“The anti-tumourigenic action of the progesterone receptor antagonist Lonaprisan (ZK230211) in breast cancer”

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“...fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza”.

(Dante, Inferno XXVI)

Abstract

Mammary gland development starts during embryogenesis and continues at puberty in response to circulating estrogen and growth hormones. Full development however only takes place during pregnancy in preparation for lactation, due to increased estrogen and progesterone levels. A role of progesterone in breast cancer has furthermore been debated for many years. Although it has been shown that some estrogen/progestagen combinations used in hormone replacement therapy may be linked to increased breast cancer risk, it is also true that the progestins used in these therapies have different mixed agonist/antagonist profiles and are not necessarily entirely selective for the PR (progesterone receptor). In addition, progesterone has a biphasic effect on cell proliferation and is difficult to study in isolation from other hormones that also contribute to breast cancer biology. Nevertheless, the presence of PR in about one third of breast cancer cases and the observed link between PR isoform dysbalance and tumor aggressiveness suggest that potent and selective PR antagonists may help in preventing tumor progression. Several antiprogestins have already been described but only few have reached the clinic for various indications including contraception, endometriosis, and uterine leiomyoma. In this thesis the effects of the new antiprogestin Lonaprisan on the T47D breast cancer cell line were studied. Strong inhibition of cell proliferation and arrest in the G0/G1 phase were observed, as well as induction of a senescence-like phenotype. This was accompanied by p21 induction through direct binding of Lonaprisan-bound PR to the promoter. In addition, the stimulation in p21 expression by Lonaprisan necessitated the presence of Sp1, and the reduction of p21 levels blunted the antiproliferative effects of Lonaprisan. Mutation analysis showed that intact PR DNA-binding properties were needed for p21 induction. Phosphorylation of PR Ser345 was stimulated by Lonaprisan, but this post-translational modification was not required for p21 promoter activation, nor was the interaction with c-Src needed. It has also been shown that PR expression is regulated by p21, leading to the hypothesis of the existence of a positive feedback mechanism between PR and p21 after Lonaprisan treatment. Altogether these results, which deepen our understanding on the relationship between PR and the cell cycle regulatory protein p21, may offer new insights for the development of novel therapy options in the treatment of hormone-dependent breast cancer and for the prevention of recurrence.

Zusammenfassung

Die Entwicklung der Brustdrüse beginnt in der Embryogenese und setzt sich während der Pubertät als Antwort auf die Stimulierung durch Estrogene und Wachstumshormone fort. Eine vollständige Ausbildung findet allerdings nur in der Schwangerschaft in Vorbereitung auf die Laktationsperiode durch Estrogen- und Progesteronstimulation statt. Eine Rolle des Progesterons im Brustkrebs wird seit Jahren diskutiert und es wurde gezeigt, dass einige Estrogen/Progestagen Kombinationen, die in der hormonellen Therapie eingesetzt werden mit einem erhöhten Brustkrebsrisiko in Zusammenhang stehen. Allerdings wurden hier unterschiedliche Progestine mit unterschiedlich gemischten agonistischen/antagonistischen Profilen verwendet, die nicht unbedingt für den Progesteron-Rezeptor (PR) selektiv waren. Ausserdem hat Progesteron einen zweiphasigen Einfluss auf die Zellproliferation und es ist schwierig die Rolle von Progesteron getrennt von der anderer Hormone, die auch für Brustkrebs verantwortlich sein könnten, zu untersuchen. Dennoch deutet die Anwesenheit des PR in etwa einem Drittel der Brustkrebsfälle und der beobachtete Zusammenhang zwischen einem Ungleichgewicht der PR-Isoformen und Tumoraggressivität daraufhin, dass potente und selektive PR-Antagonisten in der Brustkrebsbehandlung helfen könnten. Verschiedene Antiprogestine wurden beschrieben aber nur einige werden in der Klinik für verschiedene Indikationen, wie Kontrazeption, Endometriumkarzinom und Uterine leiomyoma verwendet. In dieser Doktorarbeit wurden die Effekte des neuen Antiprogestins Lonaprisan auf die T47D Brustkrebs Zelllinie untersucht. Eine starke Proliferationshemmung und ein spezifischer Arrest in der G0/G1 Phase des Zellzyklus, sowie die Induktion des Seneszenz-Phänotyps wurden festgestellt. Dieser Arrest war abhängig von der Induktion des p21-Gens durch direkte Bindung des Lonaprisan-gebundenen PR auf dem Promotor. Darüber hinaus wurde beobachtet, dass für die Stimulation der p21-Expression der Transkriptionsfaktor Sp1 notwendig war und dass reduzierte p21-Mengen die Proliferationsinhibition von Lonaprisan vermindern. Eine Mutationsanalyse zeigte, dass eine intakte PR DNA-Bindung für die Induktion von p21 nötig war. Die Phosphorylierung des PRs an der Ser345-Stelle wurde von Lonaprisan stimuliert. Diese post-transkriptionelle Modifikation wurde aber nicht für die Aktivierung des p21-Promotors benötigt, noch war die Interaktion mit c-Src erforderlich. Es konnte auch gezeigt werden, dass die PR-Expression durch p21

reguliert wird, was für einen positiven Feedback-Mechanismus zwischen PR und p21 nach Lonaprisan Behandlung spricht. Zusammengefasst konnte in dieser Arbeit der Zusammenhang zwischen PR und dem Zellzyklus-regulatorischen Proteins p21 charakterisiert werden. Außerdem geben die Ergebnisse Hinweise zur Entwicklung neuer Therapien für die Behandlung des hormonabhängigen Mammakarzinom und für die Prävention der Rekurrenz.

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Abbreviations

ADH	Atypical ductal hyperplasia
AF-1	N-terminal domain
AF-2	C-terminal LBD
AFs	Transcription activation domains
AIs	Aromatase inhibitors
AR	Androgen receptor
BRCA1 and 2	Breast carcinoma susceptibility gene 1 and 2
BUS	B-upstream segment
cAMP	cyclic AMP or 3'-5'-cyclic adenosine monophosphate
CD	Common docking domain
ChIP	Chromatin immunoprecipitation
CPA	Cyproterone acetate
CSC	Cancer stem cell
Ct	Cycle threshold
Cx43	Connexin 43
DBD	DNA-binding domain
DCIS	Ductal carcinoma in situ
DI	Dimerisation
Dox	Doxorubicin
dsRNAs	Double-stranded RNAs
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERBB2+	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ERID-I and II	ER-interacting domains I and II
Eth-1	Ethidium homodimer
FKBP54	54-kDa PR-associated immunophilin
FXR	Bile acid receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GJIC	Gap junctions
GR	Glucorticoid receptor
HREs	Hormone response elements
HSD11β2	Hydroxysteroid 11- β dehydrogenase 2
Hsps	Heat shock proteins
LBD	Ligand-binding domain
LT	Long term
LXR	Oxysterol receptor
MAPK	Mitogen-activated protein kinase
MET	Metastasis
MR	Mineralocorticoid receptor
NCoR	Nuclear receptor corepressor
NLS	Nuclear localisation sequence
NRs	Nuclear hormone receptors
p21, p27	cyclin-dependent kinase inhibitor 1A and 1B
PG	Progesterone
PKA	Protein kinase A
PPARalpha, delta, and gamma	Peroxisome proliferator activated receptors
PR	Progesterone receptor
PREs	Progesterone response elements
RAR	Retinoic acid receptor
RISCs	RNA-induced silencing complexes
RNAi	RNA interference
RTK	Receptor tyrosine kinase
RT-PCR	Real time – polymerase chain reaction
RU486	Mifepristone
SC	Stem cells
SERD	Selective estrogen receptor destabiliser
SERM	Selective estrogen receptor modulator
SH3	Src-homology 3
SHR	Steroid hormone receptor
siRNAs	Small interfering RNAs
SPRMs	Selective PR modulator

