and that small changes in their structure can lead to different biological effects, it is possible that not all progestins would increase the risk of breast cancer. Furthermore, due to concerns that the use of progestins in HRT is associated with an increased risk of breast cancer, the compounded bio-identical hormone, bProg, is sometimes used as an alternate, "safer" hormone therapy. Considering that progestins and bProg are PR ligands, and that the PR plays an important role in breast cancer development and progression, it is crucial to understand how the actions of the compounds via the PR, specifically the most active isoform PR-B, may be differentially affecting breast cancer risk. Studies directly comparing the activity of progestins from different generations via PR-B in the same model system are lacking. Interestingly, emerging evidence shows that the activity of MPA via PR-B requires the presence of ERα (Giulianelli et al. 2012). As the ER exists as two subtypes, ERα and ERβ, a question that arises is whether ERβ is also required for PR-B mediated effects of MPA, and/or other progestins. Understanding the mechanisms whereby progestins may or may not contribute to the development of breast cancer could better inform decisions in terms of the choice

of progestin for therapeutic use or the development of new therapies for the treatment of breast cancer.

1.6. Hypothesis and aims

It is evident that increased risk of breast cancer is associated with the clinical use of some progestins. Whether this risk is applicable to all progestins is not clear. Surprisingly, the role of the PR in mediating the effects of progestins, and the subsequent effects on breast cancer is not well studied. The primary hypothesis of this study was that selected progestins from the different generations would display differential transcriptional activities and proliferative effects via PR-B, while no difference would be observed between Prog and bProg. Furthermore, knowing that Giulianelli et al. (2012) has shown that ERα is required for MPA-induced breast cancer cell proliferation, and that ERα is known to be pro-proliferative, while ERβ is anti-proliferative, it was hypothesised that ERα, but not ERβ, would be required for progestin-induced gene regulation and breast cancer cell proliferation via PR-B.

The aims of this study were as follows:

To compare PR-B mediated activation and repression of transcription by Prog, bProg and selected progestins from different generations on (i) synthetic PRE- and NFκB-containing promoters in the COS-1 cell line and (ii) endogenous PRE- and NFκB-containing genes in the T47D breast cancer cell line. Furthermore, the relative agonist efficacies and potencies of the progestogens on synthetic PRE- and NFκB-containing promoters via PR-B were also compared with each other.

To determine whether ERα and/or ERβ is required for PR-B mediated activation and repression by Prog, bProg and selected progestins, in the COS-1 cell line and T47D breast cancer cell line, respectively.

To compare the agonist efficacies and potencies of Prog, bProg and selected progestins from different generations for proliferation of the T47D breast cancer cell line.

To determine whether ERα and/or ERβ is required for Prog, bProg and/or progestin-induced proliferation of the T47D breast cancer cell line.

CHAPTER 2

MATERIALS AND METHODS

2.1. Test compounds

The progestogens used in this study are listed in Table 2.1. R5020 was obtained from PerkinElmer Life and Analytical Science, South Africa, while bProg was a kind gift from the Compounding Pharmacy of South Africa, Johannesburg. All other progestogens were purchased from Sigma-Aldrich, South Africa. The ERα selective antagonist methyl-piperidino-pyrazole dihydrochloride hydrate (MMP) and the ERβ selective antagonist 2-phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-alpha]pyrimidine (PHTPP) were purchased from Whitehead Scientific, South Africa and Sigma-Aldrich, South Africa, respectively. The inflammatory cytokine, tumor necrosis factor-alpha (TNFα) was obtained from PeproTech, USA and used at a final concentration of 0.02 µg/ml for realtime quantitative PCR (qPCR) and 0.002 µg/ml for promoter-reporter assays. The lower concentration of TNFα for promoter-reporter assays has previously been optimised in the Africander laboratory (Meghan Perkins, PhD student). All test compound stock solutions were prepared in 100% ethanol (EtOH) and stored at -20°C. These compounds were diluted 1000X in serum-free culturing medium, so that the final concentration of EtOH was 0.1% (v/v). Thus, 0.1% (v/v) EtOH served as a vehicle control in all experiments.

Table 2.1 Test compounds used in this study.

Compound	**Classification**	
Progesterone (Prog)	Natural progestogen	
Bio-identical progesterone (bProg)	Semi-synthetic	
Progestins	***Synthetic***	
Promegestone (R5020)	PR-selective agonist widely used as an experimental tool	
Medroxyprogesterone acetate (MPA)	1st	Generation
Norethisterone acetate (NET-A)		
Levonorgestrel (LNG)	2nd	
Gestodene (GES)	3rd	
Drospirenone (DRSP)	4th	

2.2. Cell culture

The PR- and ER-positive T47D human breast cancer epithelial cell line was a kind gift from Prof. Iqbal Parker (University of Cape Town, South Africa) and was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) fetal calf serum (FCS) (The Scientific Group, South Africa), 10 mM HEPES (Sigma-Aldrich, South Africa), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL Life Technologies, United Kingdom). The COS-1 monkey kidney cell line was obtained from the American Type Culture Collection (ATCC, USA) and maintained in phenol-red Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained in 75 cm^2 culture flasks (Greiner Bio-One International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO_2. Cell lines were regularly tested for mycoplasma infections using Hoechst stain (Freshney 1987), and only mycoplasma negative cells were used in experiments.

2.3. Plasmids

The pMT-hPR-B plasmid expressing human PR-B (previously described by Cairns et al. 1993), was a kind gift from Prof. Sam Okret (Karolinska Institute, Sweden), whereas the plasmids expressing the human ERα (pSG5-ERα) and ERβ (pSG5-ERβ) (Flouriot et al. 2000) were obtained from Prof. Frank Gannon (European Molecular Biology Laboratory, Germany). The pTAT-2xPRE-E1b-luciferase promoter-reporter plasmid, which is driven by the E1b promoter and contains two copies of the rat tyrosine aminotransferase (TAT)-PRE (previously described by Sui et al. 1999), was a kind gift from Prof. G. Jenster (Erasmus University of Rotterdam, Netherlands), while the 5xNFκB-luciferase promoter-reporter plasmid containing five copies of the NFκB binding site upstream of the luciferase reporter gene was purchased from Strategene (Houston, Texas, USA). The pGL2-basic vector, purchased from Promega (Madison, USA), was used as a filler plasmid to ensure that the total amount of transfected DNA remained constant in all experiments.

2.4. Plasmid DNA preparation

Calcium chloride competent DH5α *Escherichia coli* (*E. coli)* cells were transformed with the respective expression vectors mentioned in Section 2.3 using the heat shock method (Cohen *et al.* 1972). The transformed *E. coli* cells were grown overnight on Luria Bertani (LB) agar (Sigma-Aldrich, South Africa) (Addendum A) plates containing ampicillin (Sigma-Aldrich, South Africa) at a final concentration of 50 µg/ml. All the plasmids used in this study contained an ampicillin resistance gene. A single colony was selected, inoculated into 25 ml LB medium (Sigma-Aldrich, South Africa) containing ampicillin (50 µg/ml) and grown at 37°C for 6 hours. Two hundred and fifty microliters of the starter culture was transferred to 250 ml LB medium containing ampicillin (50 µg/ml), and grown for 12-16 hours at 37°C. The cultures were then centrifuged at 6 000 x g for 10 minutes at 4°C in order to collect the bacterial cells. Once the supernatant was discarded, the plasmid DNA was isolated and purified from the *E. coli* cells using the Macherey-Nagel Nucleobond® Xtra Maxi kit according to the manufacturer's instructions. Briefly, the pellets were resuspended in 12 ml resuspension buffer containing RNase A. The cells were then lysed by adding 12 ml lysis buffer, inverting the tube 5 times and incubating the suspension for 5 minutes at room temperature. Following incubation, the lysing process was stopped by adding 12 ml neutralization buffer and inverting the solution 15 times. The plasmid DNA was purified by using the Nucleobond® Xtra Maxi column with an inserted filter. Prior to applying the lysate, the filter as well as the column was first equilibrated by applying 25 ml equilibration buffer. The lysate was applied to the filter in order to remove all cell debris before the eluate was passed through the column. Thereafter, 15 ml equilibration buffer was applied to the filter and the eluate was once again passed through the Nucleobond® column. The filter was discarded and the column washed with 25 ml wash buffer, prior to the addition of 15 ml elution buffer. The eluate containing the plasmid DNA was collected in a 50 ml centrifuge tube and 10.5 ml isopropanol (room temperature) was added. To precipitate the DNA, the solution was vortexed and subsequently centrifuged at 15 000 x g for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet was washed

with 5 ml 70% (v/v) EtOH, followed by centrifugation at 15 000 x g for 5 minutes at room temperature. The DNA pellet was allowed to dry at room temperature before resuspension in 1 ml nuclease-free water. The DNA concentration was determined on a NanoDrop 1000 (Thermo Fisher Scientific, South Africa) using the 260 nm/280 nm ratio, and the size and integrity of the plasmid DNA was analysed by restriction enzyme digests and agarose gel electrophoresis.

2.5. Promoter-reporter assays

For both transactivation and transrepression assays, the COS-1 cell line was maintained as described in Section 2.2 and seeded into 10 cm dishes (Whitehead Scientific, South Africa) at a cell density of 2 x 10^6 cells. Twenty-four hours later the cells were washed with pre-warmed sterile phosphate buffered saline (PBS) (Sigma-Aldrich, South Africa) and 10 ml phenol red-free DMEM supplemented with 10% (v/v) charcoal-stripped FCS (CS-FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin (hereafter referred to as supplemented phenol red-free DMEM) was added.

For transactivation assays: Using the X-tremeGENE HP DNA transfection reagent as per the manufacturer's instructions, the COS-1 cells were transiently transfected with 0.9 µg of the pMT-hPR-B expression vector and 9 µg of the pTAT-2xPRE-E1b-luciferase promoter-reporter construct, in the presence of either 0.9 µg pSG5-hERα, pSG5-hERβ or pGL2-basic (filler plasmid). The latter ensured a constant amount of total DNA (10.8 µg/10 cm dish) in all experimental conditions. Following a 24 hour period, the cells were replated into 96-well cell culture plates (Whitehead Scientific, South Africa) at a cell density of 1 x 10^4 cells per well in supplemented phenol red-free DMEM. The next day, the cells were washed with pre-warmed PBS and treated for 24 hours with either 0.1% (v/v) EtOH (vehicle control) or increasing concentrations of R5020, Prog, bProg, MPA, NET-A, LNG, GES and DRSP (hereafter referred to as the test compounds) in unsupplemented phenol red-free DMEM. The cells were then washed with PBS, lysed by adding 25 µl passive lysis buffer (Addendum A) and stored at -20°C. The cell lysates were thawed and 5 µl lysate was used to determine the luciferase activity using the Promega Luciferase Assay System (Promega, Madison, USA) and a Veritas™ microplate luminometer (Whitehead Scientific, South Africa). To normalise

the luciferase activity, the protein concentration (mg/ml) of each lysate was determined using the Bradford protein determination method (Bradford, 1976). Normalising results in this manner for bulk transfections, followed by replating into a number of wells, is a widely accepted method (Visser et al. 2013). Expression of the steroid receptors were further validated by western blotting. When comparing transactivation activity of the test compounds via PR-B, results are expressed as relative luciferase activity with 1 µM R5020 set as 100%, and all other test compounds set relative to this. Results for PR-B in the absence and presence of ERα or ERβ are expressed as fold induction with 0.1% (v/v) EtOH for PR-B alone set as 1, and all other responses set relative to this.

For transrepression assays: The COS-1 cells were transiently transfected with 1.5 µg of the pMT-hPR-B expression vector and 3 µg of the 5xNFκB-luciferase promoter-reporter construct, in the presence of either 1.5 µg pSG5-hERα, pSG5-hERβ or pGL2-basic, using the X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. The pGL2-basic filler plasmid was transfected to ensure a constant amount of total DNA (6 µg/10 cm dish) in all experimental conditions. The next day, the cells were replated into 96-well cell culture plates at a cell density of 1 x 10^4 cells per well in supplemented phenol red-free DMEM. Following a 24 hour period, the cells were washed with pre-warmed PBS and incubated for 24 hours with unsupplemented phenol red-free DMEM containing (i) 0.1% (v/v) EtOH in the absence or presence of 0.002 µg/ml TNFα or (ii) 0.002 µg/ml TNFα in the presence of increasing concentrations of the test compounds. Cells were washed with PBS, lysed and the cell lysates analysed as described for transactivation. The luciferase values obtained for all samples were normalised to the protein concentration (mg/ml) for each sample. TNFα-induction was plotted relative to 0.1% (v/v) EtOH alone set as 1. TNFα-induction was set as 100%, and the repression by each test compound was set relative to this.

2.6. Western blot analysis

COS-1 cells were maintained and transfected as described in Sections 2.2 and 2.5, respectively, and seeded into 12-well cell culture plates at a cell density of 1 x 10^5 cells per well. Positive controls

were prepared by transiently transfecting cells with 250 ng of the expression vector for the appropriate steroid receptor, while untransfected COS-1 cells were used as a negative control. Cells were grown until confluency was reached, washed with PBS and subsequently lysed in 80 µl 2x Laemmli buffer (Addendum A). In order to denature the proteins, cell lysates were boiled at 97°C for 10 minutes before separation on a 10% SDS-polyacrylamide gel at 200 V for 45 minutes using 1x SDS-PAGE running buffer (Addendum A). The PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, South Africa) was used to determine protein sizes. After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, South Africa) by electroblotting at 180 mA for 90 minutes using ice-cold 1x transfer buffer (Addendum A). Membranes were subsequently blocked for 90 minutes using 10% (w/v) fat-free milk powder prepared in 1x TRIS buffered saline (TBS) containing 0.1% (v/v) Tween 20 (Sigma-Aldrich, South Africa) (TBST) (Addendum A). Following incubation, membranes were rinsed using 1x TBST and subsequently probed with primary antibodies specific to PR-A/B (NCL-L-PGR-312, Leica Biosystems, United Kingdom), ERβ (ab92306, Abcam), ERα (sc-543, Santa Cruz Biotechnology Inc., Europe), GR (sc-8992), MR (sc-11412), AR (sc-7305) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-47724) for 16 hours at 4°C. GAPDH expression was used as a loading control. The antibody dilutions used during this study are indicated in Table 2.2. Following one 15 minute and three 5 minute washes using 1x TBST, membranes were incubated with either the goat anti-rabbit (sc-2030) or goat anti-mouse (sc-2005) horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Europe) (see Table 2.2) for 90 minutes at room temperature. The membranes were washed as above and the proteins visualized using Clarity™ Western ECL substrate (Bio-Rad, South Africa) and the Thermo Fisher Scientific MyECL™ Imager (Separations, South Africa).

Table 2.2 Dilutions of primary and secondary antibodies used for western blotting.

Primary Antibody	Dilution	Secondary Antibody	Dilution
PR-A/B	1:1 000	Goat-anti-mouse	1:2 000
ERα	1:1 000	Goat-anti-rabbit	1:1 000
ERβ	1:500	Goat-anti-rabbit	1:1 000
AR	1:1 000	Goat-anti-mouse	1:5 000
MR	1:1 000	Goat-anti-rabbit	1:4 000
GR	1:6 000	Goat-anti-rabbit	1:4 000
GAPDH	1:4 000	Goat-anti-mouse	1:6 000

2.7. RNA isolation

The human T47D breast cancer cell line was maintained as described in Section 2.2 and seeded into 12-well cell culture plates (Whitehead Scientific, South Africa) at a cell density of 1 x 10^5 cells per well. After the cells had settled, cells were washed with pre-warmed sterile PBS before the addition of supplemented phenol red-free DMEM and incubation for 24 hours. Cells were treated with unsupplemented phenol red-free DMEM containing either (i) 0.1% (v/v) EtOH or 1 nM of the test compounds (transactivation) or (ii) 0.1% (v/v) EtOH alone in the absence or presence of 0.02 µg/ml TNFα or (iii) 0.02 µg/ml TNFα in the presence of 1 nM of the test compounds (transrepression), in the absence and presence of 10 µM receptor-selective antagonists. Following incubation for 24 hours (transactivation) or 6 hours (transrepression), the cells were lysed by adding 400 µl TriReagent® (Sigma-Aldrich, South Africa) to each well. The lysates were transferred to 1.5 ml microcentrifuge tubes and 80 µl chloroform was added. The samples were vortexed for 15 seconds,

incubated for 2-3 minutes at room temperature, and then centrifuged at 12 000 x g for 15 minutes at 4°C. The centrifugation step separated the samples into three distinct phases namely, a clear aqueous phase at the top containing RNA, a middle phase containing DNA and a bottom pink phase containing protein. The top phase containing the RNA was transferred to clean 1.5 ml microcentrifuge tubes and an equal volume of ice-cold isopropanol was added. Samples were subsequently vortexed, incubated for 15 minutes at room temperature and centrifuged at 12 000 x g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellets were washed with 500 µl 75% (v/v) EtOH in diethyl pyrocarbonate (DEPC) treated water. The samples were vortexed for 1 minute and subsequently centrifuged at 7 500 x g for 15 minutes at 4°C. The supernatant was discarded and the RNA pellets were allowed to air dry on ice for 5-10 minutes. Each RNA pellet was subsequently dissolved in 15 µl DEPC treated water. The purity of the RNA was determined by calculating the concentrations from the absorbances at 260 and 280 nm using a NanoDrop1000 (Thermo Scientific, Wilmington, USA) and the RNA concentrations were subsequently calculated, while the integrity of the RNA was assessed by the presence of intact 28S and 18S ribosomal RNA bands on a 1% (w/v) denaturing formaldehyde agarose gel. A representative gel is shown in Addendum B (Figure B1). All RNA samples were subsequently stored at -80°C.

2.8. cDNA synthesis

cDNA was synthesised by reverse transcribing the total RNA extracted from the T47D breast cancer cells using the Promega ImProm-II reverse transcription system as per the manufacturer's instructions (Anatech, South Africa). Briefly, 0.5 µg total RNA and 0.25 µl oligo$(dT)_{15}$ primer (at a final concentration of 25 ng/µl) were added into a thin-walled PCR tube. This was followed by the addition of nuclease-free water to a final volume of 2.5 µl. The mixture was incubated at 70°C for 5 minutes to denature the RNA and was subsequently placed on ice. Following incubation on ice for 5 minutes, the rest of the components needed for the reverse transcription reaction were added in the following order: nuclease-free water to a final volume of 7.5 µl per sample, 2 µl Im-Prom-II 5 x reaction buffer, 0.6 µl $MgCl_2$ (at a final concentration of 1.5 mM), 0.5 µl deoxynucleotide (dNTP)

mix with a final concentration of 0.5 nM for each nucleotide, 0.25 µl recombinant RNasin ribonuclease inhibitor (10 U per reaction), and 0.5 µl ImProm-II reverse transcriptase (80 U per reaction). For the reverse transcription reaction to take place the samples were incubated for 5 minutes at 25°C to allow annealing of the primers, followed by incubation for 1 hour at 42°C for extension. Finally, samples were incubated for a further 15 minutes at 70°C in order to inactivate the Im-Prom-II reverse transcriptase enzyme. All cDNA samples were stored at -20°C.

2.9. Realtime quantitative PCR (qPCR)

Realtime qPCR was performed using the Roche LightCycler® 96 in order to determine the relative mRNA expression levels of c-myc, interleukin (IL)-8 and GAPDH. The latter gene was used as an internal control (reference gene) since the Africander laboratory has previously shown that its expression is not regulated by the test compounds (personal communication). The details of the primers used for amplification are summarized in Table 2.3. Reagents from the LightCycler® 480 SYBR Green I Master kit (Roche Applied Science, South Africa) were used, and the reaction mixture in each well of the PCR plate was as follows: forward and reverse primers (final concentration of 0.5 µM each), 5 µl SYBR Green I, PCR-grade water to a final volume of 9 µl, and 1 µl of either cDNA or PCR-grade water (negative control). Once all the samples were prepared, the Roche FastStart Taq polymerase was activated by incubating the samples at 95°C for 10 minutes. The subsequent PCR amplification consisted of three steps: a denaturation step at 95°C for 10 seconds, a 10 second annealing step at the appropriate temperature indicated in Table 2.3, and an elongation step of 10 seconds at 72°C. The PCR cycle was repeated 45 times. Melting curve analysis (Addendum B: Figure B3) and agarose gel electrophoresis (Addendum B: Figure B4) were performed to confirm the presence of a single amplicon and the amplicon size, respectively. The primer efficiencies (Table 2.3) and relative transcript levels were determined as previously described by Pfaffl *et al.* (2001). Further details on the qPCR method can be found in Addendum B.

Table 2.3 Details of primer sets used in this study.

Gene	Primers (5' – 3')	Strand	Length of amplicon (bp)	Annealing temperature (°C)	Primer Efficiency	Reference
c-myc	GACGCGGGGAGGCTATTCTG GACTCGTAGAAATACGGCTGCACCGAGTC	Forward Reverse	236	62	1.91	Dieudonne *et al.* (2006)
IL-8	TGCCAAGGAGTGCTAAAG CTCCACAACCCTCTGCAC	Forward Reverse	197	56	2.02	Wolf *et al.* (2002)
GAPDH	TGAACGGGAAGCTCACTGG TCCACCACCCTGTTGCTGTA	Forward Reverse	307	59	1.89	Ishibashi *et al.* (2003)

2.10. Cell proliferation assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay based on the principle that mitochondrial succinate dehydrogenase reduces soluble yellow MTT to form insoluble purple formazan crystals. These crystals are then dissolved, followed by spectrophotometric analysis. The absorbance values are a measure of the number of viable cells present since the soluble MTT can only be reduced to an insoluble formazan precipitate by metabolically active cells (Mosmann 1983). The human T47D breast cancer cell line was maintained as described in Section 2.2 and seeded into 96-well cell culture plates at a cell density of 1 x 10^4 cells per well. On day two, the cells were washed with sterile pre-warmed PBS before the addition of supplemented phenol red-free DMEM. The following day the cells were treated with either 0.1% (v/v) EtOH, or increasing concentrations of the test compounds. Alternatively, cells were treated with 1 nM test compound in the absence and presence of 10 µM receptor-selective antagonists and incubated for 72 hours. Four hours before the incubation period lapsed, the assay medium was replaced with 150 µl unsupplemented phenol red-free DMEM and 50 µl 5 mg/ml MTT solution (Addendum A) (Sigma-Aldrich, South Africa). Plates were returned to the incubator for the remaining 4 hours, followed by the aspiration of the medium and the addition of 200 µl dimethyl sulfoxide (DMSO) (Merck, South Africa) (solubilising agent) to each well. The formazan crystals were dissolved by pipetting up and down and the plates were subsequently covered in foil and placed on an orbital shaker at room temperature for 5 minutes. The absorbances were subsequently measured at 550 nm using a BioTek® Power Wave 340 microplate spectrophotometer (Analytical and Diagnostic Products, South Africa). Proliferation is shown as fold induction relative to 0.1% (v/v) EtOH (vehicle control) set as 1.

2.11. Data manipulation

Data manipulations, graphical presentations and statistical analysis were performed using GraphPad Prism® version 5 (GraphPad Software, Inc., San Diego, CA). Non-linear regression and the

unpaired t-test (two-tailed) were used for statistical analysis of all data with the exception of MTT assays. For MTT assays, either non-linear regression one-way ANOVA column analysis with the Newman-Keuls (compares all pairs of columns) post-test, or two-way ANOVA analysis of variance with Bonferroni (compares all pairs of columns) post-test, was used. Statistical significance are indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, while non-statistically significant results ($p>0.05$) are indicated by ns. Furthermore, the letters a, b, c, d are also used to indicate statistically significant differences, with values that differ significantly from each other being assigned different letters. The error bars represent the standard error of the mean (SEM) of two to three independent experiments, except for realtime qPCR for which only a single experiment was performed in duplicate.

CHAPTER 3

RESULTS

3.1. Most progestins display similar relative agonist efficacies and potencies to each other for transactivation via PR-B on a synthetic PRE-containing promoter

The relative agonist efficacies (maximal response a ligand can elicit) and potencies ($EC_{50;}$ concentration of ligand that induces half the maximal response) of all the progestogens for transactivation via PR-B on a synthetic PRE were compared to each other. The COS-1 monkey kidney cell line was transiently transfected with the pMT-hPR-B expression vector, a PRE driven promoter-reporter construct containing two copies of a PRE site linked to the luciferase reporter gene, and the pGL2-basic empty vector to a total amount of 10.8 µg DNA (previously optimised in the Africander laboratory). The cells were subsequently treated with either 0.1% (v/v) EtOH (vehicle control) or increasing concentrations of R5020, Prog, bProg, MPA, NET-A LNG, GES or DRSP (hereafter referred to as either progestogens or test compounds) for 24 hours. R5020 is a well-known PR-selective agonist often used as a positive control when investigating effects via the PR (Giannoukos et al., 2001; Shatnawi et al., 2007). The results are graphically presented in figure 3.1A and 3.1B, while the values for the relative agonist efficacies and potencies of the test compounds for transactivation via PR-B are summarised in Table 3.1. The natural progestogen Prog as well as the semi-synthetic, bio-identical progestogen bProg, exhibited similar efficacies to each other, but significantly lower efficacies than R5020 (figure 3.1A; Table 3.1). Interestingly, Prog displayed a similar agonist potency to that of R5020, while bProg was significantly more potent than both R5020 ($p<0.01$) and Prog ($p<0.001$). NET-A, LNG, GES and DRSP all displayed similar efficacies to each other and R5020 (figure 3.1B; Table 3.1). Surprisingly, however, MPA displayed a much lower maximal response than R5020. In terms of potency, GES displayed a similar potency to R5020, and although MPA, NET-A, LNG and DRSP appeared to be less potent than R5020, these differences were not statistically significant. When comparing the progestins to natural Prog, the results in Table 3.1 show that MPA displayed a similar efficacy to Prog, while NET-A was more efficacious than Prog. Although not statistically significant, LNG, GES and DRSP appeared to display greater maximal responses than Prog. Interestingly, MPA, NET-A and LNG displayed

similar potencies to that of Prog, while DRSP was less potent and GES was more potent than Prog. The efficacies and potencies of the progestins were also compared to that of bProg, and results show that MPA displayed a similar efficacy to that of bProg, while NET-A displayed a greater maximal response than bProg (figure 3.1A and 3.1B; Table 3.1). Although not statistically significant, LNG, GES and DRSP appeared to be more efficacious than bProg. Surprisingly, all the progestins investigated in this study were less potent than bProg.

3.2. Neither ERα nor ERβ is required for progestogen-induced transactivation via PR-B on a synthetic PRE-containing promoter

In a recent study, Giulianelli et al. (2012) reported that ERα is required for the PR-B mediated effects of MPA. This raised the question of whether ERα is also needed for the transcriptional activation of PR-B by other progestogens. We thus transiently transfected COS-1 cells with the pMT-hPR-B expression vector and the pTAT-2xPRE-E1b-luciferase promoter-reporter construct, in the absence (pGL2-basic empty vector) and presence of the pSG5-hERα expression vector, followed by the treatment of the cells with 0.1% (v/v) EtOH or increasing concentrations of MPA for 24 hours. The results in figure 3.2 show that ERα had no effect on the relative maximal response or agonist potency of MPA via PR-B, suggesting that ERα is not required for MPA-induced PR-B-mediated gene expression in our system. Considering that two distinct ER subtypes with vastly different functions exist (Deroo & Korach 2006; Hartman et al. 2009), we also determined the effects of ERβ on the PR-B-mediated relative agonist efficacies and potencies of MPA for transactivation. COS-1 cells were thus transfected as before, except that the pSG5-hERβ rather than the pSG5-hERα expression vector was transfected. Following treatment with 0.1% (v/v) EtOH or increasing concentrations of MPA for 24 hours, the results showed that the presence of ERβ significantly decreased the maximal response of MPA via PR-B ($p<0.05$), however, statistical significance could not be obtained when comparing the maximal responses for MPA via PR-B in

the presence of ERα versus ERβ. Although it appears that the relative agonist potency of MPA decrease in the presence of ERβ, this difference was not statistically significant (figure 3.2).

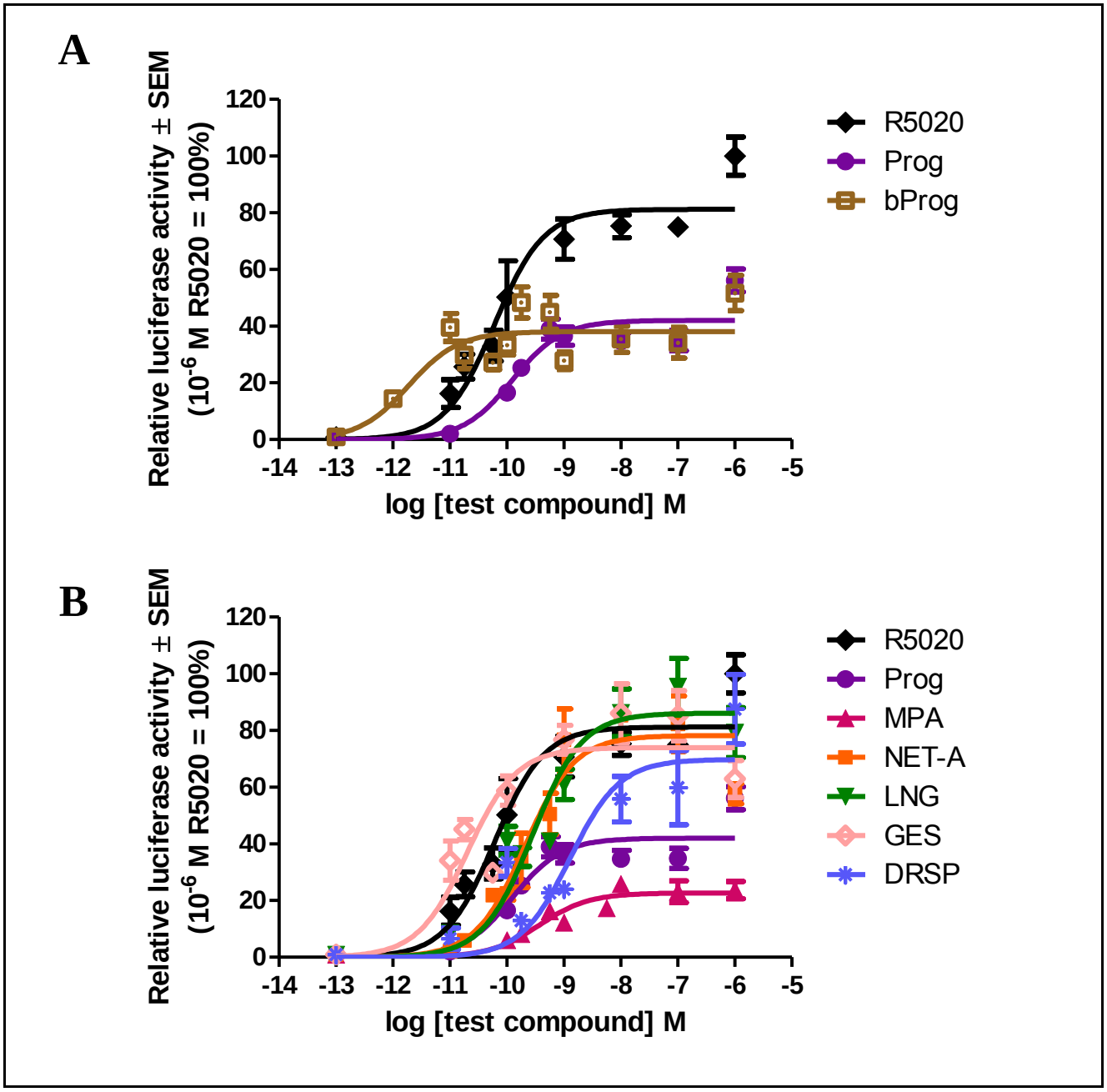


Figure 3.1. (A) Prog and bProg display similar agonist efficacies, but not potencies for transactivation via PR-B. The COS-1 cell line was transiently transfected with 900 ng of the pMT-hPR-B expression vector, 9 000 ng of the pTAT-PRE-E1b-luciferase promoter-reporter construct and 900 ng of the pGL2-basic empty vector. Cells were incubated with 0.1% EtOH or increasing concentrations of R5020 (♦), Prog (●) or bProg (□) for 24 hours. **(B) LNG, GES and DRSP appear to display greater maximal responses than Prog via PR-B, while MPA appears to be less efficacious than Prog.** COS-1 cells were transiently transfected as above and incubated with 0.1% EtOH or increasing concentrations of R5020 (♦), Prog (●), MPA (▲), NET-A (■), LNG (▼), GES (◊) or DRSP (*) for 24 hours. For both (A) and (B) induction is shown as relative luciferase activity expressed as percentage, with 10^{-6} M R5020 set as 100% and all other test compounds set relative to this. Result shown is the average of at least two independent experiments, with each condition performed in triplicate (± SEM).

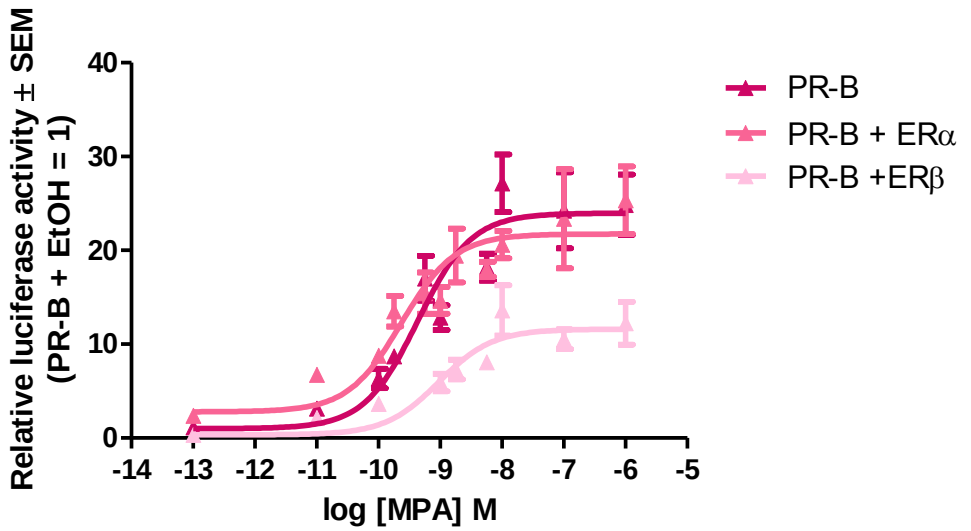
Table 3.1. Relative agonist efficacies (maximal response (MAX) in %) and potencies (expressed as EC_{50} in M) of the test compounds for transactivation via PR-B.[a]

Test compounds	Classification	MAX (%) ± SEM	EC_{50} (M) ± SEM
R5020	PR-selective agonist	100.0 ± 0.0	6.97×10^{-11} ± 2.90
Prog	Natural progestogen	43.80 ± 10.13	1.36×10^{-10} ± 0.28
bProg	Semi-synthetic (bio-identical)	37.09 ± 8.44	2.65×10^{-12} ± 1.17
MPA	1st Generation progestin	25.37 ± 9.86	4.51×10^{-10} ± 2.18
NET-A		93.57 ± 3.92	2.96×10^{-10} ± 1.27
LNG	2nd Generation progestin	86.29 ± 27.94	2.45×10^{-10} ± 1.15
GES	3rd Generation progestin	98.07 ± 28.76	3.61×10^{-11} ± 1.55
DRSP	4th Generation progestin	68.61 ± 26.83	1.36×10^{-9} ± 0.77

[a]Data shown in figure 3.1A and 3.1B were analysed to obtain the relative maximal response (MAX) ± SEM and EC_{50} ± SEM values for each test compound. MAX values are expressed as a percentage relative to 10^{-6} M R5020 = 100% and statistical analysis of these values indicated that R5020 vs. Prog and bProg ($p<0.05$); Prog vs. bProg ($p>0.05$); R5020 vs. MPA ($p<0.01$); R5020 vs. NET-A, LNG, GES and DRSP ($p>0.05$); Prog vs. NET-A ($p<0.05$); Prog vs. MPA, LNG, GES and DRSP ($p>0.05$); MPA vs. NET-A ($p<0.05$); NET-A vs. LNG, GES and DRSP ($p>0.05$); MPA vs. LNG vs. GES vs. DRSP ($p>0.05$); bProg vs. NET-A ($p<0.05$); bProg vs. MPA, LNG, GES and DRSP ($p>0.05$). Statistical analysis of EC_{50} values indicated that R5020 vs. Prog ($p>0.05$); R5020 vs. bProg ($p<0.01$); Prog vs. bProg ($P<0.001$); R5020 vs. MPA, NET-A, LNG, GES and DRSP ($p>0.05$); Prog vs. GES and DRSP ($p<0.05$); Prog vs. MPA, NET-A and LNG ($p>0.05$); GES vs. MPA, NET-A and DRSP ($p<0.05$); MPA vs. NET-A vs. LNG vs. DRSP ($p>0.05$); LNG vs. GES ($p>0.05$); bProg vs. MPA, NET-A and DRSP ($p<0.001$); bProg vs. LNG ($p<0.01$); bProg vs. GES ($p<0.05$).

Having shown that ERβ, but not ERα, can modulate the PR-B-mediated effects of MPA for transactivation, and considering that it is known that MPA does not to bind to the ER (Teulings et al. 1980), we postulated that the decrease in the PR-B-mediated effects of MPA in the presence of ERβ, and not in the presence of ERα, may be due to ERβ, but not ERα, downregulating PR-B

protein expression levels. Using western blot analysis we thus determined the effects of the ER subtypes on the protein levels of PR-B overexpressed in COS-1. Surprisingly, results in figure 3.3A and 3.3B showed that, in the absence of ligand, both ERα and ERβ significantly downregulated PR-B protein expression levels (~65.8% ($p<0.001$) and ~58.5% ($p<0.01$), respectively). Notably, co-expression of PR-B and either ERα or ERβ did not affect the protein expression of either ERα (figure 3.3C) or ERβ (figure 3.3D). Next, we determined whether the decrease in PR-B protein expression levels observed in the absence of the progestogens and presence of the ER subtypes correlated with the effects of the ER subtypes on basal PR-B-mediated transcriptional activity. Interestingly, results in figure 3.3F showed that in the absence of ligand, ERβ decreased ($p<0.001$) the basal level of transcription of PR-B on a simple synthetic PRE-containing promoter (figure 3.3E) which may be explained by the observed decrease in PR-B protein levels (figure 3.3B). Surprisingly, however, despite the decrease in PR-B protein levels observed in the presence of ERα (figure 3.3B), ERα increased ($p<0.001$) the basal level of transcription of PR-B (figure 3.3F).



	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	89.84 ± 35.03	53.10 ± 16.60
EC_{50} (M) ± SEM	4.51×10^{-10} ± 2.18	2.97×10^{-10} ± 0.81	1.04×10^{-9} ± 0.44

Figure 3.2 legend on next page.

Figure 3.2. ERα has no effect on the PR-B-mediated agonist maximal response or potency of MPA, while ERβ significantly decreases the maximal response of MPA and appears to decrease the potency. COS-1 cells were transiently transfected with 900 ng of the pMT-hPR-B expression vector and 9 000 ng of the pTAT-PRE-E1b-luciferase promoter-reporter construct, in the absence (900 ng pGL2-basic) and presence of 900 ng of either the pSG5-hERα or pSG5-hERβ expression vector. The cells were subsequently incubated with increasing concentrations of MPA for 24 hours. Induction is shown as relative luciferase activity with PR-B in the presence of 0.1% EtOH set as 1, and all other responses set relative to this. Result shown is the average of at least two independent experiments, with each condition performed in triplicate (± SEM). Data were analysed to obtain the relative maximal response (MAX) ± SEM and EC_{50} ± SEM values for MPA via PR-B or PR-B in the presence of ERα (PR-B + ERα) or ERβ (PR-B + ERβ), respectively. MAX of MPA via PR-B alone was set as 100%, and the MAX of MPA via PR-B in the presence of ERα or ERβ calculated relative to this. Statistical analysis of the MAX values indicated the following: PR-B vs. PR-B + ERα ($p>0.05$); and PR-B vs. PR-B + ERβ ($p<0.05$). Statistical analysis of the EC_{50} values indicated that PR-B vs. PR-B + ERα and PR-B vs. PR-B + ERβ ($p>0.05$).