SRCs	Steroid receptor coactivators
STASIS	Stress or aberrant signalling-induced senescence
STAT5A	Signal transducer and activator of transcription 5A
TDLU	Terminal ductal lobular unit
TGF-β	Tumour growth factor β
TR	Thyroid receptor
VDR	Vitamin D receptor
VIL2	Villin 2, also called ezrin

1 Introduction

1.1 Breast cancer

According to the American Cancer Society estimation, up to 207,090 new cases of breast carcinoma in women were reported in the U.S. in 2010 and about 40,000 women died of this devastating disease (American Cancer Society. Cancer Facts & Figures 2010. Atlanta: American Cancer Society; 2010). In Europe likewise, recent statistics on cancer occurrence and outcome have revealed breast cancer to be the third most common cause of cancer-related deaths in Europe (Ferlay et al., 2010). It is thereby clear that breast cancer is an important health issue for women in the Western hemisphere. It is therefore necessary to increase our understanding of breast cancer biology in order to help in the identification of novel therapies and in the improvement of the current therapies for the treatment and prevention of this disease.

1.1.1 The biology of breast cancer

Breast cancer should not be considered as a single disease. It represents a spectrum of diseases with diverse histopathologies, genetic variations and clinical outcomes (Vargo-Gogola and Rosen, 2007). Unlike other kinds of cancer which are typically considered as diseases of aging, breast cancer shows incidence rates which increase in young women in their late 20s (Parkin et al., 1997). This may be due to the responsiveness of breast tissues to ovarian hormones, which are made in increasing concentrations from the onset of puberty. Normally, breast tissue is characterised by lobules and ducts which consist of a bi-layered epithelium of luminal and myoepithelial cells. When a pre-malignant lesion occurs, abnormal cell layers become visible within the duct or lobule, constituting the so-called atypical ductal hyperplasia (ADH). This is the precursor of a non-invasive lesion containing abnormal cells, the ductal carcinoma *in situ* (DCIS). Once the cells have begun to invade, a malignant or invasive breast cancer has arisen with the risk of developing metastases (**Fig .1**).

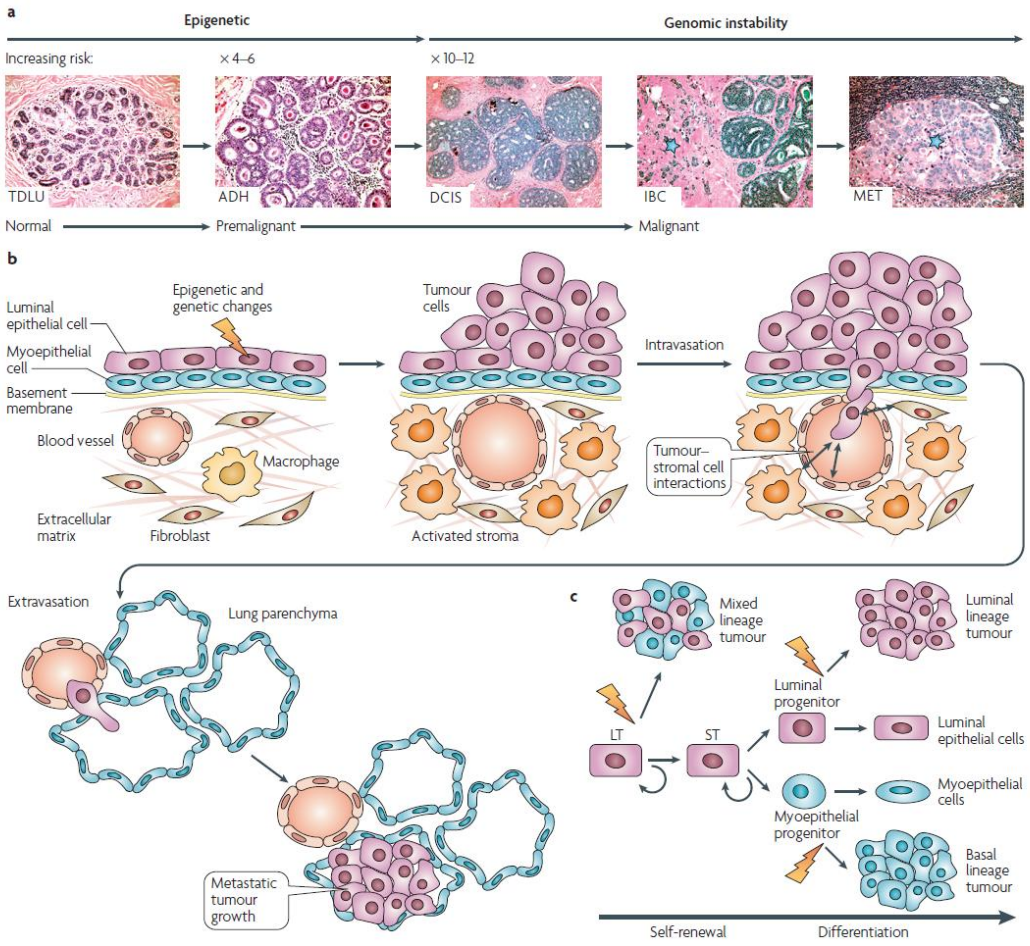


Figure 1. The biology of breast cancer.

A. The development of breast cancer from the normal breast terminal ductal lobular unit (TDLU) to the formation of metastasis (MET) is shown. **B.** Schematic of breast cancer progression. **C.** Cancer cells with stem cell-like characteristics, including the long term (LT), short term (ST) and luminal or basal progenitors, drive breast cancer initiation (Vargo-Gogola and Rosen, 2007).