Knowing that the ER and PR are co-expressed in ~70% of all breast cancers (Lange 2008), and that women not only use MPA, but also other progestins for HRT and contraception, we next determined whether ERα and/or ERβ could modulate the relative agonist efficacies and potencies of the selected progestins investigated in this study. COS-1 cells transfected with PR-B in the absence and presence of ERα or ERβ, were treated with increasing concentrations of R5020, Prog, bProg, NET-A, LNG, GES and DRSP for 24 hours (figure 3.4A-G). In contrast to the effects observed for MPA, the presence of ERα significantly decreased the efficacies of all the other progestogens tested. Interestingly, the presence of ERα caused a decrease in the relative agonist potency of Prog, whilst having no statistically significant effects on the potencies of any of the other progestogens. The results in figure 3.4A-G showed that ERβ, like ERα, decreased the maximal responses of R5020, Prog, bProg, NET-A, LNG, GES and DRSP. However, ERβ had no significant effect on the relative agonist potencies of any of the test compounds. Taken together, the results show that neither ERα, nor ERβ, is required for progestogen-induced transactivation via PR-B, but that ERβ can in fact decrease the maximal responses of all the progestogens, while ERα decreases the maximal response of all progestogens except MPA.

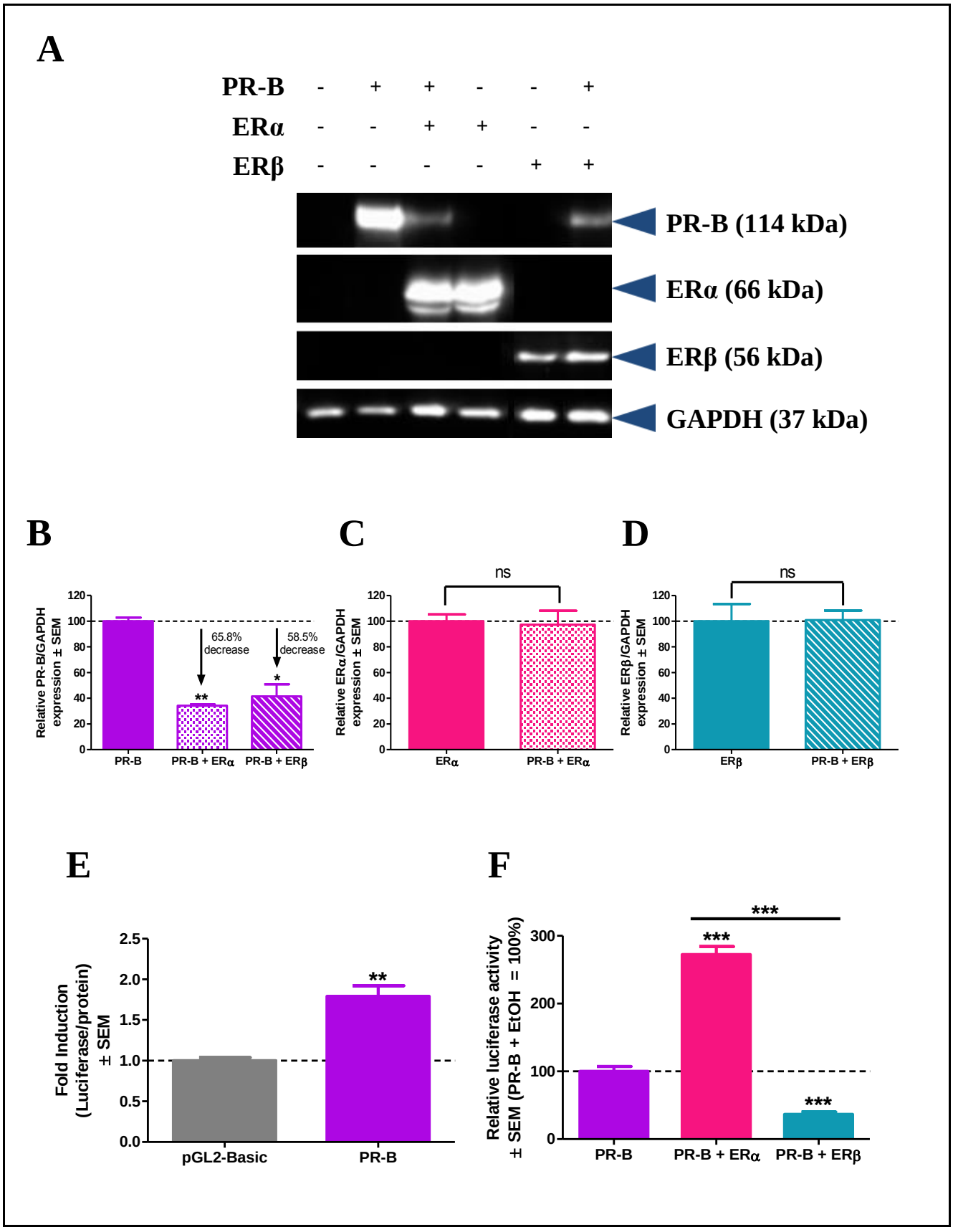


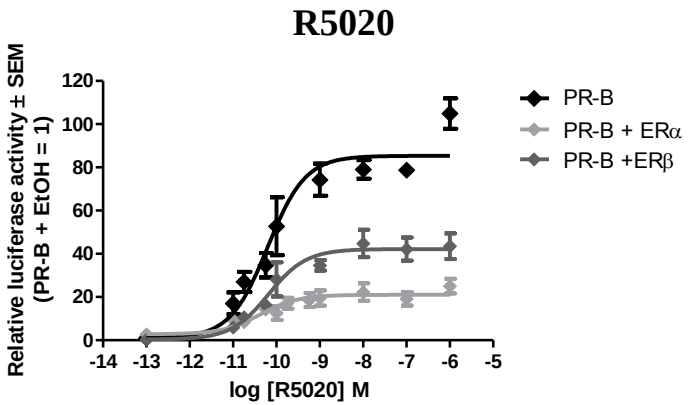
Figure 3.3 legend on next page.

Figure 3.3. ERβ decreases the basal transactivational activity of PR-B possibly by reducing the expression levels of PR-B, while ERα increases PR-B-mediated basal transactivational activity but decreases PR-B expression levels. COS-1 cells were transiently transfected with either 900 ng of the pGL2-basic empty vector (only A and E) or 900 ng pMT-hPR-B and 9 000 ng of the pTAT-PRE-E1b-luciferase promoter-reporter construct, in the absence (900 ng pGL2-basic) or presence of 900 ng of either the pSG5-hERα or pSG5-hERβ expression vector. The cells were incubated with 0.1% EtOH for 24 hours. (**A**) Whole cell extracts were prepared and protein lysates were subsequently analysed by western blotting with primary antibodies specific to PR-A/B, ERα, ERβ and GAPDH. A representative blot is shown. (**B**-**D**). Quantification of western blots from two independent experiments using the Thermo Scientific My Image Analysis software is shown. (**E**) The basal transcriptional activity of PR-B is expressed relative to the response with the empty vector (pGL2-basic) set as 1. (**F**) The effects of ERα and ERβ on the basal transcriptional activity of PR-B are expressed as percentage relative luciferase activity, with the response of PR-B alone (from E) set as 100%, and all other conditions set relative to this. The result shown is the average of at least two independent experiments with each condition performed in triplicate (± SEM).

3.3. Progestogen-induced upregulation of the c-myc gene in the human T47D breast cancer cell line is not modulated in the presence of ERα or ERβ antagonists

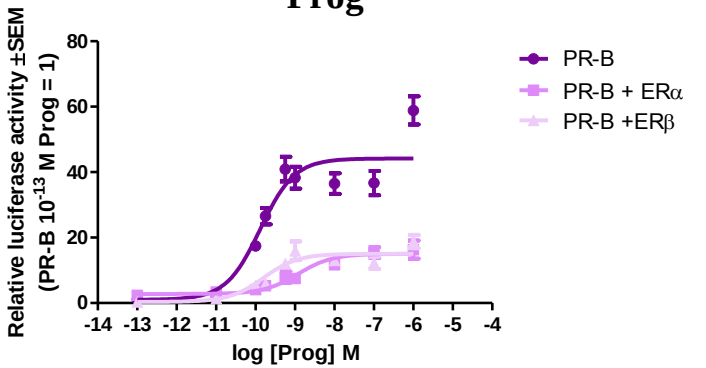
Next, we investigated whether the ER subtypes could modulate the progestogen-induced regulation of an endogenous PRE-containing gene, c-myc. The human T47D breast cancer cell line was used as model system and the western blot in figure 3.5A confirms previous reports that T47D cells endogenously express both PR isoforms (Horwitz et al., 1978; Poutanen et al., 1992; Vienonen et al., 2001) and both ER subtypes (Horwitz et al. 1978; Keydar et al. 1979). A time-course study was performed to evaluate optimal c-myc mRNA expression in the presence of MPA. Considering that Giulianelli et al. (2012) measured c-myc mRNA expression in the presence of 10 nM MPA, we incubated T47D cells with either 0.1% (v/v) EtOH or 10 nM MPA for 15 min, 30 min, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours. RNA isolation, cDNA synthesis and qPCR were subsequently performed and results show maximal MPA-induced mRNA expression at 24 hours (figure 3.5B).

A



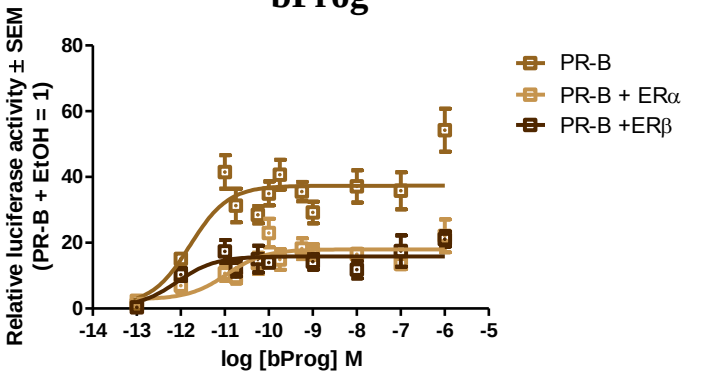
	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	17.18 ± 3.96	36.37 ± 8.30
EC_{50} (M) ± SEM	$6.97 \times 10^{-11} \pm 2.90$	$4.41 \times 10^{-11} \pm 2.70$	$7.78 \times 10^{-11} \pm 3.48$

B



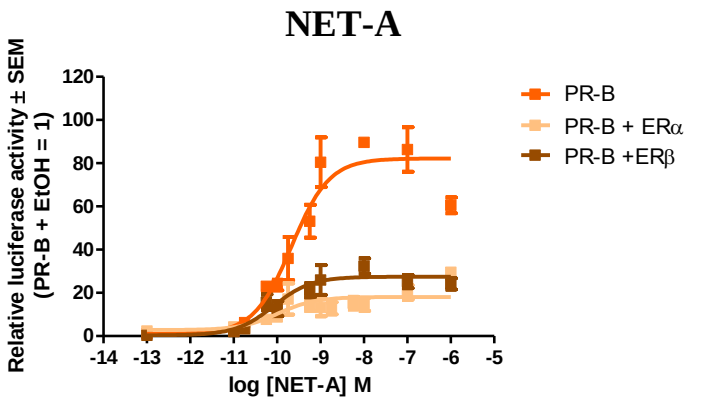
	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	33.62 ± 5.01	34.72 ± 3.26
EC_{50} (M) ± SEM	$1.36 \times 10^{-10} \pm 0.28$	$1.78 \times 10^{-9} \pm 0.99$	$1.44 \times 10^{-10} \pm 0.17$

C



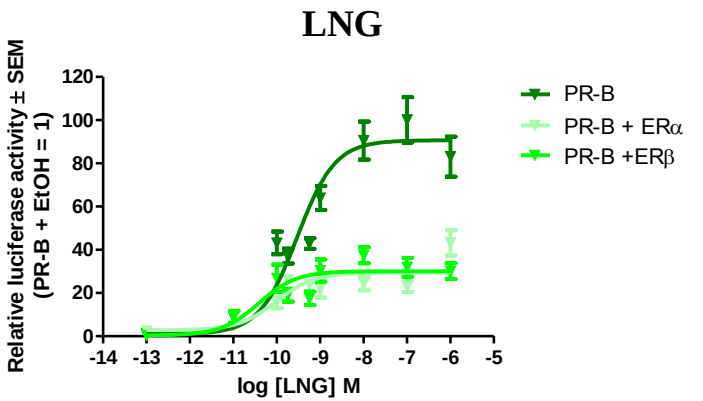
	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	31.37 ± 7.50	40.12 ± 8.49
EC_{50} (M) ± SEM	$2.65 \times 10^{-12} \pm 1.17$	$1.57 \times 10^{-11} \pm 0.72$	$3.22 \times 10^{-12} \pm 2.76$

D



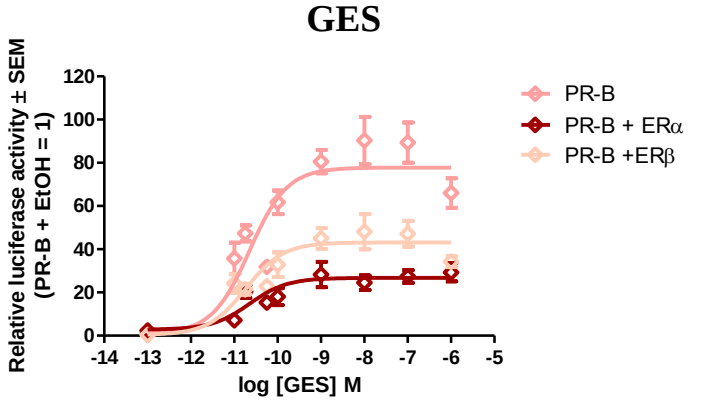
	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	18.89 ± 7.20	33.17 ± 6.63
EC_{50} (M) ± SEM	$2.96 \times 10^{-10} \pm 1.27$	$1.63 \times 10^{-10} \pm 0.97$	$1.38 \times 10^{-10} \pm 0.84$

E



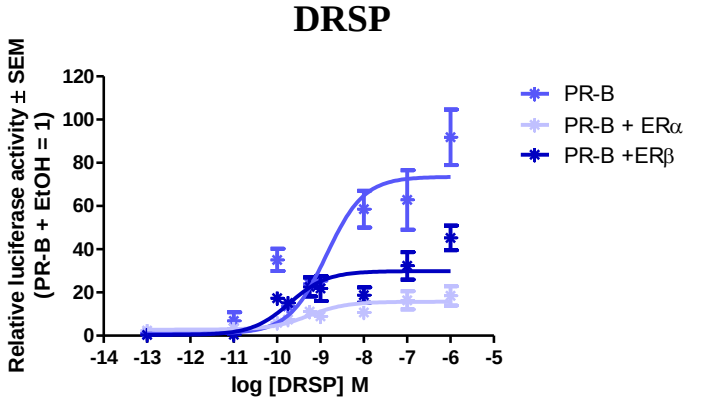
	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	29.83 ± 10.23	35.25 ± 2.04
EC_{50} (M) ± SEM	$2.45 \times 10^{-10} \pm 1.15$	$1.00 \times 10^{-10} \pm 0.09$	$1.04 \times 10^{-10} \pm 0.96$

F



	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	26.27 ± 9.04	45.50 ± 3.52
EC_{50} (M) ± SEM	$3.61 \times 10^{-11} \pm 1.55$	$2.52 \times 10^{-11} \pm 1.10$	$1.93 \times 10^{-11} \pm 0.37$

G



	PR-B	PR + ERα	PR + ERβ
MAX (%) ± SEM	100 ± 0.0	27.25 ± 12.79	43.19 ± 18.34
EC_{50} (M) ± SEM	$1.36 \times 10^{-9} \pm 0.77$	$4.68 \times 10^{-9} \pm 4.57$	$3.84 \times 10^{-10} \pm 1.46$

Figure 3.4 legend on next page.

Figure 3.4. ERα and ERβ decrease the PR-B-mediated efficacies of all progestogens tested, while the relative agonist potency of only Prog is decreased by ERα. COS-1 cells were transiently transfected with 900 ng of the pMT-hPR-B expression vector and 9 000 ng of the pTAT-PRE-E1b-luciferase promoter-reporter construct, in the absence (900 ng pGL2-basic) and presence of 900 ng of either the pSG5-hERα or pSG5-hERβ expression vector. The cells were subsequently incubated with increasing concentrations of (**A**) R5020 (♦), (**B**) Prog (●), (**C**) bProg (□), (**D**) NET-A (■), (**E**) LNG (▼), (**F**) GES (◊) and (**G**) DRSP (*) for 24 hours. Induction is shown as relative luciferase activity with PR-B in the presence of 0.1% EtOH set as 1, and all other responses set relative to this. Result shown is the average of at least two independent experiments, with each condition performed in triplicate (± SEM). Data were analysed to obtain the relative maximal response (MAX) ± SEM and EC_{50} ± SEM values for PR-B or PR-B in the presence of ERα (PR-B + ERα) or ERβ (PR-B + ERβ), respectively. MAX of each test compound via PR-B alone was set as 100%, and the MAX of each test compound via PR-B in the presence of ERα or ERβ calculated relative to this. Statistical analysis of the MAX values indicated the following: PR-B vs. PR-B + ERα: R5020, Prog and NET-A ($p<0.001$); bProg, LNG and DRSP ($p<0.01$); GES ($p<0.05$); and PR-B vs. PR-B + ERβ: Prog, NET-A and LNG ($p<0.001$); R5020, bProg and GES ($p<0.01$); DRSP ($p<0.05$). Statistical analysis of the EC_{50} values indicated the following: PR-B vs. PR-B + ERα: Prog ($p<0.05$); R5020, bProg, NET-A, LNG, GES and DRSP ($p>0.05$); and PR-B vs. PR-B + ERβ: R5020, Prog, bProg, NET-A, LNG, GES, DRSP ($p>0.05$).

T47D cells were next treated for 24 hours with either 0.1% (v/v) EtOH or 1 nM of the progestogens, in the absence and presence of 10 µM of the ERα- or ERβ-specific antagonists, MPP (Sun et al. 2002) and PHTPP (Aguirre et al. 2010), respectively. The concentration of 1 nM progestogen was chosen as the serum concentrations of at least three progestins, MPA, NET and DRSP, in contraceptive users have previously been reported to be in the nanomolar range (reviewed in Africander, et al. 2011a; Blode et al. 2012; Ito et al. 2016). We did not include bProg in these experiments as we showed that Prog and bProg displayed similar PR-B-mediated effects at 1 nM on a simple synthetic PRE-containing promoter (figure 3.1A). Figure 3.6A shows that all the progestogens, except R5020, upregulated the mRNA expression of the c-myc gene. Although inductions of more than 100-fold were observed, statistical analysis indicated that c-myc gene expression was not significantly upregulated by any of the progestogens.

Next we set out to determine whether the ERα- or ERβ-specific antagonists MPP and PHTPP, respectively, could modulate progestogen-induced c-myc mRNA expression. Notably, R5020 was excluded from any further analysis as no induction was obtained with this ligand. In addition, results for LNG in the presence of the ER antagonists are not shown as these samples could not be detected. Results in figure 3.6B and 3.6C show that both MPP and PHTPP appeared to increase c-

myc mRNA expression. Furthermore, neither MPP nor PHTPP had a significant effect on Prog-, MPA-, NET-A-, GES- and DRSP-induced c-myc mRNA expression (figure 3.6B and 3.6C).