The multistage process leading to the transformation of breast epithelial cells into metastatic breast cancer is the result of a deregulation of proliferation control, survival, differentiation and migration beside aberrant tumour-stromal cell interactions. According to the clonal evolution theory, a term first coined by Peter Nowell (1976), cells with genetic and epigenetic alterations of tumour suppressor genes and other oncogenes become able to invade through the basement membrane, intravasate, survive in absence of adhesion, extravasate and establish a new tumour in a distal location. Recently, a new theory for the emergence and maintenance of different cancer forms has been proposed, the cancer stem cell (CSC) theory. Stem cells (SC) are present both in the normal and in the malignant breast, are usually in the resting state, committed to

differentiation and are very long-lived (Russo and Russo, 2004; Villadsen et al., 2007). During their lifetime, the SCs can be exposed to radiation and chemical mutagens for a prolonged period of time. They are also very resistant to most current therapies which are aimed at rapidly dividing cells (Fillmore and Kuperwasser, 2008; O´Brien et al., 2009; Tanei et al., 2009). The transformation of the breast may result from a multiplicity of factors including peptide growth factors, oncogenes, loss of tumour suppressor gene activity and steroid hormones and their receptors (Clarke et al., 1992; Elledge and Fuqua, 2000; Clark, 2000). An increasing number of gene expression profiling studies has shown that there are at least five subtypes of invasive breast cancer forms named luminal subtype A, luminal subtype B, ERBB2+ (V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog, avian), basal subtype and normal breast-like, which constitute approximately 80% of all breast cancers (**Fig. 2**) (Perou et al., 2000; Sorlie et al., 2001). In the next session a selection of well-established risk factors for breast cancer will be reviewed.

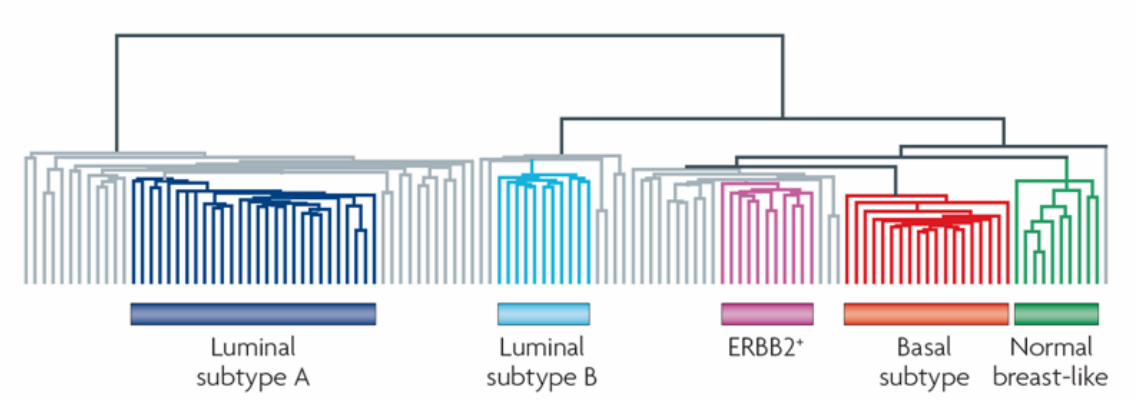


Figure 2. Breast cancer subtypes.
A large number of gene expression profiles has allowed the identification of at least five subtypes of invasive ductal carcinoma, constituting approximately 80% of all breast cancers (Vargo-Gogola and Rosen, 2007).

1.1.2 Factors involved in breast carcinogenesis

1.1.2.1 Peptide growth factors

In the normal mammary development as well as in tumours, peptide growth factors and their receptors such as the HER/erbB, tumour growth factor β (TGF-β), and insulin-like

growth factor families, have been shown to play a crucial role. HER-1 and HER-2 are members of the subclass I of the receptor tyrosine kinase (RTK) superfamily and found overexpressed in about 25% of invasive breast tumours, usually as a result of gene amplification (Slamon et al., 1987). HER-2 overexpression has been associated with a poor prognosis, even if no correlation has been reported with tumour size, degree of differentiation or metastatic potential. This led to the conclusion that the HER family members may contribute to the overall outcome, but are not involved in the pathway causing the transformation (Slamon et al., 1989; Thor et al., 2000; Emi et al., 2002).

1.1.2.2 Tumour suppressor genes and breast carcinoma susceptibility genes

Several tumour suppressor genes including p53 are thought to play a key role in the transformation of the breast. Indeed, somatic cell mutation in the p53 nuclear phosphoprotein is observed in 20-30% of primary breast carcinoma cases (Kern et al., 1991; Sullivan et al., 2002) and these tumours tend to be highly invasive, poorly differentiated and of high grade (Bosari et al., 1993). Interestingly, a physical association between p53 and the estrogen receptor (ER) has been observed. ER protects p53 from MDM2-mediated degradation, resulting in p53 up-regulation and mediation of the G1 arrest (Fabian et al 2000; Liu et al., 2000; Hurd et al., 1997; Zheng et al., 2001). When ER is overexpressed however, an overexpression of MDM2 together with a reduced p53 transcriptional activity, which leads to an increased cellular proliferation, is observed (Kato et al., 2002; Hori et al., 2002). Moreover, mutations in two other genes, the breast carcinoma susceptibility genes BRCA1 and BRCA2, have been associated with the development of hereditary breast carcinoma. It has been shown that women with a mutation in one of these genes have a 60-80 % risk of developing breast cancer (Lee and Boyer 2001).

1.1.2.3 Steroid hormone receptors

Steroid hormones are responsible for the regulation of development, reproduction, metabolism, and response to environmental factors (Mangeldsdorf et al., 1995). The signalling pathways activated by steroid hormones converge at a small family of cellular and nuclear receptors, the nuclear hormone receptors (NRs). NRs are ligand-dependent or -independent transcription factors and are highly evolutionarily conserved from invertebrates to higher organisms (Keay and Thornton, 2009). Within the NR

superfamily the steroid receptors constitute a subfamily that responds to hormones based partially on steroid scaffolds. These include the ER, progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), thyroid receptor (TR), vitamin D receptor (VDR), and mineralocorticoid receptor (MR) (**Fig. 3A**). Other NRs, such as the peroxisome proliferator activated receptors (PPARalpha, delta, and gamma), the bile acid receptor (FXR), and the oxysterol receptor (LXR), respond to well- and less-well-characterised natural products, often representing endogenous metabolites derived from nutrients, xenobiotics, and lipids (Moore et al., 2006). Indeed, NRs altogether appear to represent important "lipid sensors" (Besinger and Tontonoz, 2008). The members of the steroid hormone receptor (SHR) family share a similar, modular architecture, consisting of a number of independent functional domains (**Fig. 3B**).

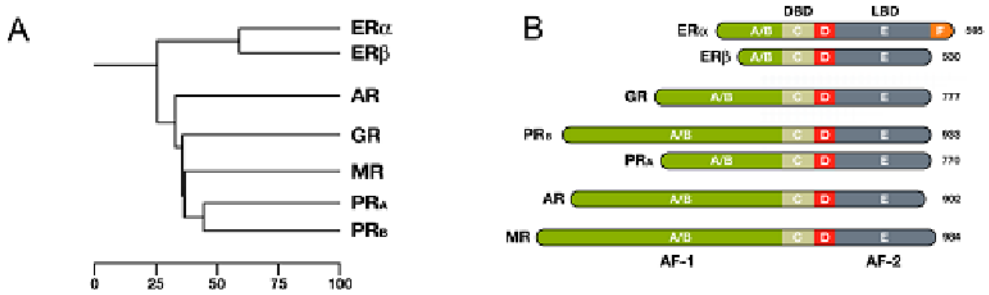


Figure 3. Overview of the steroid hormone receptor family.

A. Phylogenetic tree of the SHR family showing the evolutionary interrelationships and distance between the various receptors. **B.** Structural overview of the SHR family. They are composed of a variable N-terminal domain (A/B) containing the AF-1 transactivation region, a highly conserved DNA-binding domain (DBD), a flexible hinge region (D), and a C-terminal ligand-binding domain (LBD, E) containing the AF-2 transactivation region. The ERα also contains an additional C-terminal F domain. Numbers refer to the length of the receptor in amino acids (Griekspoor et al., 2007).

Most conserved is the centrally located DNA-binding domain (DBD) containing the characteristic zinc-finger motifs. The DBD is followed by a flexible hinge region and a moderately conserved ligand-binding domain (LBD), located at the carboxy-terminal end of the receptor. The ERα is unique in that it contains an additional F domain of which the exact function is unclear (Griekspoor et al., 2007). Upon entering the cell by passive diffusion, the hormone (H) binds the receptor, which is subsequently released from heat shock and immunophilin proteins, and translocates into the nucleus. There, the receptor dimerizes, binds to specific sequences in the DNA, called hormone response elements (HREs), to affect the expression of downstream genes. (**Fig. 4**).

There is considerable variation on this theme, with some receptors bound to the HRE even in the absence of hormone, sometimes functioning to suppress gene expression (Kininis and Kraus, 2008). In the nucleus, coregulators catalyze or mediate chromatin remodeling, epigenetic modifications, receptor recycling, and ultimately gene expression (Lonard et al., 2007).

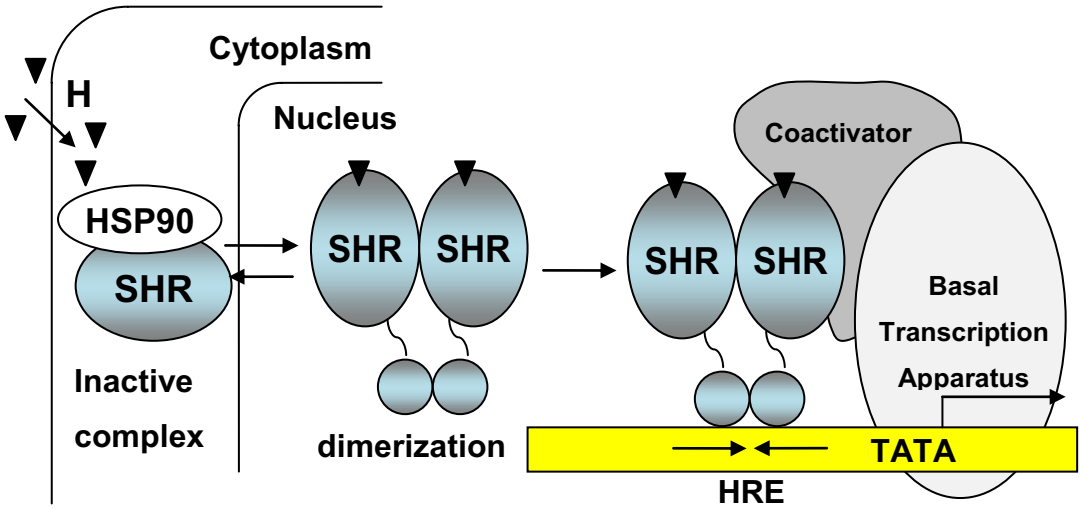


Figure 4. Steroid hormone receptor signalling.
Upon hormone (H) binding, the receptor dissociates from the chaperone proteins (e.g. HSP90), translocates into the nucleus, dimerises, binds to specific semi-palindromic sequences in the DNA called Hormone Responsive Elements (HREs), thereby regulating the expression of downstream genes (Adapted from Leonhardt et al., 2003).

The ER and PR are transcription factors normally required for breast development. The first factor stimulates ductal elongation while the second one induces ductal side-branching and alveologenesis with the help of the epidermal growth factor (EGF) (Hovey et a. 2002). PR and ER positive cells normally represent only about 7-10% of the luminal epithelial cell population, and are usually in a non-proliferative status, although they can induce the proliferation of nearby or adjacent cells by expressing and secreting pro-proliferative molecules such as wnts, IGF-II (Lange 2008; Robinson et al., 2000). In contrast to what is observed in the normal breast, nearly 70% of breast cancers express ER and PR at the time of diagnosis (Lange and Yee 2008). It is therefore believed that the steroid receptor positive cells undergo an early switch to autocrine stimulation and continue to divide. Thus, it is likely that signalling pathways involved in normal mammary gland growth and development are reactivated during breast

carcinogenesis (Lange et al., 2008); (**Fig. 5**). The correlation between early menarche, late menopause, nulliparity and risk of developing breast cancer suggests that prolonged exposure to estrogen and progesterone may contribute to the carcinogenesis. Indeed, already in the 1896, Thomas Beatson described that removal of endogenous estrogen via oophorectomy in advanced breast cancer patients often resulted in remarkable improvement (Beatson, 1896). High bone density and obesity in postmenopausal women are two further parameters associated with elevated breast cancer risk. High bone density is linked to elevated exposure to estrogens, whereas fat tissue may increase the level of circulating estrogen metabolites such as estrone (Cauley et al., 1989).