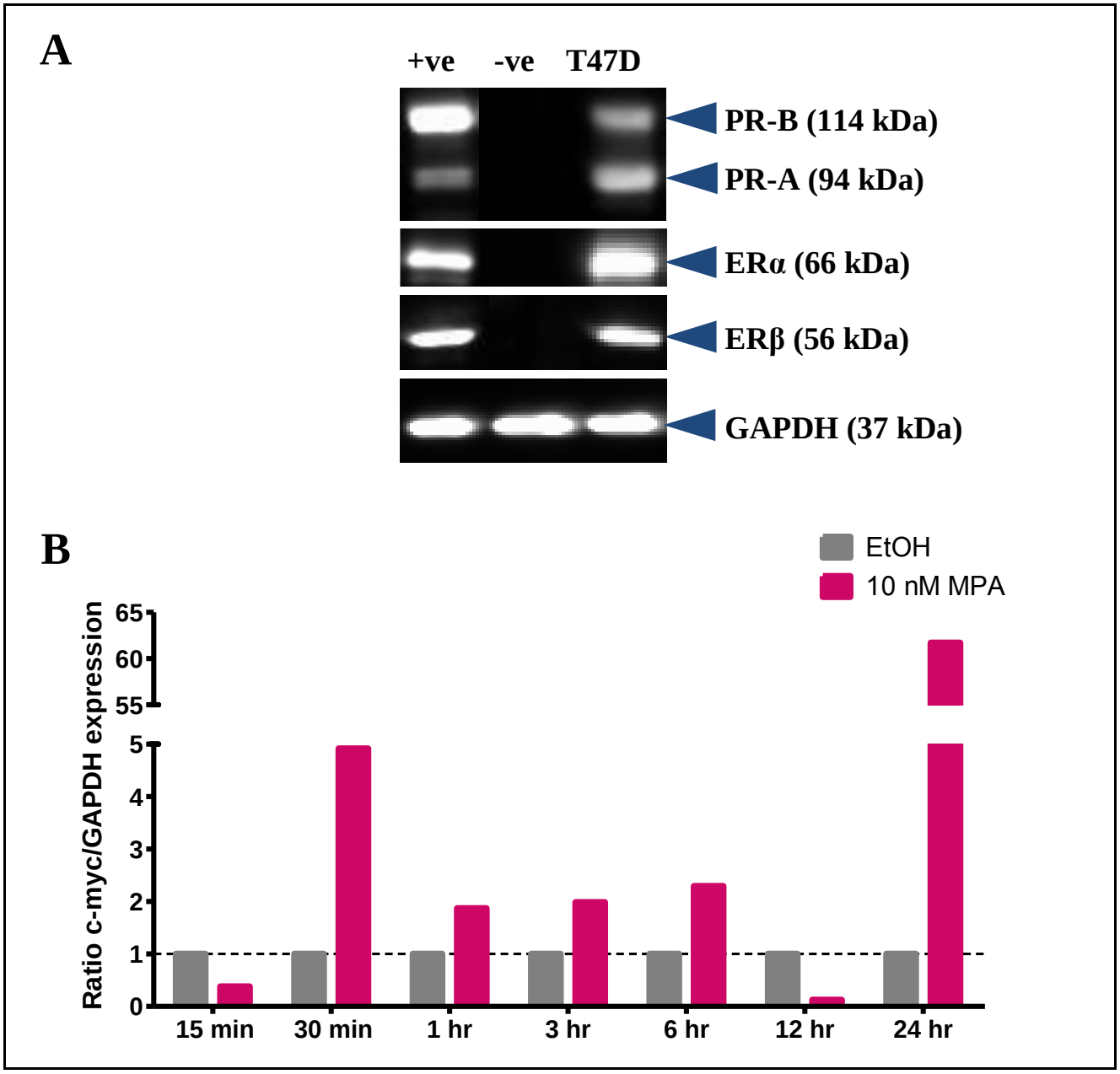


Figure 3.5. (A) Both PR isoforms and ER subtypes are expressed in the T47D breast cancer cell line. Whole cell extracts were prepared from the T47D cell line. Untransfected COS-1 cells were used as the negative control (-ve), while COS-1 cells transfected with expression vectors for PR-A, PR-B, ERα or ERβ respectively, were used as positive controls (+ve). Protein lysates were analysed by western blotting with primary antibodies specific to PR-A/B, ERα, ERβ and GAPDH. A representative blot is shown. **(B) Time-course analysis showing optimal MPA-induced c-myc mRNA expression at 24 hours.** T47D cells were treated with either 0.1% EtOH (vehicle control) or 10 nM MPA for 15 min, 30 min, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours. Total RNA was isolated and reverse transcribed. Relative c-myc mRNA expression was measured by realtime qPCR analysis and normalised to GAPDH mRNA expression. Results are expressed as a ratio of c-myc/GAPDH expression with 0.1% EtOH set as 1. The result shown is from a single experiment.

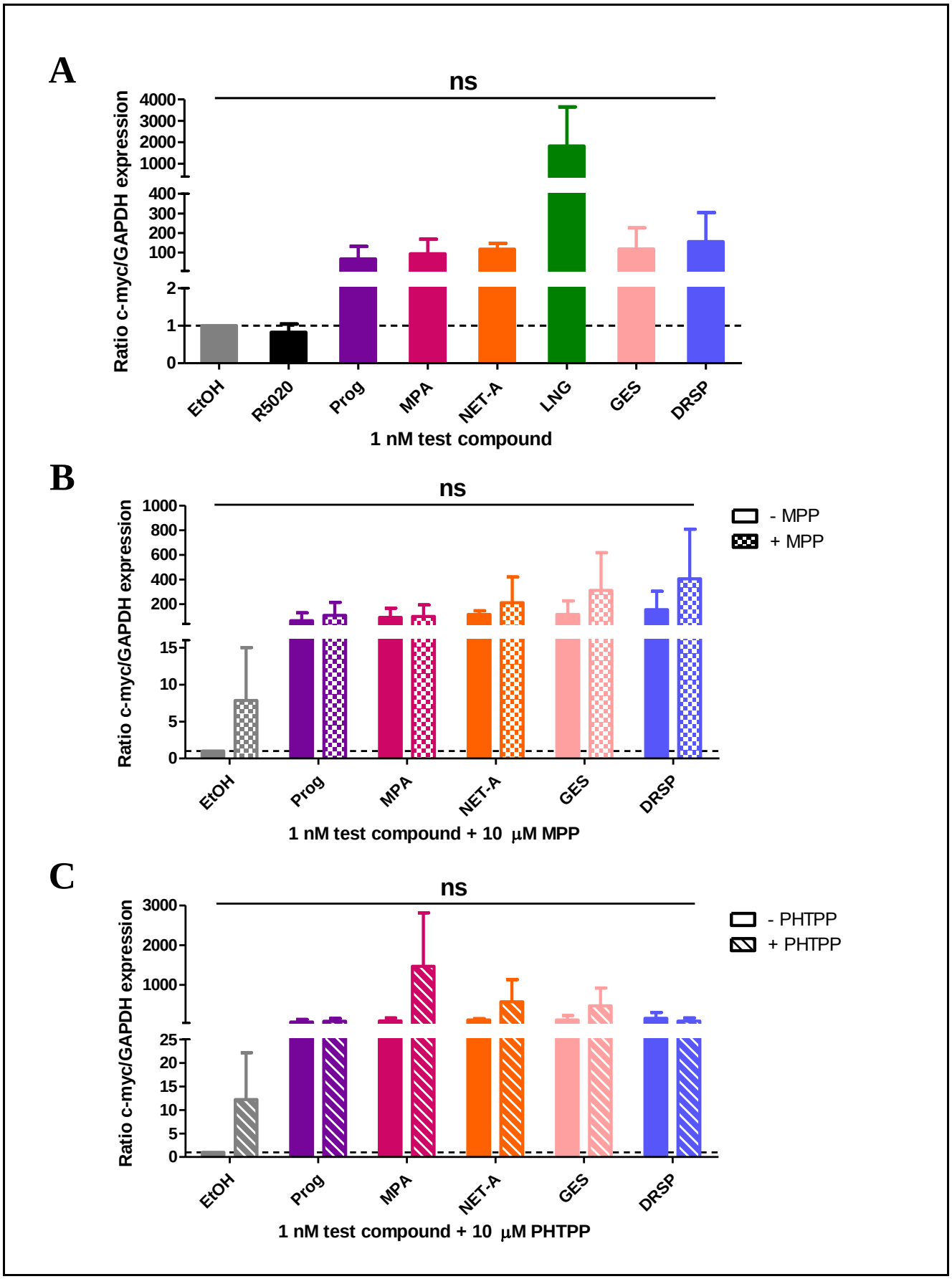


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Figure 3.6. MPP and PHTPP do not influence the upregulation of c-myc mRNA expression in response to the progestogens. T47D breast cancer cells were incubated for 24 hours with either 0.1% EtOH (vehicle control) or 1 nM of each of the test compounds in the (**A**) absence and (**B** and **C**) presence of the receptor specific antagonists for (**B**) ERα (MPP) or (**C**) ERβ (PHTPP). Total RNA was isolated and reverse transcribed. Relative c-myc mRNA expression was measured by realtime qPCR and normalised to GAPDH mRNA expression. Results are expressed as a ratio of c-myc/GAPDH expression with 0.1% EtOH set as 1. The result shown is from a single experiment performed in duplicate.

3.4. Progestogens display similar relative agonist efficacies for transrepression via PR-B, while appearing to display differential agonist potencies

The PR regulates gene expression by either increasing (transactivation) or decreasing (transrepression) transcription of target genes (Griekspoor 2007; Kalkhoven et al. 1996; Kobayashi et al. 2010). Having shown that progestins mostly display similar progestogenic activity for transactivation via PR-B, we next evaluated the progestogenic properties for PR-B-mediated transrepression. The COS-1 cell line was transiently transfected with the pMT-hPR-B expression vector, a luciferase reporter plasmid linked downstream to five copies of a NFκB site (5xNFκB-luciferase) and the pGL2-basic empty vector to a total amount of 6 µg DNA (previously optimised in the Africander laboratory). The cells were subsequently incubated with 0.1% (v/v) EtOH in the absence and presence of 2.0 ng/ml TNFα, or 2.0 ng/ml TNFα in the presence of increasing concentrations of the test compounds for 24 hours. As shown in figure 3.7A, treatment with TNFα resulted in a 12-fold induction on the synthetic 5xNFκB promoter-reporter construct. The results in figure 3.7B and Table 3.2 clearly show that Prog and bProg display similar agonist efficacies to each other and the PR-selective agonist R5020 for transrepression via PR-B. Furthermore, bProg was shown to be equally potent to R5020 at repressing the TNFα-induced response. Although it appeared that Prog was less potent than both R5020 and bProg, this difference was surprisingly not statistically significant. Results in 3.7C showed similar maximal repression of the TNFα-induced response by MPA, NET-A, LNG GES, and DRSP and that the percentage repression observed by the progestins was not significantly different from R5020, Prog and bProg (figure 3.7B; Table 3.2). In terms of relative agonist potencies, R5020, bProg, NET-A, LNG, GES and DRSP were equally

potent to each other and Prog, while MPA was significantly less potent than all these progestogens with the exception of Prog.

3.5. Both ER subtypes can modulate the PR-B-mediated transrepressive activities of some progestogens

Having shown that all the progestogens displayed similar maximal repression on the NFκB promoter-reporter construct via PR-B, whilst appearing to display differential agonist potencies, and that the ER subtypes significantly decreased the PR-B-mediated maximal responses of most progestogens for transactivation, we next evaluated whether ERα and/or ERβ could modulate the PR-B-mediated relative agonist efficacies and potencies of the test compounds for transrepression. The COS-1 cell line was transiently transfected with the pMT-hPR-B cDNA expression vector and the 5xNFκB-luciferase promoter-reporter construct, in the absence (pGL2-basic empty vector) and presence of an expression vector for either pSG5-hERα or pSG5-hERβ. The cells were subsequently treated as in Section 3.4.

Results showed that the presence of ERα did not significantly affect the relative agonist efficacies of Prog (figure 3.8C), bProg (figure 3.8D), MPA (figure 3.8B) and NET-A (figure 3.8E) for transrepression via PR-B, while completely abolishing the PR-B-mediated transrepressive activities of R5020 (figure 3.8A) and the newer generation progestins, LNG (figure 3.8F), GES (figure 3.8G) and DRSP (figure 3.8H). Furthermore, although it appeared as though the PR-B-mediated relative agonist potencies of bProg (figure 3.8D) and NET-A (figure 3.8E) on the NFκB-containing promoter-reporter construct increased in the presence of ERα, only the increased potencies of Prog (figure 3.8C) and MPA (figure 3.8B) were significantly different ($p<0.05$).

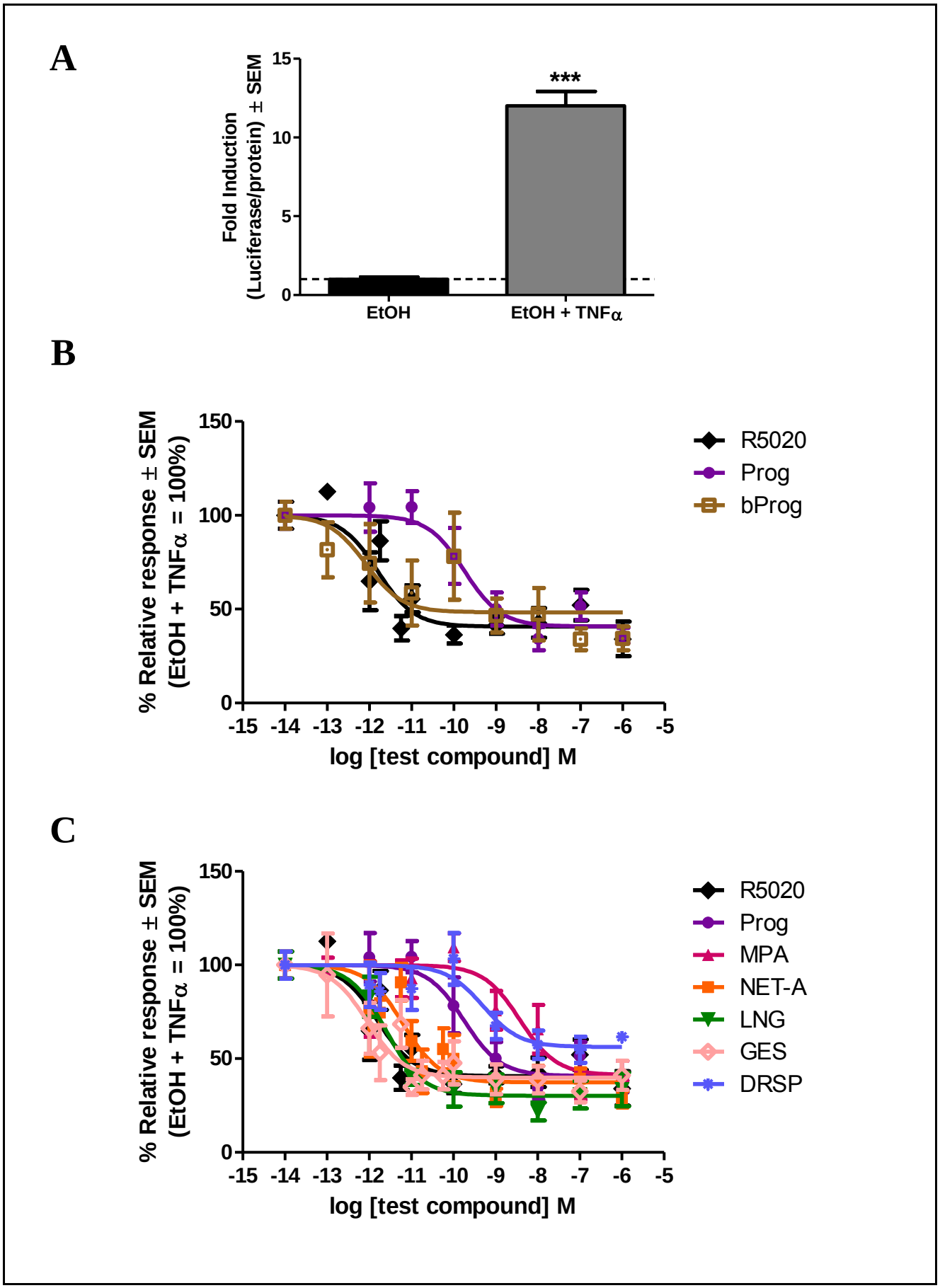

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Figure 3.7. Transrepression activity of Prog, bProg and select progestins from different generations via PR-B. COS-1 cells were transiently transfected with 1 500 ng of the pMT-hPR-B expression vector, 3 000 ng of the 5xNFκB-luciferase promoter-reporter construct and 1 500 ng of the pGL2-basic empty vector. Cells were treated with (**A**) 0.1% EtOH in the absence and presence of 2.0 ng/ml TNFα, or (**B**) 2.0 ng/ml TNFα in the presence of increasing concentrations of R5020 (♦), Prog (●) and bProg (□), or (**C**) 2.0 ng/ml TNFα in the presence of increasing concentrations of R5020 (♦), Prog (●), MPA (▲), NET-A (■), LNG (▼), GES (◊) and DRSP (*) for 24 hours. (**A**) TNFα induction was plotted relative to 0.1% EtOH alone set as 1. (**B** and **C**) TNFα-induction in (**A**) was set as 100%, and the repression by each test compound was set relative to this. The result shown is the average of at least two independent experiments with each condition performed in triplicate (± SEM).

Table 3.2. Relative agonist efficacies (maximal repression (MAX) in %) and potencies (expressed as EC_{50} in M) of the test compounds for transrepression via PR-B.[b]

Test compounds	Classification	MAX (%) ± SEM	EC_{50} (M) ± SEM
R5020	PR-selective agonist	100.00 ± 0.0	1.64×10^{-12} ± 0.81
Prog	Natural progestogen	101.60 ± 2.10	5.39×10^{-10} ± 4.69
bProg	Semi-synthetic (bio-identical)	84.81 ± 16.43	2.65×10^{-12} ± 2.62
MPA	1st Generation progestin	99.85 ± 15.45	3.63×10^{-9} ± 3.20
NET-A		103.70 ± 11.39	1.33×10^{-11} ± 1.28
LNG	2nd Generation progestin	115.70 ± 7.73	2.18×10^{-12} ± 0.72
GES	3rd Generation progestin	105.80 ± 17.15	2.62×10^{-12} ± 1.29
DRSP	4th Generation progestin	75.67 ± 12.52	5.11×10^{-10} ± 5.08

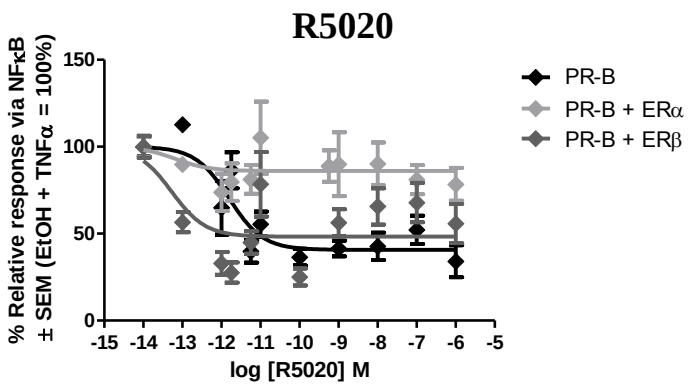
[b]Data depicted in figure 3.7B and 3.7C were analysed and the relative maximal repression (MAX) ± SEM and EC_{50} ± SEM values were obtained. MAX values for all test compounds are expressed as a percentage of the R5020 response (set as 100% repression). Statistical analysis of the MAX values indicated R5020 vs. Prog vs. bProg vs. MPA vs. NET-A vs. LNG vs. GES vs. DRSP ($p>0.05$). Statistical analysis of the EC_{50} values indicated that R5020 vs. Prog vs. bProg vs. NET-A vs. LNG vs. GES vs. DRSP ($p>0.05$); R5020 vs. MPA ($p<0.05$); Prog vs. bProg, MPA, NET-A, LNG, GES, DRSP ($p>0.05$); MPA vs. LNG and GES ($p<0.05$); MPA vs. NET-A vs. DRSP ($p>0.05$); NET-A vs. LNG vs. GES vs. DRSP ($p>0.05$); bProg vs. MPA ($p<0.05$); bProg vs. NET-A, LNG, GES and DRSP ($p>0.05$).

Considering that R5020, LNG, GES and DRSP were unable to repress the TNFα-induced response on the synthetic NFκB-containing promoter in the presence of ERα, relative agonist efficacies and potencies could not be determined for these compounds. In contrast to ERα, ERβ had no significant effect on the relative efficacies of any of the progestogens investigated in this study, while significantly increasing the potencies of Prog and MPA. Although ERβ also appeared to increase the potencies of R5020, NET-A and DRSP, it was surprisingly not statistically significant.

3.6. ERβ, but not ERα, appears to modulate Prog-, MPA-, NET-A-, LNG-, GES- and DRSP-induced repression of IL-8 gene expression

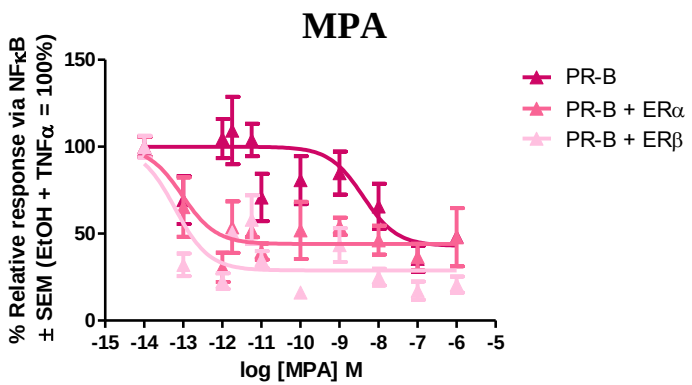
Having shown that ERα, but not ERβ, modulates the PR-B-mediated transrepressive efficacies of R5020, LNG, GES and DRSP on a simple synthetic NFκB-containing promoter, whilst the potencies of Prog and MPA were shown to be significantly increased in the presence of both ER subtypes, we were interested in investigating whether the ER subtypes could also modulate the progestogenic properties for transrepression on an endogenous NFκB-containing promoter. First, we determined the optimal time for TNFα-induced expression of the endogenous IL-8 gene in the human T47D breast cancer cell line. Cells were incubated with 0.1% (v/v) EtOH in the absence and presence of 20.0 ng/ml TNFα for 30 min, 2 hours, 6 hours and 24 hours. It should be noted that the concentration of TNFα used for realtime qPCR is higher than that used in the promoter-reporter assays as this concentration has previously been reported to significantly induce the expression of endogenous cytokine genes (Africander, et al. 2011b; Verhoog et al. 2011; Louw-du Toit et al. 2014). Results in figure 3.9 showed that TNFα upregulated IL-8 mRNA expression at 30 min, 6 hours and 24 hours, with optimal induction observed at 6 hours (23.3-fold).

A



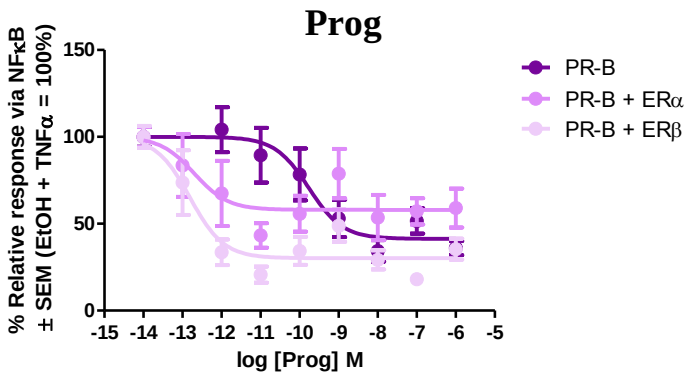
	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	ND	91.43 ± 6.92
EC_{50} (M) ± SEM	$1.64 \times 10^{-12} \pm 0.81$	ND	$1.20 \times 10^{-13} \pm 0.70$

B



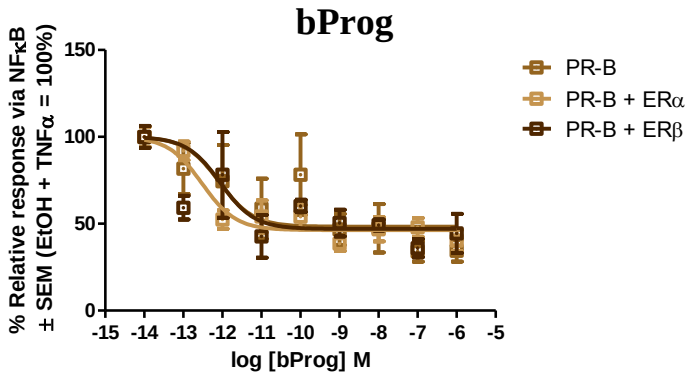
	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	113.60 ± 8.06	111.00 ± 32.95
EC_{50} (M) ± SEM	$3.63 \times 10^{-9} \pm 3.20$	$1.25 \times 10^{-13} \pm 0.88$	$1.53\times 10^{-13} \pm 1.06$

C



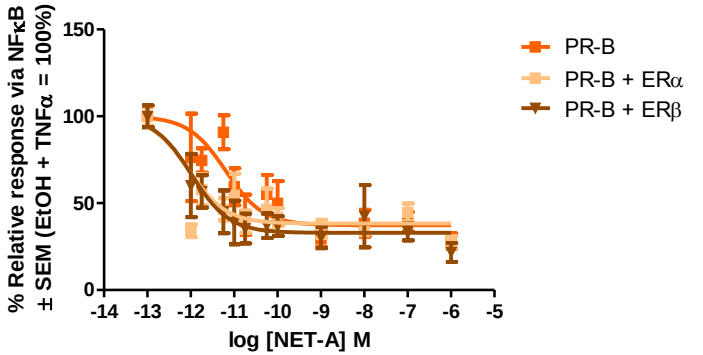
	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	92.46 ± 7.96	102.7 ± 14.74
EC_{50} (M) ± SEM	$5.39 \times 10^{-10} \pm 4.69$	$1.91 \times 10^{-13} \pm 0.75$	$3.66 \times 10^{-13} \pm 0.22$

D



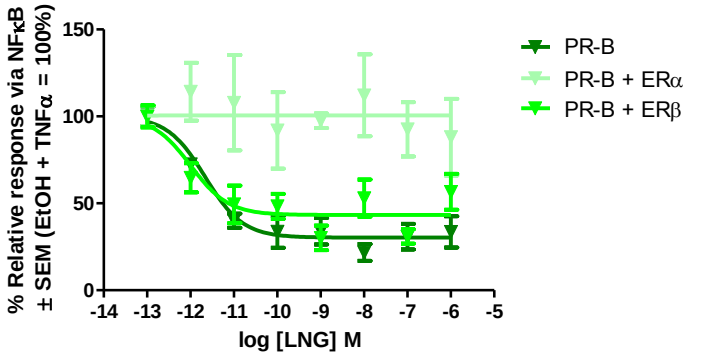
	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	107.6 ± 37.10	108.3 ± 22.36
EC_{50} (M) ± SEM	$2.65 \times 10^{-12} \pm 2.62$	$3.03 \times 10^{-13} \pm 2.79$	$1.58 \times 10^{-12} \pm 1.54$

E



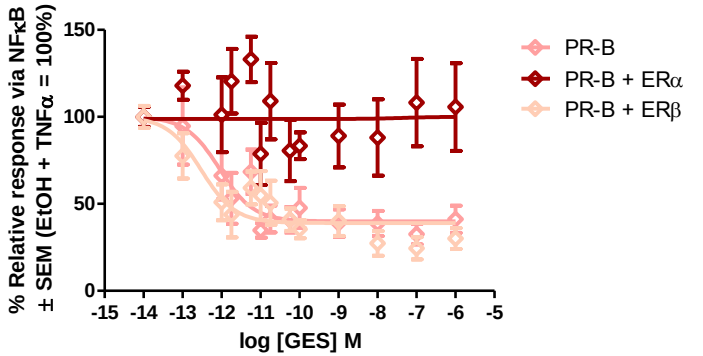
	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	108.90 ± 25.59	120.00 ± 30.43
EC_{50} (M) ± SEM	$1.33 \times 10^{-11} \pm 1.28$	$1.90 \times 10^{-12} \pm 1.34$	$1.91 \times 10^{-12} \pm 0.72$

F



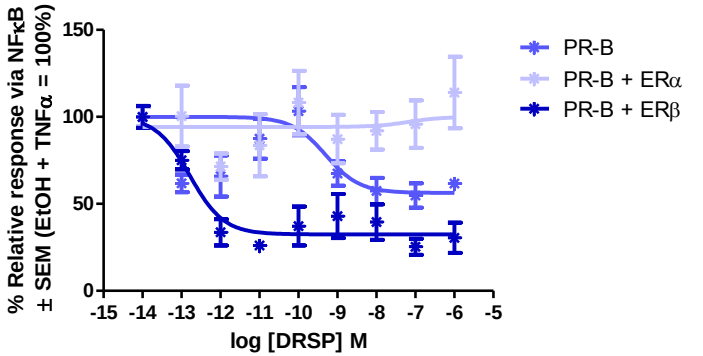
	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	ND	97.03 ± 3.23
EC_{50} (M) ± SEM	$2.18 \times 10^{-12} \pm 0.72$	ND	$1.51 \times 10^{-12} \pm 0.80$

G



	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	ND	102.3 ± 12.80
EC_{50} (M) ± SEM	$2.62 \times 10^{-12} \pm 1.29$	ND	$2.83 \times 10^{-13} \pm 0.77$

H



	PR-B	PR+ ERα	PR+ ERβ
MAX (%) ± SEM	100.00 ± 0.0	ND	88.74 ± 9.22
EC_{50} (M) ± SEM	$5.11 \times 10^{-10} \pm 5.08$	ND	$2.49 \times 10^{-13} \pm 1.06$

Figure 3.9 legend on next page.

Figure 3.8. ERα, but not ERβ, abolishes the agonist efficacies and potencies of R5020, LNG, GES and DRSP for transrepression via PR-B. COS-1 cells were transiently transfected with 1 500 ng of the pMT-hPR-B expression vector and 3 000 ng of the 5xNFκB-luciferase promoter-reporter construct, in the absence (1 500 ng pGL2-basic) and presence of 1 500 ng of either the pSG5-hERα or pSG5-hERβ expression vector. Cells were treated with 0.1% EtOH in the absence and presence of 2.0 ng/ml TNFα, or (**B**) 2.0 ng/ml TNFα in the presence of increasing concentrations of (**A**) R5020 (♦), (**B**) MPA (▲), (**C**) Prog (●), (**D**) bProg (□), (**E**) NET-A (■), (**F**) LNG (▼), (**G**) GES (◊) and (**H**) DRSP (*) for 24 hours. TNFα-induction (from figure 3.7A) was set as 100%, and the repression by each test compound was set relative to this. The result shown is the average of at least two independent experiments with each condition performed in triplicate (± SEM). Relative maximal repression (MAX) ± SEM and EC_{50} ± SEM values were obtained. MAX values indicated in the tables for all test compounds are expressed as a percentage, with the maximal repression obtained for each test compound via PR-B set as 100%, and maximal repression for PR-B in the presence of either ERα or ERβ set relative to this. ND denotes that the value could not be determined using non-linear regression and sigmoidal dose response analysis. Statistical analysis of the MAX values indicated the following: PR-B vs. PR-B + ERα: Prog, bProg, MPA and NET-A ($p>0.05$), and PR-B vs. PR-B + ERβ: R5020, Prog, bProg, MPA, NET-A, LNG, GES and DRSP ($p>0.05$). Statistical analysis of the EC_{50} values indicated the following: PR-B vs. PR-B + ERα: Prog and MPA ($p<0.05$); bProg and NET-A ($p>0.05$), and PR-B vs. PR-B + ERβ: Prog ($p<0.05$); MPA ($p<0.01$); R5020, bProg, NET-A, LNG, GES and DRSP ($p>0.05$).

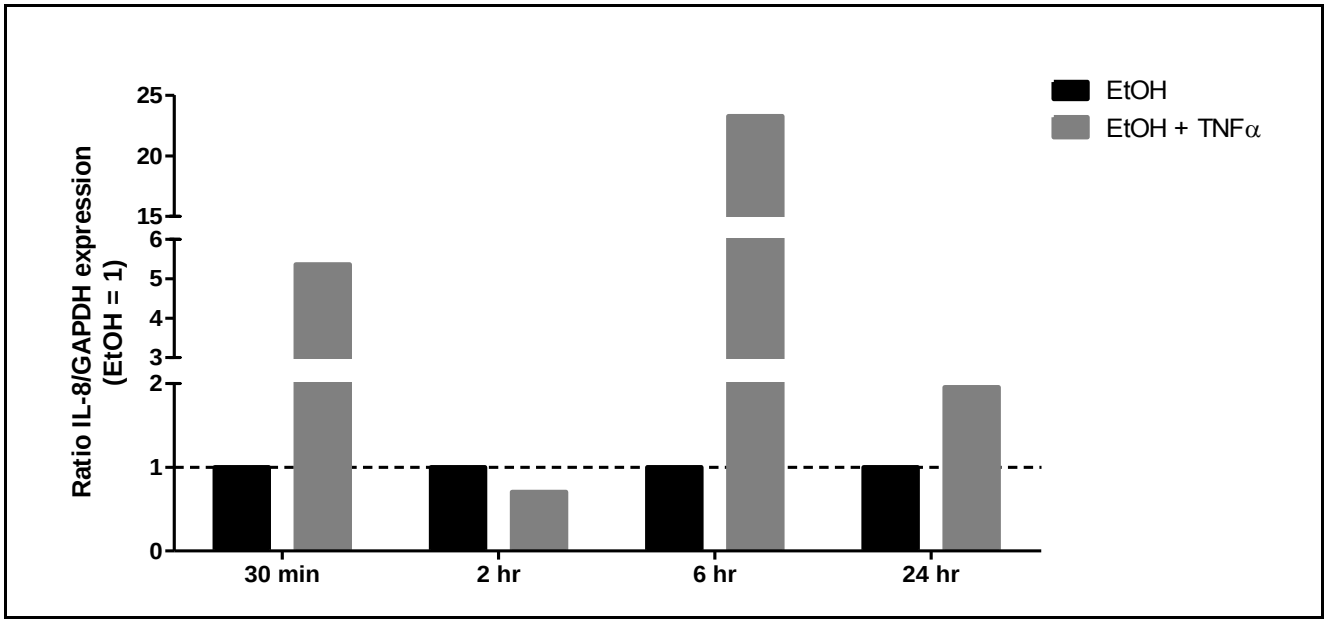


Figure 3.9. Optimal TNFα-induced expression of the endogenous IL-8 gene in the T47D cell line occurs at 6 hours. T47D cells were incubated with either 0.1% EtOH (vehicle control) or stimulated with 20.0 ng/ml TNFα for 30 min, 2 hours, 6 hours and 24 hours. Total RNA was isolated and reverse transcribed. Relative IL-8 mRNA expression was measured by realtime qPCR and normalised to GAPDH mRNA expression. Results are expressed as a ratio of IL-8/GAPDH expression with 0.1% EtOH set as 1. The result shown is from a single experiment.

To compare the effects of the progestogens on IL-8 mRNA expression in the human T47D cell line, and to determine how these effects may be modulated by either ERα or ERβ, cells were subsequently treated for 6 hours with 0.1% (v/v) EtOH in the absence and presence of 20.0 ng/ml TNFα, or 20.0 ng/ml TNFα and 1 nM of the progestogens, in the absence and presence of 10 µM of the ERα- and ERβ-selective antagonists, MPP and PHTPP, respectively. Results in figure 3.10A showed a ~26-fold increase in IL-8 mRNA expression in the presence of TNFα. As shown in figure 3.10B, Prog, NET-A, LNG, GES and DRSP significantly repressed TNFα-induced IL-8 gene expression. Even though MPA appeared to repress TNFα-induced IL-8 gene expression to a lesser extent than the other progestogens, this difference was not statistically significant and probably due to large experimental error.

We next determined whether the ERα- and/or ERβ-specific antagonists could modulate the progestogen-induced repression of IL-8 gene expression. Results in figure 3.10C show that the ERα-specific antagonist (MPP) had no significant effect on the progestogen-induced repression of IL-8 mRNA expression. Although we could not obtain statistical significance in this single experiment, the ERβ-specific antagonist (PHTPP) appeared to completely abolish repression of IL-8 gene expression by all progestogens, except R5020 (figure 3.10D). Surprisingly, MPP significantly repressed TNFα-induced IL-8 gene expression (figure 3.10C), while PHTPP did not affect the expression (figure 3.10D).

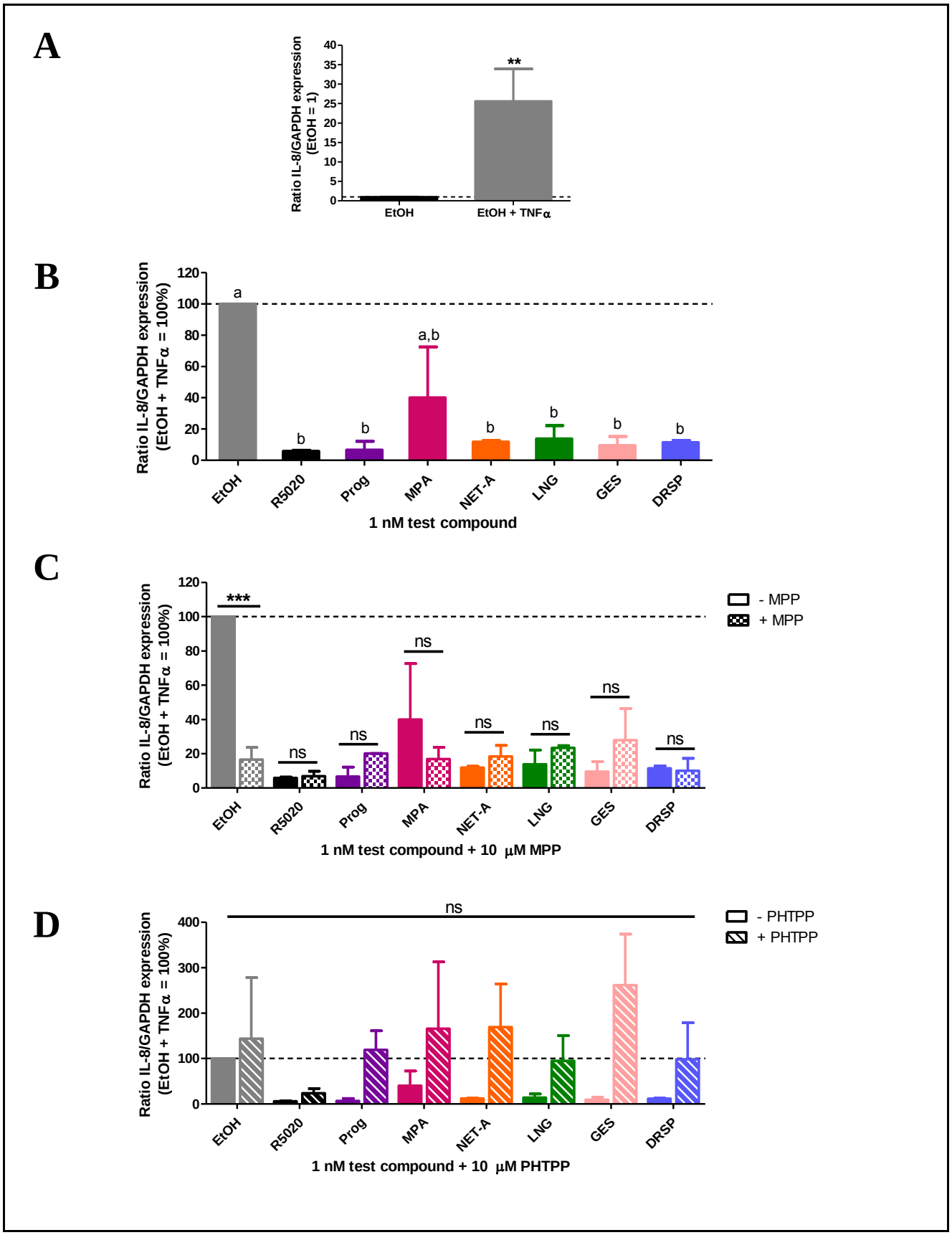


Figure 3.10 legend on next page.

Figure 3.10. The ERα-specific antagonist, MPP, has no effect on progestogen-induced repression of IL-8 gene expression, while it appears to abolished by the ERβ-specific antagonist, PHTPP. T47D breast cancer cells were incubated with (**A**) 0.1% EtOH (vehicle control) in the absence and presence of 20.0 ng/ml TNFα, or (**B**) 20.0 ng/ml TNFα in the absence and presence of 1 nM of each test compound or (B) in the presence of the **(C)** ERα-specific antagonist MPP, and **(D)** ERβ-specific antagonist PHTPP for 6 hours. Total RNA was isolated and reverse transcribed. Relative IL-8 mRNA expression was measured by realtime qPCR analysis and normalised to GAPDH mRNA expression. Results are expressed as a ratio of IL-8/GAPDH expression with (A) 0.1% EtOH set as 1 or (B, C and D) TNFα induction in (A) set as 100% and the repression by each test compound in the absence and presence of MPP or PHTPP set relative to this.

3.7. R5020, MPA, LNG and GES are the most potent agonists for proliferation of theT47D breast cancer cell line

Having shown that all progestogens can activate and repress gene expression in the presence of overexpressed PR-B, and knowing that both transactivation and transrepression of genes contribute to the phenotype of cell proliferation (Moore et al. 1997; Kalkhoven et al. 1996), we next wanted to compare the relative agonist efficacies and potencies of the different progestogens for proliferation via PR-B. However, we were unable to optimise proliferation assays in the PR- and ER-negative MDA-MB-231 breast cancer cell line (Horwitz et al. 1978; Leo et al. 2004) transfected with the pMT-hPR-B expression vector. We thus evaluated the proliferative effects of the progestogens in the T47D breast cancer cell line endogenously expressing the PR isoforms and ER subtypes using the MTT assay. The cells were treated with either 0.1% (v/v) EtOH or increasing concentrations of the test compounds for 72 hours. Results in figure 3.11A, 3.11B and Table 3.3 show that Prog, bProg, MPA, LNG, GES and DRSP displayed similar proliferative efficacies to each other and the PR-specific agonist, R5020. While NET-A appeared to be more efficacious than all the other progestogens, statistically significant differences could only be determined for NET-A vs. bProg and NET-A vs. DRSP. In terms of potencies, Prog and bProg displayed similar potencies to each other, but were significantly less potent than R5020 (figure 3.11A). Results also indicated that MPA, LNG and GES displayed similar potencies to each other and to R5020, while NET-A and DRSP were less potent than R5020, MPA, LNG and GES. Furthermore, MPA and GES were significantly more potent than bProg but not Prog. Although NET-A was more efficacious than

DRSP, these two progestins displayed similar potencies to each other. In summary, all the progestogens investigated in this study, except NET-A and DRSP, displayed similar proliferative efficacies and potencies.