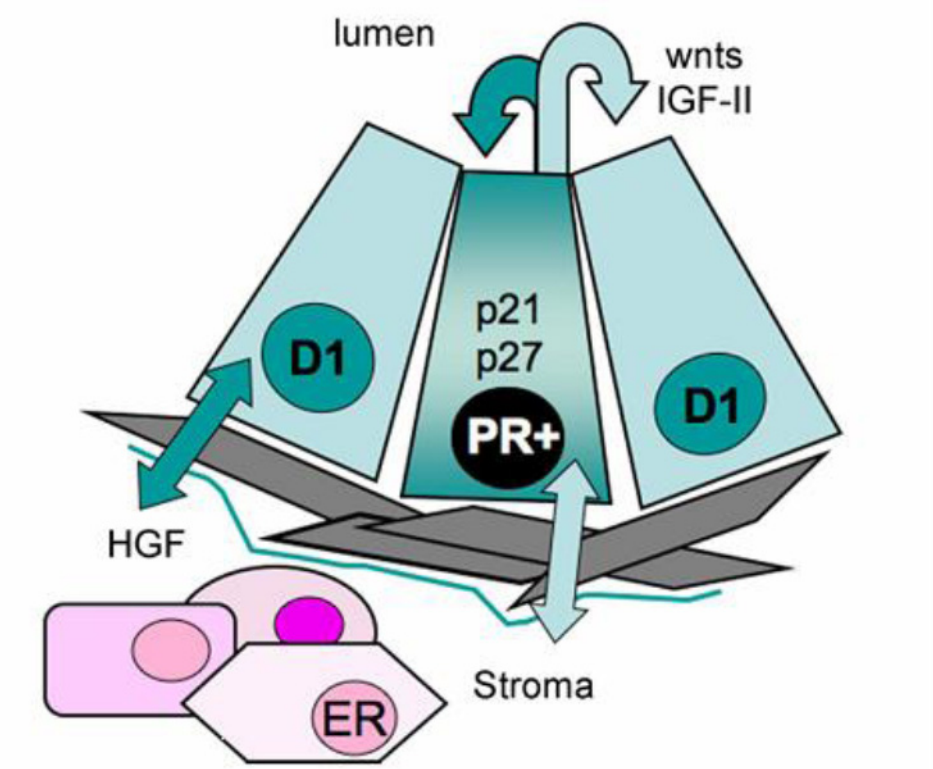


Figure 5. Mammary gland structure.
In the acinus, the functional unit of the mammary gland, steroid receptor positive (ER+/PR+) cells are found adjacent to proliferating cells. The proliferation of the ER/PR-negative cells is mediated by paracrine signalling between the epithelial and the stromal compartments. In breast cancer a switch from paracrine to autocrine mechanism of proliferation in ER+/PR+ cells is observed (Lange, 2008).

The steroid hormone positive tumours usually grow more slowly, are better differentiated and are characterised by a better prognosis (Maruyama et al., 2001; Paech et al., 1997; Barkhem et al., 1998). Indeed, a 10% survival advantage has been shown for patients with receptor-positive disease (Grann et al, 2005). In addition, another

steroid receptor seems to play a crucial role in the transformation of the breast, the retinoic-acid receptor (RAR). RARs are ligand-activated transcription factors which regulate the expression of target genes through binding to the retinoic acid response elements present in the promoter of target genes. In the normal mammary epithelial tissue, RARs are highly expressed and inhibit cell proliferation while inducing differentiation and apoptosis (Widschwendter et al., 2001). On the contrary, down-regulation of RARs, as a result of either allelic deletion (Deng et al., 1996; Yang et al., 2001) or epigenetic regulation (Widschwendter et al., 2001; Yang et al., 2001; Liu et al., 1996; Arapshian et al., 2000; Bovenzi et al., 2001; Sirchia et al., 2000), is found in malignant tumours, leading to uncontrolled cell proliferation. A more detailed description of the role of the PR in breast carcinogenesis will be given in the next paragraph.

1.2 Progesterone receptor

Demonstrating a clear role of progesterone in breast cancer is a challenge, due to its complex role in normal breast physiology. I will now focus on the role of progesterone and PR in the pathophysiology of the breast cancer describing its structure and functions.

1.2.1 PR structure

The PR isoforms are member of the steroid hormone receptor family of ligand-activated transcription factors. Upon binding with the ovarian steroid ligand progesterone, PRs become activated and bind to specific sites in the DNA, thus regulating the expression of target genes. Three PR isoforms are generated by a single gene located on chromosome 11 at q22-23 which is transcribed using three independent translational start sites (**Fig. 6**). A “distal” and a “proximal” region have been described in the PR promoter (Kraus et al., 1993). PR-B is the longest protein isoform (116 kDa), whereas the PR-A form is truncated at the N-terminus (94kDa) and the PR-C isoform is even shorter (60kDa). While PR-C seems to be mainly uterine-specific (Condon et al., 2006), PR-A and PR-B are coexpressed in the breast, and in several other progesterone target organs. Normal development of the mammary gland requires the PR-B isoform (Mulac-Jericevic et al., 2003), while the PR-A isoform is necessary for uterine development and the reproductive function (Mulac-Jericevic et al., 2000). Despite similar hormone and

DNA-binding activities, the two isoforms differ with regard to their transcriptional activities (Richer et al., 2002; Giangrande and McDonnell, 1999). PR-B is considered a stronger activator than PR-A, possibly because of its third transactivation domain (AF-3) present in the N-terminal region (Sartorius et al., 1994). The short PR-C isoform does not show any transcriptional activity, but it has been reported that once expressed, PR-C can enhance PR activity in breast cancer cells (Wei et al., 1997) or function as dominant inhibitor of PR-B in the uterus (Condon et al 2006). The region of PR-B upstream of the PR-A start site has been named the B-upstream segment (BUS), since this region is specific for PR-B. Both PR-B and PR-A isoforms contain the characteristic regions found in other nuclear receptors: a C-terminal ligand-binding domain (LBD); a hinge region (H); a centrally located DNA-binding domain (DBD); and two activating function domains.

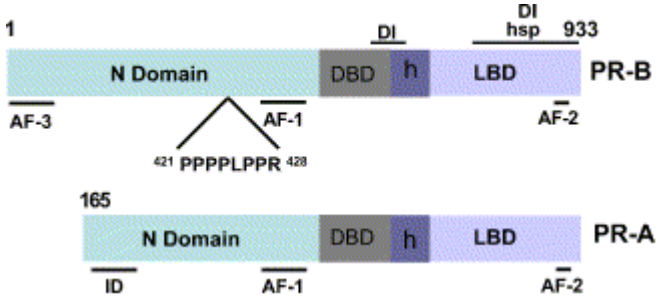


Figure 6. Schematic representation of the PR

PR functional domains N-Domain, DNA-binding domain (DBD), hinge region (H), ligand-binding domain are shown together with the transcription activation domains (AF-1, AF-2 and AF-3) (Leonhardt et al., 2003).

The N-domain has an important function, since it is responsible for full transcriptional activity and for several cell- and target gene-specific responses. Moreover, other determinants have been identified within the PR domains. The ligand-binding domain, for example, is not only necessary for binding to steroid hormones, but it also contains determinants for dimerisation (DI) in the absence of DNA, for binding of heat shock proteins (hsps) and for nuclear localisation. A second nuclear localisation sequence (NLS) and a dimerisation domain, independent from DNA binding, are contained in the DBD. Moreover, steroids receptor are characterised by two transcription activation domains (AFs), one in the N-terminal domain (AF-1) and one in the C-terminal LBD (AF-2) (**Fig. 4**). Through these domains, which function as specific binding sites for coactivators, the DNA-bound receptor can activate the transcription of target genes. The

ligand-dependent AF-2 is activated following the conformational change induced by the steroid hormone. This creates a hydrophobic binding pocket for the p160 family of steroid receptor coactivators (SRCs). The coactivators binding to and mediating the activity of the AF-1 domain are far less well understood.