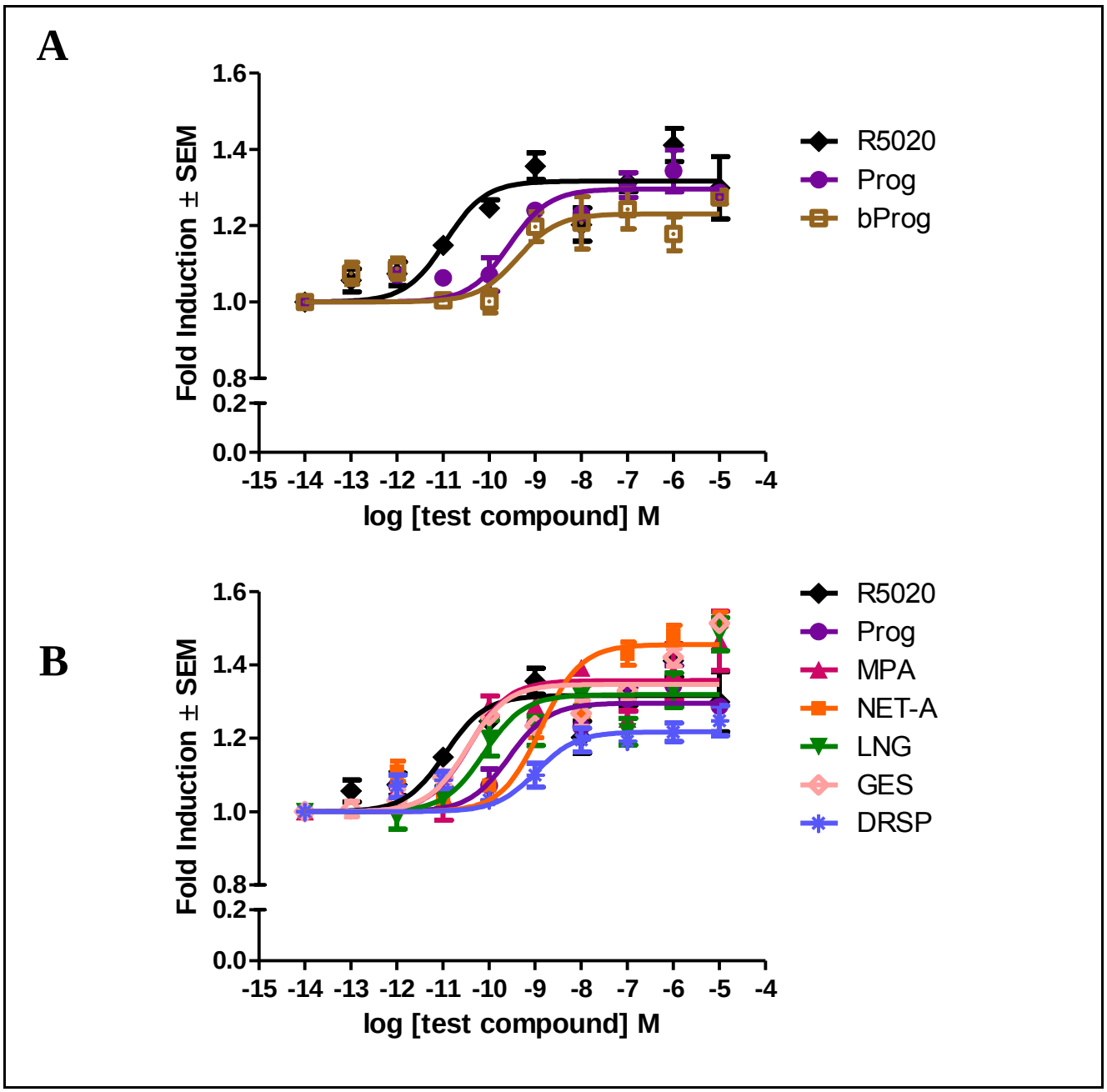


Figure 3.11. (A) Prog and bProg display similar proliferative efficacies and potencies to each other. T47D breast cancer cells were incubated with either 0.1% EtOH or increasing concentrations of R5020 (♦), Prog (●) or bProg (□) for 72 hours. (**B**) **All progestogens, except NET-A and DRSP, display similar proliferative maximal responses and relative agonist potencies.** T47D breast cancer cells were incubated with either 0.1% EtOH or increasing concentrations of R5020 (♦), Prog (●), MPA (▲), NET-A (■), LNG (▼), GES (◊) or DRSP (*) for 72 hours. **(A and B)** Four hours before the 72 hour incubation period lapsed, MTT solution was added and the cells incubated for a further 4 hours. Absorbances were measured at 550 nm and proliferation is shown as fold induction with 0.1% EtOH set as 1. The result shown is the average of at least two independent experiments with each condition performed in triplicate (± SEM).

Table 3.3. Relative efficacies (maximal response (MAX) in %) and potencies (expressed as EC_{50} in M) of the test compounds for proliferation.[c]

Ligand	Classification	MAX (%) ± SEM	EC_{50} (M) ± SEM
R5020	PR-selective agonist	100.00 ± 0.0	1.10×10^{-11} ± 0.11
Prog	Natural progestogen	95.09 ± 0.59	3.00×10^{-10} ± 1.63
bProg	Semi-synthetic (bio-identical)	90.41 ± 1.39	5.93×10^{-10} ± 3.10
MPA	1st Generation progestin	99.87 ± 0.93	3.71×10^{-11} ± 2.50
NET-A		112.3 ± 3.84	3.95×10^{-9} ± 2.57
LNG	2nd Generation progestin	97.23 ± 5.80	8.07×10^{-11} ± 1.21
GES	3rd Generation progestin	102.6 ± 5.56	3.09×10^{-11} ± 0.86
DRSP	4th Generation progestin	89.60 ± 2.45	1.15×10^{-9} ± 0.43

[c]Data shown in figure 3.11A and 3.11B were analysed to obtain relative maximal response (MAX) ± SEM and EC_{50} ± SEM values for each of the test compounds. MAX values are expressed as a percentage relative to 10^{-5} M R5020 = 100% and statistical analysis of the MAX values indicated that R5020 vs. Prog vs. bProg vs. MPA vs. LNG vs. GES vs. DRSP ($p>0.05$); NET-A vs. Prog, MPA, LNG and GES ($p>0.05$); NET-A vs. bProg and DRSP ($p<0.05$). Statistical analysis of the EC_{50} values indicated that R5020 vs. MPA vs. LNG vs. GES ($p>0.05$); R5020 vs. Prog ($p<0.05$); R5020 vs. bProg and DRSP ($p<0.01$); R5020 vs. NET-A ($p<0.001$); Prog vs. bProg, MPA, NET-A, LNG, GES and DRSP ($p>0.05$); MPA vs. NET-A ($p<0.01$); MPA vs. DRSP ($p<0.05$); NET-A vs. GES ($p<0.001$); NET-A vs. LNG ($p<0.01$); NET-A vs. DRSP ($p>0.05$); LNG vs. DRSP ($p<0.05$); GES vs. DRSP ($p<0.01$); bProg vs. NET-A, LNG and DRSP ($p>0.05$); bProg vs. MPA and GES ($p<0.05$).

3.8. ERα- and ERβ-specific antagonists do not modulate progestogen-induced proliferation of the T47D breast cancer cells

Having shown that neither the ERα-specific antagonist (MPP) nor the ERβ-specific antagonist (PHTPP) modulated the progestogen-induced upregulation of c-myc gene expression, while the

progestogen-induced downregulation of IL-8 gene expression appears to completely reversed by PHTPP, but not MPP, we next investigated whether the ER specific antagonists could modulate the progestogen-induced proliferation of the T47D breast cancer cell line. T47D cells were thus treated with 0.1% (v/v) EtOH or 1 nM test compound in the absence and presence of 10 µM MPP or PHTPP, and cell proliferation quantified using the MTT assay. As shown in figure 3.12A and 3.12B all the progestogens could induce proliferation of the T47D breast cancer cells at 1 nM, albeit to different extents. Furthermore, although the result in figure 3.12A show that cell proliferation induced by the progestogens was increased in the presence of MPP, this is probably not an increase in progestogen-induced proliferation, but rather due to the fact that MPP itself increases proliferation. In contrast, PHTPP itself had no effect on proliferation and increased LNG- and GES-induced breast cancer cell proliferation (figure 3.12B).

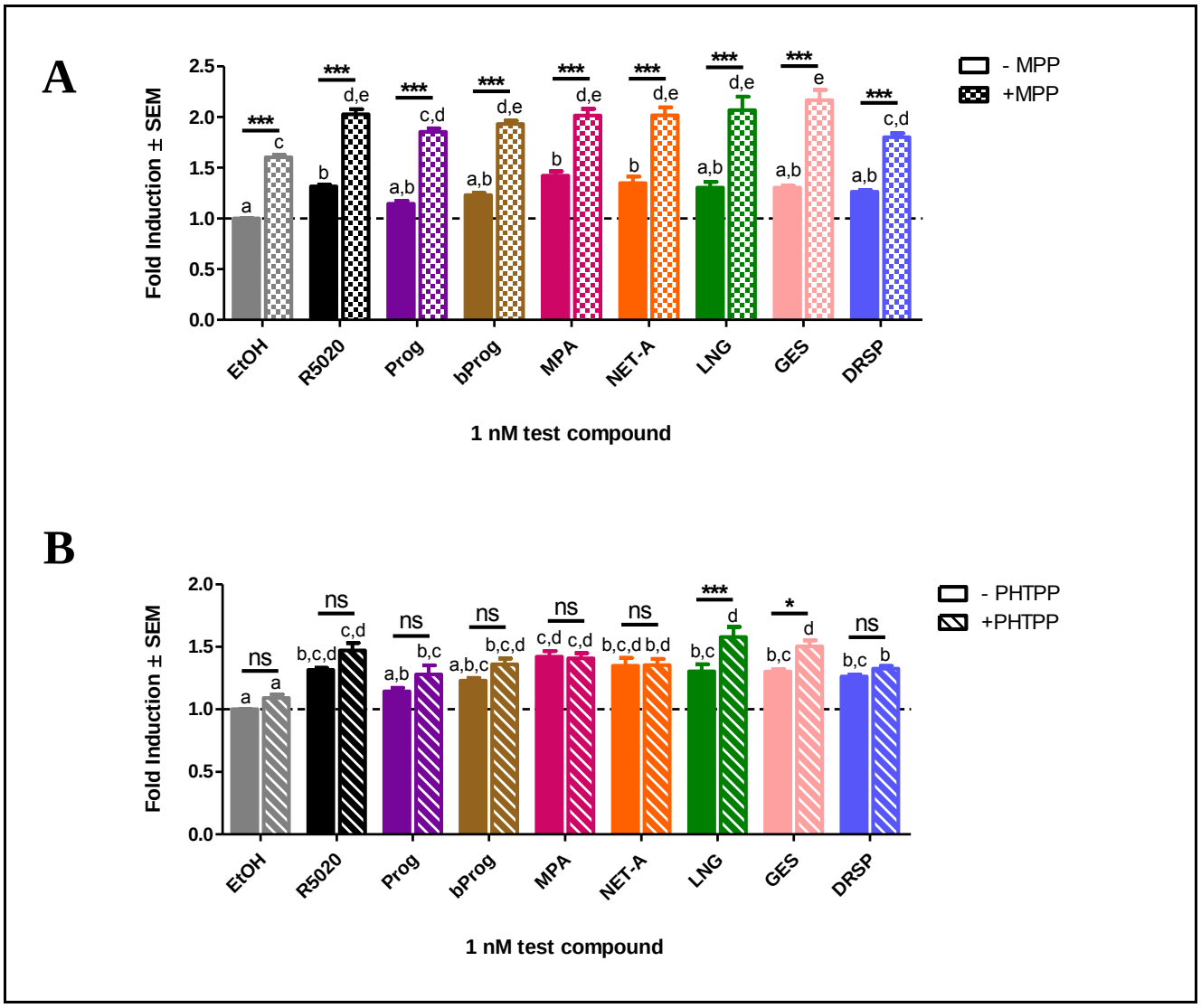


Figure 3.12. The ERα-specific antagonist, MPP, has no effect on progestogen-induced breast cancer cell proliferation, while LNG- and GES-induced breast cancer cell proliferation is significantly increased by the ERβ-specific antagonist, PHTPP. T47D breast cancer cells were incubated with (**A** and **B**) 0.1% EtOH (vehicle control) or 1 nM of the test compounds in the absence and presence of 10 μM of the receptor specific antagonists for **(A)** ERα (MPP) or **(B)** ERβ (PHTPP) for 72 hours. **(A and B)** Four hours before the 72 hour incubation period lapsed, MTT solution was added and the cells were incubated for a further 4 hours. Absorbances were measured at 550 nm and proliferation is shown as fold induction with 0.1% EtOH set as 1. The result shown is the average of at least two independent experiments with each condition performed in triplicate.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1. Introduction

A wide variety of progestins are extensively used in various therapeutic applications, including HRT (Whitehead et al. 1979; Greendale et al. 1999; Hickey et al. 2005) and contraception (Sitruk-Ware 2005a; Sitruk-Ware & Nath 2010; Africander, et al. 2011a). Progestins can be classified into four consecutive generations (Sitruk-Ware 2004a), with the first two generations often referred to as the older progestins, while the third and fourth generations are considered the newer progestins (Sitruk-Ware 2004a; Sitruk-Ware & Plu-Bureau 2004). These newer progestins were designed to be more specific for the PR (Sitruk-Ware 2004a) and more closely mimic the biological effects of Prog (Sitruk-Ware 2006). Evidence from several clinical trials, population-based case control studies and experimental studies suggest an association between the use of some of the older generation progestins like MPA, NET-A and LNG, and increased risk of developing breast cancer (Lee et al. 1987; Kalkhoven et al. 1994; Ross et al. 2000; Newcomb et al. 2002; Rossouw et al. 2002; Althuis et al. 2003; Beral 2003; Hunter et al. 2010; Li et al. 2012; Giulianelli et al. 2012; Ruan et al. 2012; Beaber et al. 2014; Wargon et al. 2014). This has however led to the misconception that all progestins increase breast cancer risk, whilst in reality there has been a lack of research investigating the association between breast cancer and the use of newer generation progestins. The concerns of increased breast cancer risk have however resulted in women searching for safer HRT alternatives. The use of compounded bio-identical HRT has thus gained popularity amongst women as compounded bio-identical hormones such as bProg, have been claimed to be “safer” than the synthetic hormones used in conventional HRT (Boothby et al. 2004; Boothby & Doering 2008; Holtorf 2009; Panay & Fenton 2010; Files et al. 2011). However, these claims have not been substantiated by scientific evidence.

Progestins, natural Prog and bProg (progestogens) exert their biological effects by binding to PRs (Conneely et al. 2001; Conneely et al. 2003), namely PR-A and PR-B (Kastner et al. 1990), with PR-B being the more transcriptionally active isoform in the presence of ligand and the more proliferative PR isoform in breast cancer (Conneely et al. 2003; Diep et al. 2015). Surprisingly,

even though progestins are PR ligands, comparative agonist efficacy and potency values for progestins for transactivation and transrepression via PR-B is lacking. Furthermore, emerging evidence suggests that crosstalk between PR and ERα signalling pathways plays an important role in breast cancer, and may be a potential mechanism whereby MPA increases breast cancer risk. This thus raised the question of whether ERα is required for PR-B-mediated gene regulation and breast cancer cell proliferation induced by other progestogens. Given that the ER exists as two subtypes, ERα and ERβ, a further question was whether ERβ would also be needed for progestogen-induced gene regulation via PR-B. In the light of the above, in Chapter 3 we thus pharmacologically characterised the progestogens in terms of their efficacies and potencies for transactivation and transrepression via PR-B on synthetic promoters containing PRE and NFκB binding sites, respectively, while also evaluating the progestogen-induced transactivation and transrepression of endogenous PRE- and NFκB-containing promoters. We also determined the efficacies and potencies of the progestogens for proliferation of the T47D breast cancer cell line. Subsequently, we investigated whether ERα and/or ERβ modulates progestogen-induced gene regulation and cell proliferation.

4.2. Most progestogens are agonists for transactivation on both a synthetic and endogenous PRE-containing promoter

In the first part of this thesis (Section 3.1), we aimed to compare the relative agonist efficacy and potency (EC_{50}) values of select progestogens for transactivation via PR-B on a synthetic PRE-containing promoter (figure 3.1; Table 3.1). PR-B was overexpressed in COS-1 cells as these cells express negligible amounts of endogenous steroid receptors (Africander et al. 2014), which allowed the accurate determination of the transcriptional activity of the test compounds via PR-B. Although a number of studies have examined the relative agonist efficacies and potencies of progestogens via the PR, these studies often do not distinguish between PR-isoforms or they examine activities in cell lines that also express other steroid receptors to which these ligands may bind (Markiewicz &

Gurpide 1994; Bray et al. 2005). Our study is thus the first to directly compare the relative agonist efficacy and potency values of the progestogens for transactivation via PR-B within the same model system. When comparing the relative efficacy values of Prog, bProg and select progestins from different generations to the PR-specific agonist R5020 (positive control), we were able to characterize the agonist properties of the progestogens. Agonists that display similar, lower or higher maximal responses than the positive control, are referred to as full-, partial or supra-agonists, respectively (Africander, et al. 2011a). We show that NET-A, LNG, and GES are full PR-B agonists, which is in agreement with a previous study showing full agonist activity for these progestins in the HeLa cervical carcinoma cell line transfected with PR-B and the PRE-E1b-chloramphenicol acetyltransferase (CAT) reporter construct (García-Becerra et al. 2004). However, in contrast to our findings that Prog and MPA are partial PR-B agonists, Markiewicz and co-workers (1994) have previously reported full agonist activity for Prog and MPA in T47D cells (Markiewicz & Gurpide 1994). It is not surprising that the results differ between these two systems, as the T47D cell line endogenously expresses equimolar concentrations of PR-B and PR-A (Horwitz et al. 1978; Keydar et al. 1979; Wargon et al. 2014), and it is likely that these progestogens are eliciting their transcriptional effects via both PR-B and PR-A. Considering that the newer generation progestins were designed to be more PR specific (Sitruk-Ware 2004a), it was surprising that GES and DRSP displayed similar relative agonist efficacies to NET-A and LNG. Furthermore, our results also indicate that the two first generation progestins, NET-A and MPA, differ in their maximal responses, suggesting that progestins from the same generation will not necessarily elicit the same effects.

In terms of the relative agonist potencies (EC_{50}) of the progestogens via PR-B our results showed that there was no significant difference between Prog, R5020, the first (MPA and NET-A) and the second (LNG) generation progestins, while the third generation progestin GES was shown to be more potent than Prog and the fourth generation progestin DRSP less potent (figure 3.1; Table 3.1). The EC_{50} value of 0.036 nM determined for GES in the present study is similar to the EC_{50} value of

0.018 nM reported by García-Becerra and co-workers (2004) in the HeLa cervical carcinoma cell line transfected with PR-B and the PRE-E1b-chloramphenicol acetyltransferase (CAT) reporter construct (García-Becerra et al. 2004). Furthermore, the EC_{50} value of 0.45 nM obtained for MPA in our study is in line with that reported by Sasagawa et al. (2008) showing an EC_{50} value of 0.1 nM for MPA in COS-1 cells transfected with PR-B and the PRE2-tk-luciferase reporter (Sasagawa et al. 2008).

We next determined whether the progestogens display similar transactivation activities on the endogenous c-myc gene which contains a functional PRE sequence in its promoter region (Moore et al. 1997), in the T47D breast cancer cell line (figure 3.6A). This cell line was chosen as an *in vitro* model system as it endogenously expresses high levels of the PR isoforms, and is often used to study the activity of the PR in breast cancer (Keydar et al. 1979; Wargon et al. 2014). The c-myc gene was used as it is overexpressed in many different forms of cancers including breast cancer, while the c-myc protein has been shown to regulate a number of genes involved in cellular processes such as proliferation, apoptosis, differentiation and cell growth (Liao & Dickson 2000). Moreover, c-myc gene expression has previously been shown to be upregulated by the first generation progestin MPA (Giulianelli et al. 2012; Wargon et al. 2014). Surprisingly, the results in figure 3.6A show that the PR-specific agonist R5020 had no effect on the mRNA expression of c-myc. In contrast to our results, Moore and co-workers (1997) have previously shown that c-myc mRNA expression is upregulated (1.9-fold) by 1 nM R5020 in T47D cells (Moore et al. 1997). However, it should be noted that the results in figure 3.6 are representative of a single experiment performed in duplicate, and thus the discrepancy between our result for R5020 and that of Moore et al. (1997) may simply be ascribed to experimental error on our part. We also showed that Prog, MPA, NET-A, LNG, GES and DRSP all appeared to upregulate c-myc mRNA expression (figure 3.6A). Our results for MPA showing upregulation of c-myc mRNA expression, although not statistically significant, is in agreement with results from three previous studies performed in T47D cells (Wong & Murphy 1991; Giulianelli et al. 2012; Wargon et al. 2014). Considering that

overexpression of the c-myc gene is associated with increased breast cancer cell proliferation, our results showing increased expression of the c-myc gene and increased cell proliferation in the presence of all the progestogens, except R5020, may suggest that these progestogens promote breast cancer via a mechanism involving PR-B.

4.3. Both ER subtypes decreased the PR-B-mediated maximal responses of all the progestogens, except MPA, on a synthetic PRE-containing promoter, while ER subtype-specific antagonists have no effect on progestogen-induced transactivation of an endogenous PRE-containing promoter

Giulianelli and co-workers (2012) showed that ERα is required for the PR-B-mediated effects of MPA on gene regulation in the MDA-MB-231 breast cancer cell line (Giulianelli et al. 2012), but did not investigate the requirement of ERα for any other progestins, or whether ERβ, may also be required. Having shown that all progestogens investigated in this study are agonist for transactivation via PR-B on a synthetic PRE-containing promoter and that the progestogens upregulated the mRNA expression of the endogenous PRE-containing gene, we next determined whether the ER subtypes could modulate the progestogen-induced effects. First, we investigated whether effects observed by Giulianelli et al. (2012) could be mimicked in our model system and results in figure 3.2 show that it could not, as we found that ERα is not required for the MPA-induced transcriptional activation via PR-B on a synthetic PRE-containing promoter. This discrepancy could possibly be attributed to the use of different cell lines and/or promoter reporter constructs as we transiently transfected COS-1 cells with PR-B, ERα and the pTAT-2xPRE-E1b-luciferase promoter-reporter construct, while Giulianelli and co-workers used MDA-MB-231 breast cancer cells stably transfected with PR-B, and transiently transfected with ERα as well as a (PRE)2pGL3p promoter-reporter construct (Giulianelli et al. 2012). Furthermore, considering that the MDA-MB-231 breast cancer cell line, unlike the COS-1 cell line, endogenously express both GR (Horwitz et al. 1978) and MR (Leo et al. 2004), and that MPA has previously been shown to

bind to both these receptors (Koubovec et al. 2005; Africander et al. 2013), it is possible that the GR and/or MR may have contributed to the difference observed between our result and that of Giulianelli et al. (2012). Two other factors which may also have contributed to the difference between the two studies, could be that the PR-B and ERα protein expression levels vary between these two *in vitro* model systems, or that the transcriptional activity is influenced by co-factors which are differentially expressed in different cell lines. Furthermore, we show for the first time that ERβ decreased the PR-B-mediated maximal response of MPA (figure 3.2). The exact reason for the difference in the effect of ERα and ERβ on the relative maximal response of MPA via PR-B is not clear. We speculated that the significant decrease in the maximal responses of MPA in the presence of ERβ, but not ERα, may be due to ERβ affecting the expression levels of PR-B. Indeed, our results showed that ERβ significantly downregulates PR-B protein levels (figure 3.3A and 3.3B), while simultaneously decreasing basal PR-B-mediated transcriptional activity (figure 3.3F) in COS-1 cells co-expressing PR-B and ERβ. However, to our surprise, ERα also downregulated the protein expression levels of PR-B (figure 3.3A and 3.3B), and even more unexpected, this decrease in the PR-B protein levels was associated with an increase in the basal PR-B-mediated transcriptional activity (figure 3.3F). This does however not explain our results in figure 3.2 indicating that ERα had no effect on the PR-B-mediated maximal response of MPA. Considering that MPA is associated with an increased risk of breast cancer, this result suggests that ERβ may protect against MPA-induced breast cancer when co-expressed with PR-B.

The exact mechanism whereby unliganded ERα downregulates PR-B protein expression whilst also increasing basal PR-B-mediated transcriptional activation is unclear, however some speculations can be made. PR-B has previously been shown to be activated in the absence of ligand when Ser400 is phosphorylated by cyclin-dependent protein kinase 2 (CDK2). Considering that increased CDK2 activity can stimulate PR-B downregulation (Pierson-mullany & Lange 2004), we hypothesize that ERα may elevate CDK2 activity, which would lead to the downregulation of PR-B, but also increased phosphorylation, and hence activity, of PR-B. To the best of our knowledge, this is the

first study to show that ERα upregulates, while ERβ downregulates, the basal transcriptional activity of PR-B on a synthetic PRE-containing promoter. Considering that unliganded PR-B can be activated through a non-genomic signalling mechanism (Pierson-Mullany et al. 2003; Pierson-mullany & Lange 2004) and can subsequently activate the expression of PR-regulated genes that play a role in cell proliferation (Sartorius et al. 2003; Giulianelli et al. 2012; Wargon et al. 2014), our results suggest that ERα may promote PR-positive breast cancer while ERβ may protect against it.

We continued investigating the effects of the ERs on PR-B-mediated agonist efficacies and potencies of select progestins from different generations, natural Prog and bProg for transactivation. In contrast to the results for MPA, our results showed that ERα significantly lowered the PR-B-mediated maximal response of R5020, Prog, bProg, NET-A, LNG, GES and DRSP on a synthetic PRE-containing promoter, while also decreasing the relative agonist potency of Prog (figure 3.4A-G). We also show for the first time that ERβ decreased the PR-B-mediated maximal response of all the progestogens investigated in this study, including MPA, while having no significant effect on the relative agonist potencies of any of the progestogens (figure 3.4A-G). Understanding why ERβ would modulate the maximal response of all progestogens, including MPA, while ERα decreased the maximal response of all progestogens except MPA is not straightforward. Considering that structurally different progestogens can lead to slightly different ligand-receptor conformations (Wagner et al. 1996; Rękawiecki et al. 2011; Hapgood et al. 2014), it may be possible that ERβ can interact with the MPA-bound conformation of PR-B, while ERα cannot. As mentioned earlier for MPA, a limitation of this study was that the effects of the ER subtypes on PR-B protein expression was not investigated in the presence of any of the progestogens investigated in the present study. We next determined whether the ERα-specific antagonist MPP and/or the ERβ-specific antagonist PHTPP, could modulate progestogen-induced upregulation of the c-myc gene. Results showed that neither MPP (figure 3.6B) nor PHTPP (figure 3.6C) had any effect on progestogen-induced c-myc mRNA expression, suggesting that neither ERα nor ERβ could modulate the progestogen-induced

upregulation of the c-myc gene. A major limitation of our study was that we did not use the ER antagonist ICI 182 780. This antagonist acts by binding to the ER subtypes and leading to the subsequent degradation of the ER subtypes (Alarid et al. 1999; Long & Nephew 2006), while MPP and PHTPP exert their effects by binding to ERα and ERβ, respectively, and preventing the ER subtypes from activating transcription whilst having no effect on ER protein levels (Hartman et al. 2009; Chan et al. 2014). Thus, ERα and ERβ are still present in our model system making it difficult to ascertain whether the individual ER subtypes would modulate the progestogen-induced effects.

In summary, our results indicating that neither ERα nor ERβ is required for progestogen-induced transcriptional activation on a synthetic PRE-containing promoter, and that both MPP and PHTPP do not modulate the effects of the progestogens on the endogenous PRE-containing c-myc gene, refuted our hypothesis that ERα, but not ERβ, would be required for progestogen-induced gene regulation. In fact, we showed that the PR-B-mediated maximal response of all progestogens, except MPA, was decreased by the expression of ERα, whereas ERβ decreased the maximal response of all the progestogens on the synthetic PRE-containing promoter-reporter construct.

4.4. All progestogens are agonists for transrepression on both synthetic and endogenous NFκB-containing promoters

Apart from the transactivation mechanism whereby the PR can positively regulate gene expression by directly binding to PREs, the PR can also negatively regulate gene expression by repressing the activity of the transcription factor NFκB (Kalkhoven et al. 1996; Kobayashi et al. 2010). NFκB plays an integral role in the inflammatory response and cell proliferation by regulating the expression of pro-inflammatory genes (Ben-Neriah & Karin 2011; Niu et al. 2012). An increase in pro-inflammatory gene expression leads to inflammation, and while acute inflammation is considered beneficial in that it is a natural mechanism used by the body to fight infection (Ben-Neriah & Karin 2011), continual expression of high levels of pro-inflammatory genes could lead to

chronic inflammation. Chronic inflammation has been shown to be a major etiologic factor in promoting breast cancer development and progression, as it contributes to proliferation, angiogenesis, metastasis and reduces responsiveness to cancer treatments (Colotta et al. 2009; Culig 2011; Niu et al. 2012). We investigated the potential PR-mediated anti-inflammatory effects of the progestogens, by determining the potential of the progestogens to repress TNFα-induced transcription. Specifically, the transrepressive properties of the progestogens via overexpressed PR-B were investigated on a synthetic NFκB-containing promoter in COS-1 cells, while transrepressive properties were also investigated on an endogenous NFκB-containing promoter in T47D cells endogenously expressing both PR isoforms. This study is the first to directly compare the relative agonist efficacies and potencies for transrepression of R5020, Prog, bProg and select progestins from different generations via PR-B. While all progestogens displayed similar relative maximal repression to each other and Prog, differences were observed between the potencies of the first (MPA), second (LNG) and third (GES) generation progestins (figure 3.7B and 3.7C; Table 3.2).

To investigate the potential of the progestogens to repress TNFα-induced transcriptional activation on an endogenous NFκB-containing promoter, we determined the effects of the different progestogens on the expression of the endogenous IL-8 gene in the T47D breast cancer cell line. The IL-8 gene was selected as it contains a NFκB binding site in its promoter (Roebuck 1999) and has been shown to promote angiogenesis in various different forms of cancer which in turn may increase the risk of metastasis (Xie 2001; Benoy et al. 2004; Lin et al. 2004). Furthermore, patients with metastatic breast cancers have also been shown to express elevated IL-8 serum levels (Xie 2001; Benoy et al. 2004). Results from the current study showed that 1 nM R5020 significantly downregulates TNFα-induced IL-8 gene expression, which is consistent with the findings of a study by Kobayashi and co-workers (2010) showing that 10 nM R5020 downregulated IL-1β-induced IL-8 gene expression in T47D cells engineered to express either PR-A or PR-B alone (Kobayashi et al. 2010). Interestingly, all progestogens except MPA significantly repressed TNFα-induced IL-8 mRNA expression (figure 3.11B). However, it should be noted that these results, like those for c-

myc mRNA expression (figure 3.6A), were from a single experiment, and thus no definitive conclusions can be drawn at this time.

Taken together, our results show for the first time that all the progestogens investigated in this study are full PR-B-agonists for transrepression, and the progestins display differential transrepressive potencies on a synthetic NFκB-containing promoter via PR-B. Although the progestogens could also repress gene expression on the endogenous NFκB-containing promoter of the IL-8 gene, we did not establish which PR isoform is involved in the progestogen-induced effects on endogenous IL-8 gene expression, and can therefore not specifically pinpoint the effects to PR-B.

4.5. Both ERα and ERβ modulate progestogen-induced transcriptional repression on a synthetic NFκB-containing promoter, while the ERβ-specific antagonist PHTPP appeared to modulate progestogen-induced repression on the endogenous NFκB-containing IL-8 gene promoter

Having shown that all progestogens repress TNFα-induced transcriptional activation on a synthetic NFκB-containing promoter via PR-B, we next investigated whether ERα and/or ERβ could influence the progestogen-induced transcriptional repression. Interestingly, we showed that ERα prevented the ability of the PR-specific agonist R5020, as well as the second (LNG), third (GES) and fourth (DRSP) generation progestins to repress gene expression via PR-B, while both ERα and ERβ modulated the PR-B-mediated agonist potencies of the natural progestogen Prog and the first generation progestin MPA (figure 3.9). These results suggest that ERα may promote inflammation-induced breast cancer by inhibiting the PR-B-mediated anti-inflammatory effects of R5020, LNG, GES and DRSP. In trying to understand the mechanism whereby ERα abolishes the transrepressive effects of some, but not all progestogens, it can be speculated that R5020, LNG, GES and DRSP induce a conformational change in PR-B which allows ERα to interact with the progestogen-bound PR-B, thereby preventing PR-B from interacting with NFκB and subsequently preventing PR-B-

mediated transrepression. Alternatively, considering that ERα can also interact with NFκB (Ray et al. 1997), ERα may be competing with the progestogen-bound PR-B for binding to NFκB.

Our next aim was to determine whether the ERα-specific antagonist MPP, and the ERβ-specific antagonist PHTPP, could modulate progestogen-induced transrepression on the endogenous NFκB-containing IL-8 gene. We showed that the R5020-, LNG-, GES- and DRSP-induced repression of IL-8 gene expression is not modulated in the presence of the ERα antagonist MPP (figure 3.10C). However, it should be noted that MPP itself significantly downregulated the TNFα-induced IL-8 mRNA expression. Considering that the IL-8 gene contains NFκB and AP-1 binding sites in its promoter (Roebuck 1999), and that the ER subtypes has also been shown to interact with the DNA-bound NFκB transcription factor to negatively regulate gene transcription (Ray et al. 1997), it is possible that the antagonist bound-ERα may form a complex with the PR at the NFκB cis-elements upon addition of the progestogens, where it then modulates expression of the IL-8 gene. Results in figure 3.10D showed that PHTPP alone had no significant effect on TNFα-induced transcriptional activation of the IL-8 gene, while the antagonist-bound ERβ can modulate progestogen-induced repression of the IL-8 gene (figure 3.10D). However, as mentioned before, no definitive conclusions can be drawn from the endogenous IL-8 gene expression data as this is the result of a single experiment which needs to be repeated.

4.6. Progestogen-induced breast cancer cell proliferation is not modulated by the ER subtypes

The transcriptional regulation of several genes, whether it is via a transactivation (Nass & Dickson 1997), transrepression (Badache & Hynes 2001) or non-genomic mechanism (Saitoh et al. 2005), have been shown to contribute to cellular processes such as proliferation. Numerous *in vitro* studies have investigated whether progestins can lead to breast cancer cell proliferation (Horwitz & Freidenberg 1985; van der Burg et al. 1992; Botella et al. 1994; Kalkhoven et al. 1994; Catherino et al. 1993; Krämer et al. 2006; Ruan et al. 2012), with the results often contradictory. Furthermore,

the majority of these studies did not directly compare the proliferative effects of progestins from different generations. To the best of our knowledge, our study is thus the first to directly compare the proliferative efficacies and potencies of select progestins from different generations to each other and natural Prog in the T47D breast cancer cell line. Results from colorimetric MTT assays showed that all progestogens, with the exception of NET-A and DRSP, displayed similar proliferative efficacies and potencies (figure 3.11; Table 3.3). These results are in agreement with a previous study showing that NET, LNG and GES stimulate cell proliferation of the HCC1500 (Krämer et al. 2006) breast cancer cell line at concentrations of 10- and 100-fold more than used in our study. Additionally, 10 nM GES and MPA has previously been shown to increase cell proliferation in the MCF-7 (Catherino et al. 1993) and T47D (Giulianelli et al. 2012; Wargon et al. 2014) breast cancer cell lines.

We also investigated whether the ERα-specific and ERβ-specific antagonists could modulate progestogen-induced T47D breast cancer cell proliferation, and showed that the ERα-specific antagonist had no effect on progestogen-induced proliferation of the T47D breast cancer cells (figure 3.12A), while the ERβ-specific antagonist significantly increased LNG- and GES-induced T47D breast cancer cell proliferation (figure 3.12B). The latter result suggests that the ERβ may play a protective role in LNG- and GES-induced T47D breast cancer cell proliferation.

In summary, considering that the progestogens appear to upregulate expression of the proliferation-related c-myc gene which is often overexpressed in cancer, while exhibiting anti-inflammatory effects by repressing the expression of the pro-inflammatory cytokine, IL-8 which is often upregulated in breast cancer, the progestogens appear to be exerting their proliferative effects via the transactivation function of the PR. In addition to the fact that the results on c-myc and IL-8 gene expression is from a single experiment, it is however important to note that the regulation of a large number of genes, and not only individual genes, are involved in the regulation of cellular processes such as proliferation.

4.7. Future work

The present study has a number of shortcomings which should be addressed in future studies. The major limitation was that only a single realtime qPCR experiment was performed to investigate the effects of the different progestogens on the expression of the endogenous c-myc and IL-8 genes, and that the requirement of the ER was not investigated using the ER antagonist ICI 182 780. Thus, future studies should include at least two repeats of these experiments, in the absence and presence of ICI 182 780, so as to confirm the effects of the progestogens on these genes, while also evaluating the contribution of the ERs. However, ICI 182 780 will not discriminate between the ER subtypes, thus we suggest that the individual role of ERα and ERβ should also be addressed using siRNA technology to silence the expression of the specific ER subtype. Furthermore, as the T47D cells express both PR isoforms, siRNA technology could also be performed to investigate the roles of PR-B and PR-A in mediating the effects of the progestogens on gene expression and breast cancer cell proliferation.

In terms of understanding the mechanism whereby ERα and ERβ decreased the maximal responses of the progestogens via PR-B on a synthetic PRE-containing promoter, we suggest that future research should be devoted towards investigating whether the ER subtypes form a complex with PR-B and whether this PR-B/ER complex co-localises to the PRE *cis*-element of the c-myc promoter. To determine whether a protein-protein interaction occurs between PR-B and ERα or ERβ, co-immunoprecipitation assays (Co-IP) could be performed, while chromatin immunoprecipitation (ChIP) assays could be used to evaluate whether the PR-B/ER complex occupies the PRE *cis*-element in the promoter of the c-myc gene. Similarly experiments can be performed to delineate the mechanism whereby ERα abolishes the ability of some progestogens to transrepress the TNFα-induced activity on the synthetic NFκB-containing promoter. Lastly, as we speculated that ERα downregulates PR-B protein expression while simultaneously increasing the basal transcriptional activity of PR-B by a mechanism involving an increase in the activity of

CDK2, this should be evaluated by performing an enzymatic assay to determine CDK2 activity and western blot analysis using primary antibodies to detect PR-B phosphorylation at Ser400.

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ADDENDUM A

BUFFERS AND SOLUTIONS

1. *Media*

Luria Bertani (LB) medium

10 g NaCl

10 g Tryptone

5 g yeast extract

Adjust to final volume of 1 L using reverse osmosis (RO) water.

Sterilize by autoclaving and store at room temperature.

LB Agar plates

LB medium

15 g/L bacterial agar

Ampicillin (final concentration 50 µg/ml)

2. *Cell lysis*

10X Tris-phosphate-EDTA (TPE) buffer

108 g Tris (hydroxymethyl) aminomethane

15.5 ml 85% phosphoric acid

40 ml 0.5 M EDTA (pH 8.0)

Adjust to final volume of 1 L using RO water.

Sterilize by autoclaving and store at room temperature.

Passive Lysis Buffer

0.5 ml Triton X-100

25 ml Glycerol

7 ml 1X TPE buffer

720 µl 0.5 M EDTA

Adjust to final volume of 250 ml using RO water and store at 4°C.

3. *Electrophoresis and western blotting solutions*

10% (w/v) Sodium dodecyl sulphate (SDS)

Dissolve 10 g SDS in 100 ml RO water at 68°C.

Store at room temperature.

2X Laemmli buffer

1 ml 1M Tris-HCl (pH 6.8)

5 ml 10% (w/v) SDS

2 ml Glycerol

500 µl β-mercaptoethanol

0.01 g bromophenol blue

Adjust to final volume of 25 ml with RO water and store at -20°C.