1.2.2 Classical action of PR

In the absence of their cognate ligand, PRs are complexed with several chaperone molecules including heat-shock protein (hsp) such as hsp90, hsp 70, hsp 40, Hop and p23. These interactions are necessary for proper protein folding and assembly of stable PR-hsp90 heterocomplexes that are able to bind ligand (Pratt and Toft, 2003). Another function of hsps is to connect PRs to protein trafficking systems. Upon binding to progesterone, the receptors undergo a conformational change, dimerisation and hsp dissociation. Activated receptors translocate into the nucleus and associate with numerous coregulatory molecules, including histone acetyltransferases (CBP/p300, p160/SRC family steroid receptor coactivators 1–3), chromatin-remodeling machines (SWI/SNF), and TRAP/DRIP complexes that recruit RNA polymerase II. The complexes of receptor and co-activator bind directly to specific progesterone response elements (PREs) and PRE-like sequences in the regulatory regions of target genes, such as c-myc (Moore et al, 1997), fatty acid synthetase (Chalbos et al., 1987) and MMTV (Krusekopf et al., 1991) (**Fig. 7A**). On the contrary, in presence of a PR antagonist, co-repressors are preferentially recruited which will result in down-regulation of transcription (Chabbert-Buffet et al., 2005). Progestin treatment can also result in an upregulation of genes devoid of classical PREs in their proximal promoter regions. This is the case for the epidermal growth factor receptor (Lange et al., 1998), c-fos (Richer et al., 1998), p21 (Owen et al., 1998), IRS-2 (Cui et al., 2003) and cyclin D1 (Groshong et al., 1997). Since these genes do not possess canonical PREs, their regulation by PR can occur through PR tethering to other DNA-binding transcription factors, such as specificity protein 1 (Owen et al., 1998), activating protein 1 (Tseng et al., 2003) or signal transducers and activators of transcription (Stats) (Proietti et al., 2005; Richer et al., 1998) (**Fig. 7D**).

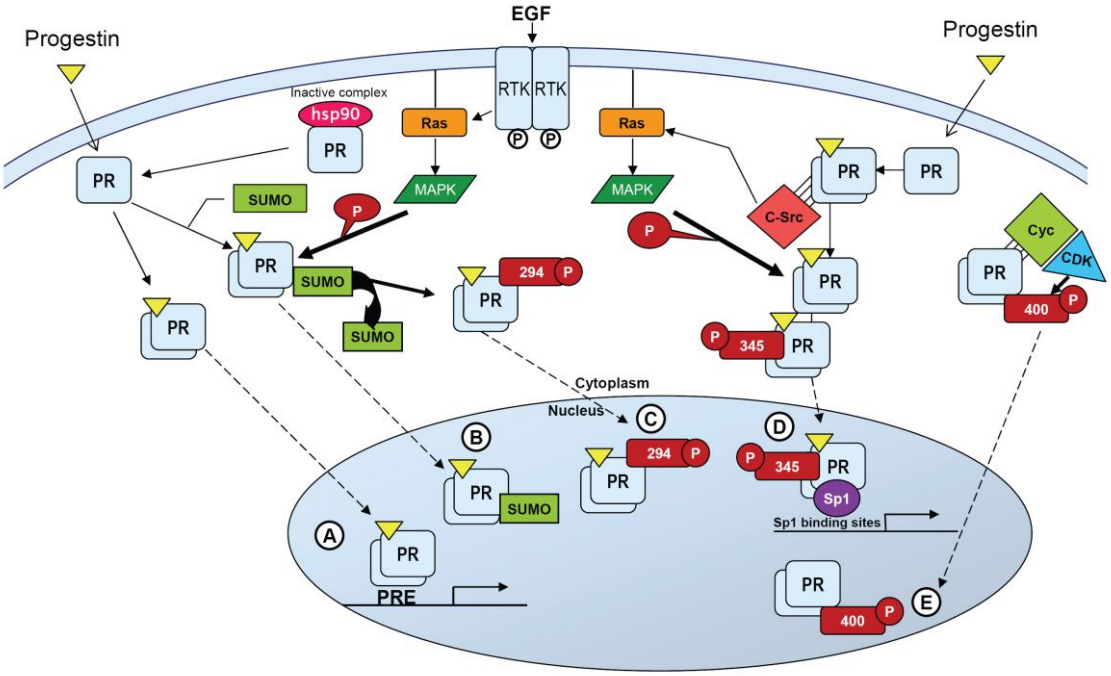


Figure 7. PR signalling pathway.

A. Classical genomic action of the PR. **B.** Sumoylation of PR on Lys388. **C.** In response to growth factors (e.g. EGF) MAPK signalling is activated and results in PR phosphorylation at Ser294 and desumoylation at Lys388. **D.** Progestin-induced c-Src activation results in MAPK signalling and phosphorylation at Ser345, binding to Sp1 and activation of Sp1-dependent genes. **E.** PR interactions with CDK2 result in increased liganded or unliganded PR activity on unknown PR target gene promoters (adapted from Dressing et al., 2009).

1.2.3 Membrane-associated rapid signalling

While the genomic actions of steroid hormone receptors take several minutes to hours to take place, which is the time necessary for transcription and translation of target genes, rapidly occurring (within a few minutes) extranuclear or nongenomic effects of cell membrane-localised steroid hormone receptors have recently been described. It has been observed for example that after progestin treatment of breast cancer cells a rapid and transient (2–10 min) activation of cytoplasmic protein kinases, including mitogen-activated protein kinase (MAPK), PI3K and p60-Src kinase takes place (Boonyaratanakornkit et al., 2001; Migliaccio et al., 1998) (**Fig. 7D**). Similar findings have been reported for membrane-associated ER and AR (Shupnik, 2004). PRs contain multiple distinct domains (proline-rich, ERID-I and –II, and CD domain) that facilitate

interactions with signalling molecules placed in or in close proximity to the plasma membrane and growth factor receptors and their effectors. A proline-rich (PXXP) motif in the PR is responsible for the direct ligand-dependent binding of PR to the Src-homology 3 (SH3) domains of signalling molecules of the p60-Src kinase family (Boonyaratanakornkit et al., 2001). It has been demonstrated in vitro that upon progestin treatment purified PR-A and PR-B activate the c-Src-related protein kinase, Hck. In vivo however only PR-B but not PR-A activates c-Src and MAPKs. In line with this, PR-B mutated in the proline-rich motif is not able to interact with c-Src anymore and the activation of c-Src (or Hck) and p42/p44 MAPKs by progestin is prevented. On the contrary, mutation of the PR-B DBD inhibits PR transcriptional activity without influencing progestin-induced c-Src or MAPK activation. Likely, the nongenomic MAPK activation by progestin/PR-B/c-Src complexes is a c-Src-dependent mechanism which involves Ras activation of the MAPK pathway. According to a second model of PR-induced activation of c-Src/MAPK, the presence of ER is required (Migliaccio et al., 1998). A further study has shown that two regions within the PR N-terminus directly interact with ER, namely ERID-I and –II (ER-interacting domains I and II; Ballare et al., 2003). Another domain responsible for PR binding to MEK1 is the common docking (CD) domain identified in the N-terminal BUS region. The functional significance of the PR/MEK1 association has not been clarified yet. It probably allows MEK1 to position itself in close proximity to key components of the MAPK-signalling pathways. The importance of the rapid PR actions as important contributors to the regulation of cell proliferation after hormone treatment has been evidenced by several groups (Migliaccio et al., 2005; Skildum et al., 2005). Although the role of steroid hormone receptor-mediated activation of cytoplasmic signalling molecules has not been entirely clarified in human physiology yet, it is believed that it may potentiate the nuclear activity of these receptors.