10X SDS-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer

20 g SDS

60.6 g Tris

288.2 g glycine

Adjust to final volume of 2 L using RO water and store at room temperature.

1X Transfer buffer

6.06 g Tris

28.83 g glycine

200 ml methanol

Adjust to final volume of 2 L using RO water and store at 4°C.

10X Tris buffered saline (TBS) (pH 7.5)

Dissolve 60.5 g Tris and 87.6 g NaCl in 800 ml RO water.

Adjust the pH to 7.5

Adjust to final volume of 1 L using RO water and store at 4°C.

TBS-Tween (TBST)

100 ml 10X TBS buffer

1 ml Tween 20

Adjust to final volume of 1 L using RO water and store at 4°C.

50X Tris-acetate-EDTA (TAE) buffer

Dissolve 242.2 g Tris in 700 ml RO water.

Add 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.2).

Adjust to final volume of 1 L using RO water and store at room temperature.

The buffer is used for preparing and performing agarose gel electrophoresis.

10X Morpholinopropanesulfonic acid (MOPS) running buffer

Dissolve 83.71 g MOPS in 800 ml DEPC-treated H_2O.

Add 33.4 ml 3 M NaOAc and 20 ml 0.5 M EDTA RNase-free stock solutions.

Adjust pH to 7.0

Adjust to final volume of 1 L using DEPC-treated H_2O.

Sterilize by autoclaving and store in a dark place at 4°C.

The buffer is mainly used for the electrophoresis of denaturing formaldehyde agarose gels.

Formaldehyde RNA loading buffer (2x):

0.071 g bromophenol blue

1 ml Glycerol

1.5 ml 10X MOPS

2.6 ml formaldehyde

7.3 ml formamide

Adjust to final volume of 15 ml using DEPC-treated H_2O.

Prepare 1 ml aliquots and store at -20°C.

Add 2.5 µl ethidium bromide to each 1 ml aliquot before use.

The buffer is used for preparation of RNA samples for denaturing formaldehyde agarose gel electrophoresis.

4. *Cell proliferation assay*

MTT Solution (5 mg/ml)

Dissolve 5 mg MTT in 1 ml sterile PBS.

Filter sterilize.

ADDENDUM B

REALTIME QUATITATIVE POLYMERASE CHAIN REACTION (qPCR)

Introduction

Realtime qPCR is a powerful technique in which the amplification of DNA can be monitored in realtime (Arya et al. 2005). This is in contrast to conventional PCR where the amplified product can only be seen when the PCR is completed. In qPCR, the amplified DNA is labelled with fluorescent dyes such as SYBR Green, which binds to the minor groove of double stranded DNA (Lekanne Deprez et al. 2002). It is thus possible to monitor the amplified DNA in realtime by measuring fluorescence throughout the PCR process. The amount of fluorescence detected during amplification is directly proportional to the amount of amplified DNA (Arya et al. 2005). The cycle number at which fluorescence is first observed is called the quantification cycle (C_q) (Pfaffl 2001).

The current study used qPCR to detect and quantify changes in gene expression upon treatment with the test compounds. Before qPCR analysis could be performed it was important to isolate intact RNA that was of a high quality as RNA of poor quality can negatively influence the subsequent qPCR results. RNA purity was evaluated by measuring the optical density (OD) of the RNA, followed by agarose gel electrophoresis on a denaturing formaldehyde agarose gel to assess the quality of the RNA. An OD 260/280 ratio of 1.9 or greater is indicates pure RNA (Sambrook et al. 1989), while two clear 28S and 18S RNA bands, with the 28S band being approximately twice the intensity of the 18S band, indicates intact RNA (Krebs et al. 2009). A representative 1% denaturing formaldehyde agarose gel showing intact RNA is shown in Figure B1.

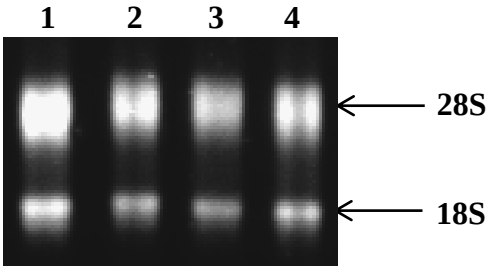

Figure B1. A representative 1% denaturing formaldehyde agarose gel indicating intact RNA. T47D breast cancer cells were treated with 0.1% (v/v) EtOH (Lane 1 and 3) or 10 nM MPA (Lane 2 and 4). Total RNA was isolated as described in Chapter 2 Section 2.7 and 1 µg RNA was loaded onto the agarose gel. The RNA was visualised by ethidium bromide staining.

The intact total RNA isolated from the T47D cells was used to synthesize cDNA as described in Chapter 2 Section 2.8 and subsequently realtime qPCR was performed as described in Section 2.9 of Chapter 2. Considering that PCR efficiency is dependent on the efficiencies of the primer sets, we first determined the primer efficiencies of the c-myc, IL-8 and GAPDH primer pairs. In theory, PCR efficiency is assumed to be two as the amount of DNA present in a sample should double with each PCR cycle (Pfaffl 2001; Wong & Medrano 2005). However, this is not always the case since the efficiency of each reaction is dependent on the efficiency of the primer set (Wong & Medrano 2005), which may vary. Therefore, the efficiency of the respective primer pairs for c-myc, IL-8 and GAPDH was determined before calculating the relative expression of these genes.

Determining primer pair efficiency

To determine the amplification efficiency of each of the primer sets used in this study, a dilution series of a single cDNA sample was prepared to generate a standard curve and each dilution was analysed in triplicate. A no template control was also included in order to confirm that no contamination or primer self-amplification was present. A standard curve (Figure B2) was generated with the C_q values for each dilution plotted against log cDNA concentration. Using the following equation (Pfaffl, 2001), the slope obtained from the standard curve was used to calculate the exponential amplification value (E, primer efficiency) of each primer set:

$$E = 10^{[-1/slope]} \quad (1)$$

The primer efficiencies reported for the c-myc, IL-8 and GAPDH primer pairs are the averages of two independent experiments. The efficiencies of the primer sets used in this study can be found in Table 2.3 in Chapter 2.

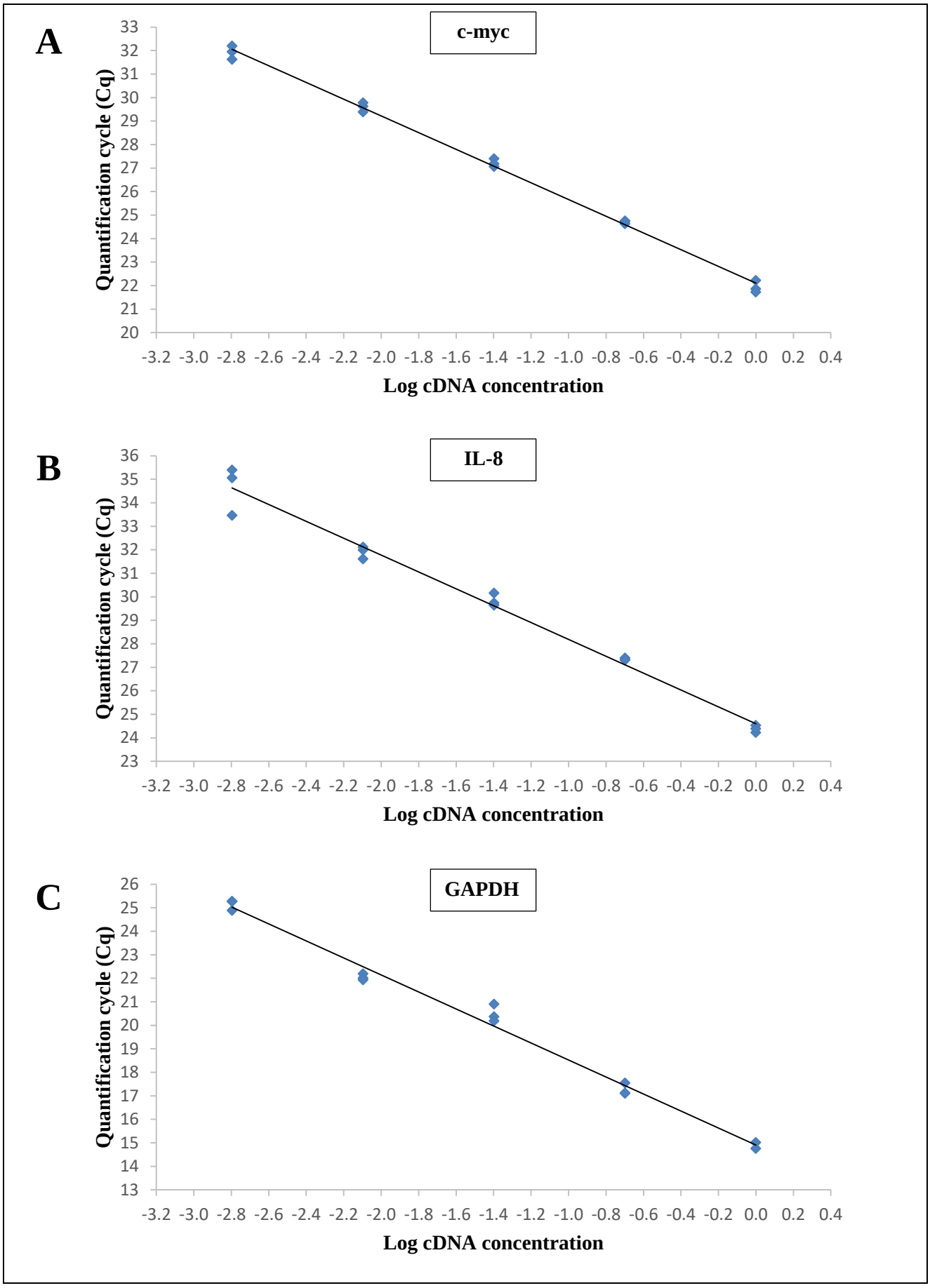

Figure B2 legend on next page.

Figure B2. Representative standard curves generated for the (A) c-myc, (B) IL-8 and (C) GAPDH genes, respectively. The standard curves were obtained by plotting the cycle number (C_q) relative to log cDNA concentration.

Melting curve analysis

Each PCR product has a unique melting temperature which is dependent on the nucleotide content and the size of the amplicon (Ririe et al. 1997). A single melting peak per sample confirms the presence of only one PCR amplified product. If more than one peak occurs, it indicates the presence of primer-dimers or other non-specific products (Ririe et al. 1997; Fraga et al. 2008). Considering that SYBR Green binds to double stranded DNA, and that the total fluorescence measured using SYBR Green is the sum of the fluorescence from all double stranded DNA products formed during the PCR reaction, it is important to ensure that the fluorescence measured is from a single product. A melting curve analysis was performed by varying the temperature between 65°C and 95°C, while gradually increasing the temperature by 2.2°C for each step and continuously measuring the fluorescence. Using the LightCycler® software, a melting curve was generated by plotting the negative derivative of fluorescence over temperature (-dF/dT) versus temperature (^{o}C) (Figure B3). The melting temperatures for the c-myc, IL-8 and GAPDH primers were determined to be 86.5°C, 81.1°C and 88.0°C, respectively. Apart from performing melting curve analysis to ensure that a single product was amplified, agarose gel electrophoresis was performed to confirm amplicon size. Representative agarose gels are shown in Figure B4.

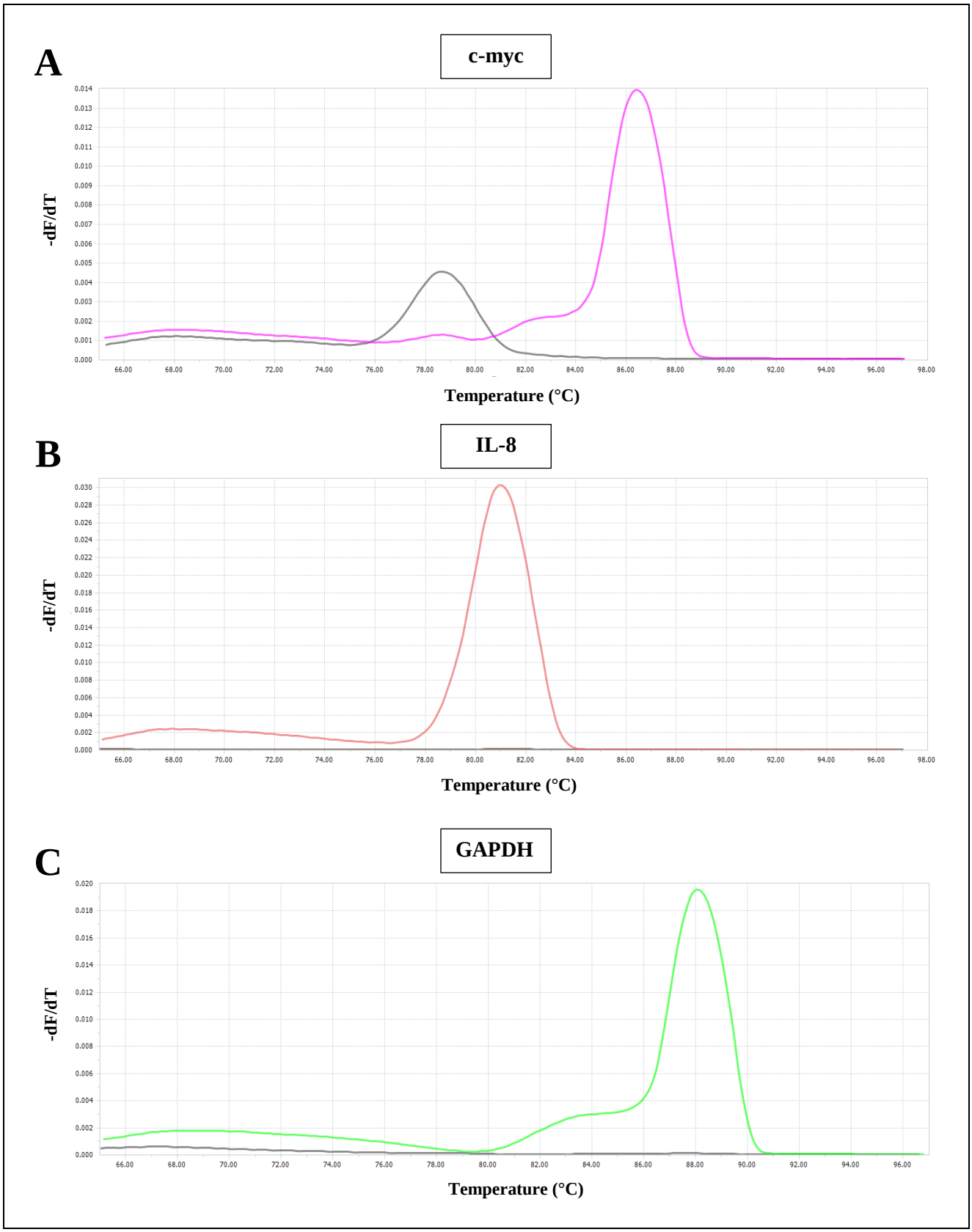

Figure B3. Melting curves generated for realtime qPCR products. The melting curves were generated for (**A**) c-myc, (**B**) IL-8 and (**C**) GAPDH using the LightCycler® 96 software. The negative controls containing no template are represented by the grey curves.

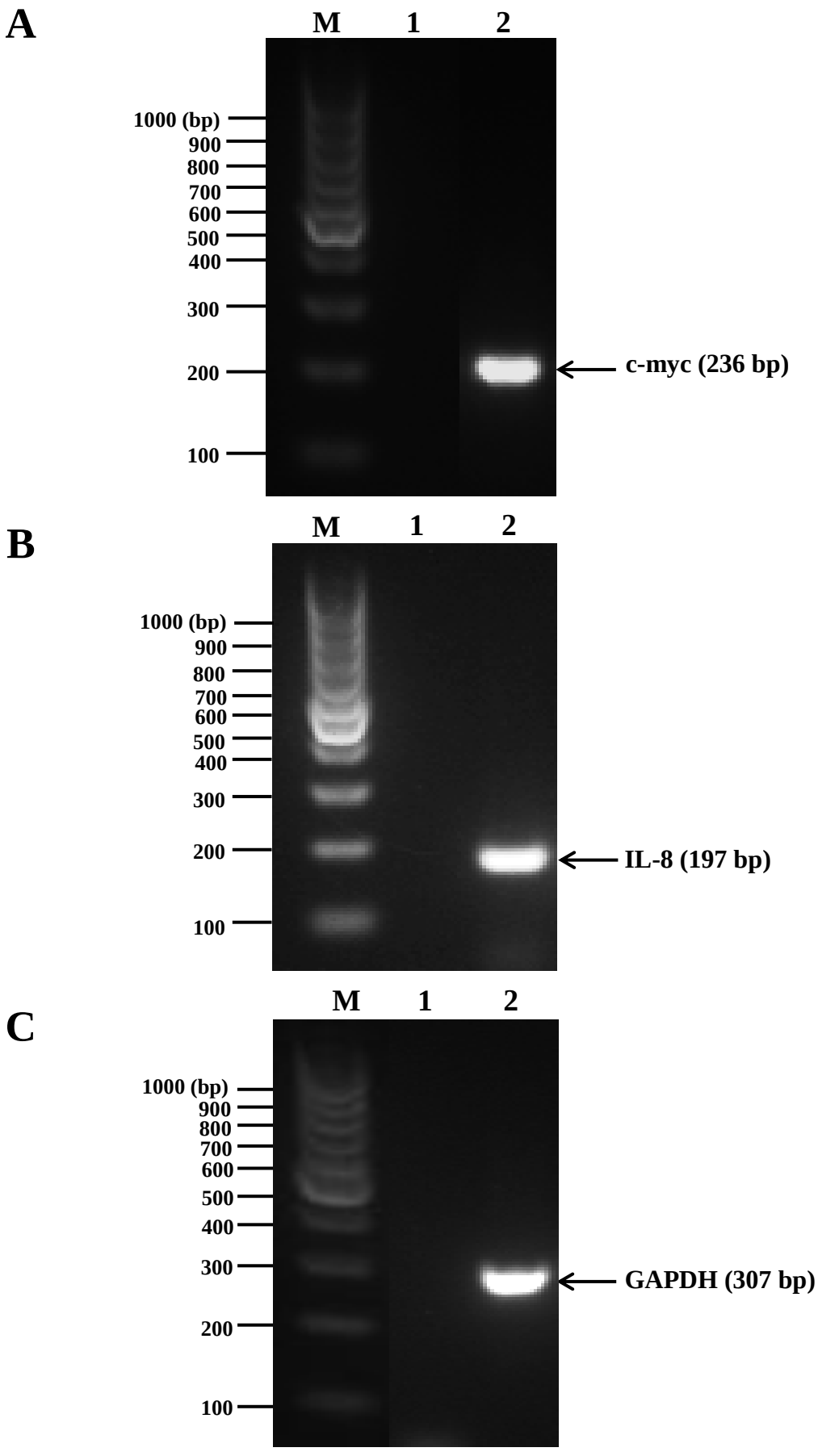

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Figure B4. Representative agarose gels indicating the realtime qPCR end products of (A) c-myc, (B) IL-8 and (C) GAPDH. The products were subjected to electrophoresis on a 2% agarose gel and visualised using the Nancy-520 (Sigma-Aldrich, South Africa) nucleic acid stain. M: GeneRuler 100 bp DNA Ladder (Thermo Scientific, USA); Lane 1: no template control; Lane 2: sample treated with 1 nM R5020.

Determining the relative expression levels of the genes of interest

Having shown that a single amplicon of the correct size was amplified for all three genes, the relative expression levels (R) of the target genes, c-myc and IL-8, were determined by using the mathematical model for relative quantification described by Pfaffl (2001) in equation 2, and expressed as a relative ratio of target gene expression relative to the expression levels of the reference gene.

$$R = \frac{(E_{\text{target gene}})^{\Delta Cq_{\text{target}}\,(\text{control - sample})}}{(E_{\text{reference gene}})^{\Delta Cq_{\text{reference}}\,(\text{control - sample})}} \qquad (2)$$

The R value is dependent on the primer efficiency (as calculated using equation 1) and the change in quantification cycle (ΔC_q). The ΔC_q can be determined by subtracting the C_q value of the sample treated with the test compound from the C_q value of the EtOH-treated (vehicle control) sample. A R value of one indicates no difference between the treated and untreated samples, while a R value of more than one indicates upregulation of the target gene and less than one indicate inhibition of the target gene (Pfaffl 2001).