1.2.4 PR post-translational modifications

Post-translational modifications have been described for all steroid receptors, including the PR (Faus and Haendler, 2006). Several of these modifications have been linked to rapid, non-genomic effects. Up to 14 residues in the PR-B are known to be phosphorylated in vitro and in vivo (Lange, 2004). Eight out of these 14 residues are cyclin/CDK2 targets (Moore et al., 2007). One CDK2 phosphorylation site in the PR is

Ser400 but the functional significance of PR/cyclin/CDK interaction is not fully understood (**Fig. 7E**). Besides phosphorylation, sumoylation has been best studied (Daniel and Lange, 2009). Sumoylation at Lys388 leading to repression of PR transcriptional activity has been evidenced (Daniel et al., 2007) (**Fig. 7B**). This is prevented by phosphorylation of Ser294 which contributes to the regulation of hormone responsiveness. Activation of the MAPK (ERK1/2) signalling pathway by growth factor receptor tyrosine kinases (e.g. via EGF; Qiu et al., 2003) or progestin-dependent PR/c-Src rapid signalling results in PR Ser294 phosphorylation (Shen et al., 2001, Skildum et al., 2005) (**Fig. 7C**). Ser345 is also phosphorylated by MAPK, following activation of the pathway by c-Src, which is directly triggered by liganded PR (Boonyaratanakornkit et al., 2001); (Faivre et al., 2008). Ser345 phosphorylation leads to binding of the PR to the Sp1 transcription factor and activation of Sp1-controlled target genes (**Fig. 7D**). These post-translational modifications play decisive roles in the fine expression control of specific gene subsets (Daniel and Lange, 2009).

1.2.5 Progesterone receptor and breast cancer

Initial development of the mammary gland takes place during embryogenesis. Further important modifications occur at puberty but full development only takes place during pregnancy, in preparation for lactation (Conneely et al., 2007). These changes require progesterone, which has essential roles in the proliferation and differentiation of the mammary epithelium (Lange et al., 2008). This was confirmed in PR knockout mice which are characterised by delayed lobuloalveolar development (Mulac-Jericevic et al., 2003), while PR-B transgenic animals exhibited hyperplasia (Shyamala et al. 2000). In addition, in the postpubertal nulliparous animal, progesterone stimulates ductal side branching and is required for the massive epithelial cell expansion and alveologenesis that occurs during early pregnancy (Fernandez-Valdivia et al., 2005; Lydon et al., 1995; Fernandez-Valdivia et al., 2008). A role of progesterone in mammary cancer has been debated for many years and is still controversial (Lange and Yee, 2008). Since the publication of the WHI and Million Women reports which show that some estrogen/progestagen combinations used in hormone replacement therapy are linked to increased breast cancer risk (Rossouw et al., 2002);(Beral, 2003), many studies have been performed to clarify the role of the PR in mammary cell carcinoma (Lange and Yee, 2008); (Gadducci et al., 2009). One reason for the controversy is that progestins

used in hormone replacement therapy have different mixed agonist/antagonist profiles and are not necessarily entirely selective for the PR (Spitz, 2006). Also, progesterone has a biphasic effect on cell proliferation and is difficult to study in isolation from other hormones that also contribute to breast cancer biology. One example is estrogen which directly stimulates PR expression upon binding to its cognate receptor, another one is prolactin, which is essential for epithelial mammary cell proliferation during lactation (Lange, 2008). Studies with a mouse model deficient in PR show a lower incidence of carcinogen-induced mammary tumour (Lydon et al., 1999); (Ismail et al., 2003). Furthermore, the presence of PR in the primary tumour is an independent marker of favorable prognosis and the tumour is characterised by a more differentiated phenotype, suggesting that progesterone has a protective role against progression and invasion once a tumour has developed (Creighton et al., 2009; Cui et al., 2005). Mice lacking BRCA-1 and p53 expression in their mammary gland overexpress PR, due to a defect in the proteasome-dependent degradation pathway, and develop aggressive tumours which are highly responsive to antiprogestins (Poole et al., 2006). A transgenic mouse model overexpressing the PR-A isoform shows abnormal mammary development and alterations in growth potential (Chou et al., 2003). This is not observed in mice overexpressing PR-B. In vitro, numerous experiments performed with breast cancer cell lines document the importance of PR in proliferation and colony formation (Musgrove et al., 1993);(Faivre and Lange, 2007);(Afhuppe et al., 2010), and crosstalk with growth factor signalling pathways have been evidenced (Skildum et al., 2005); (Daniel et al., 2007); (Faivre and Lange, 2007). In addition, protective effects of progestins against apoptosis have been reported (Moore et al., 2000). Furthermore, the extranuclear actions of steroid hormone receptors, including PR, are thought to contribute to deregulate breast cancer cell growth and increase breast cancer risk. Breast cancers often exhibit increased c-Src and MAPK activities relative to normal breast tissue (Gee et al., 2001; Wilson et al., 2006). Steroid hormone receptors including ER, AR, and PR may contribute to the constitutive signalling of these mitogenic kinases via their membrane-associated activities, thereby circumventing endocrine-based therapies.

1.3 Therapies of breast cancer

Because of the complexity of breast cancer biology, with multiple factors contributing to its development and progression, different therapies directed to specific targets have been developed.

1.3.1 HER/erbB therapies

Since HER-1 and HER-2 are found overexpressed in a significant portion of breast carcinoma cases, therapies targeting the HER family members have been tested. ZD1839, a member of the anilinoquinazoline class of RTK1 blockers works as HER-1 and -2 inhibitor, decreasing their expression by interfering with phosphorylation of PI3K, activation of AKT, and phosphorylation of the MAPK cascade (Moasser et al., 2001; Moulder et al., 2001). ZD1839 inhibits proliferation and induces apoptosis in HER-2-positive cell lines (Moulder et al., 2001).

Trastuzumab is a monoclonal antibody that was raised against the ectodomain of HER-2 and that blocks cell proliferation, inhibits cell growth and induces apoptosis in breast carcinoma cells (Kita et al., 1996; Kunisue et al., 2000). For this reason, the presence of overexpressed HER-2 serves as a good predictive factor of clinical response to trastuzumab. Trastuzumab inhibits both PI3K activation of the AKT pathway and activation of the MAPK pathway. Women with advanced breast tumours overexpressing HER-2 receive trastuzumab in combination with cytotoxics as treatment (Slamon et al., 2001). Preclinical studies suggest that ZD1839 and trastuzumab may work synergistically to inhibit tumour progression via inhibition of both the AKT and MAPK pathways.

1.3.2 Anti-hormone therapy

Over a century ago, surgical oophorectomy was shown to be effective in the therapy of breast cancer. After the discovery of steroid hormones and the steroid hormone receptors, it became clear that merely inhibiting of steroid hormone receptor function by an antagonist could also prevent tumour growth. Cyproterone acetate (CPA) and tamoxifen were the first steroid receptor antagonists discovered and the large progress made in the field since the cloning of the receptor cDNAs, allowed a deeper biological

understanding and enabled rational, structure–activity relationship-based drug discovery programs (Hoffmann and Sommer, 2007).

Endocrine therapy of breast cancer is based on one of the following principles: hormone deprivation, hormone antagonism, and hormone interference. The deprivation of endogenous hormones can be achieved by the inhibition of the hormone´s biosynthesis or by removal or inactivation of the hormone producing tissue. To antagonize an hormone, drugs which are able to bind and inhibit the steroid hormone receptors or different types of releasing hormone receptors have to be applied. The hormone interference can be obtained by applying high doses of hormones that either directly or through negative feedback mechanisms inhibit tumour growth (Hoffmann and Sommer, 2007).

The main endocrine therapies for the treatment of breast cancer will be reviewed in the next paragraphs.

1.3.2.1 Aromatase inhibitors

Development of aromatase inhibitors (AIs) began in the early 1970s and is till the object of intensive work. One way of interfering with ER signalling is to reduce the circulating level of its ligand estradiol by inhibiting the enzyme aromatase. Aromatisation is the last step in the synthesis of estradiol. This reaction is catalysed by the P450 aromatase mono-oxygenase complex that is present in the smooth endoplasmic reticulum of placenta and granulosa cells of ovarian follicles.

Because of the limited specificity of the first- and second-generation AIs, this class of compounds has been abandoned therapeutically for a long time. However, clinical studies with the selective and potent third-generation AIs have revealed that AIs are an alternative endocrine therapy for treating patients with advanced breast cancer. Several clinical trials demonstrated that AIs are superior to tamoxifen in the neo-adjuvant and first-line treatment of advanced breast cancer in post-menopausal women (Brueggemeier et al., 2005). In particular, three relevant clinical studies showed that the third-generation AIs, anastrozole, letrozole and exemestane, are superior to tamoxifen in patients with advanced disease. On the base of these results, a series of clinical trials comparing AIs with tamoxifen in the adjuvant setting has been initiated, and ultimately led to the approval of these compounds (Goss, 2003).

1.3.2.2 Anti-estrogens

The introduction of the anti-estrogen tamoxifen has modified the treatment of breast cancer. It constitutes the preferential method for the treatment of advanced disease in pre- and post-menopausal women and for prevention in women at high risk for developing breast cancer (Fisher, 1999). The occurrence of positive side-effects first identified for Tamoxifen has led to the term selective ER modulator (SERM). A partial ER agonistic activity has been shown for tamoxifen, besides its anti-estrogenic effects. The partial estrogenicity of tamoxifen is shown in the maintenance of bone density in post-menopausal women and in the decreased incidence in hip, wrist and spinal fractures (Leverson and Jordan, 1999). A problem associated with the use of tamoxifen is however the development of resistance to the drug, probably due to its estrogenicity. For this reason in the past 25 years much effort has been put in the identification of a compound with increased anti-tumour activity and with reduced proliferative effects on the endometrium. A second generation of structurally related triphenyl-ethylenes like droloxifene, toremifene and idoxifene has been developed but these compounds were not superior to tamoxifen (Robertson, 2004). The same was also true for raloxifen, which showed however a more effective prevention of osteoporosis (Cauley et al., 2001). Based on the strong anti-tumour activity of tamoxifen and the improved safety profile and bone protection of raloxifen, a third generation of SERM compounds is currently under development. Furthermore, in 1991, the first prototype of a so-called pure anti-estrogen known as selective ER destabiliser or SERD (fulvestrant, or ICI 182,780) was introduced (Wakeling et al., 1991). This compound has no agonistic activity but rather destabilises the ERα protein and completely blocks ERα-mediated growth stimulation. It may therefore represent a therapeutic option for breast cancer patients being probably effective even for tamoxifen-resistant breast cancer. A number of pure anti-estrogens were synthesised and characterised more recently (Bohlmann et al., 2001). These new steroidal anti-estrogens are highly active in pre-clinical tumour models in mice and rats and show a high potency with regard to growth inhibition of ERα-positive breast cancer (Hoffmann et al., 2004).

1.3.2.3 Progesterone receptor antagonists

The presence of PR in about one third of breast cancer cases (Sorlie et al., 2001) and the observed link between PR isoform dysbalance and tumour aggressiveness (Mote et al.,

2007); (Kariagina et al., 2008) suggest that potent and selective PR antagonists may help preventing tumour progression. After the discovery of progesterone in the early 1930s, first, synthetic progesterone analogues were synthesised with the aim of developing oral contraceptives. Later, pure PR antagonists and selective PR modulator (SPRMs) were identified. All these compounds are on the whole referred to as PR ligands. The first PR antagonist, mifepristone (RU-486), was discovered by the French company Roussel-Uclaf in the 1980s (Baulieu, 1987). In postmenopausal women, two studies with mifepristone as second- or third-line treatment for metastatic breast cancer showed an objective response rate of 10 and 13% and stable disease in 54 and 40% of patients, respectively. A third study was conducted using mifepristone as first-line treatment. An objective response rate of 11% and a stable disease rate of 39% was reported (Klijn et al., 1994). Unfortunately, this compound shows in addition to its PR antagonistic activity, also antagonistic effects towards the GR. For this reason, considerable effort has been made in the optimisation of the receptor selectivity. Therefore several steroidal PR antagonists were further developed. The fact that an anti-progestin such as mifepristone is linked to drug-induced abortion by blocking ovulation (Luukkainen et al., 1988) and preventing implantation (Batista et al., 1991) has however restricted the involvement of the major pharmaceutical companies in the development of PR antagonists and SPRMs. Onapristone was investigated in the clinic for the treatment of advanced breast cancer where efficacy could be shown in Phase II clinical trials. This drug was the first PR antagonist investigated as an alternative endocrine agent for the treatment of advanced breast cancer. In a phase II study, onapristone was given at a dose of 100 mg/day to 118 patients with metastatic breast cancer resistant to tamoxifen. The objective response rate was 10%, and in 39% of the patients there was stable disease for at least 3 months. The overall time to progression was 4 months (Jonat et al., 2002). Unfortunately, this compound caused hepatotoxicity that necessitated discontinuation of the trial (Robertson et al., 1999). Nevertheless, these clinical results suggest a potential benefit of adding PR antagonists to the panel of options for the treatment of endocrine-responsive breast cancer, especially in order to extend the therapeutic options in antiestrogen refractory diseases. Other compounds that entered the clinical trials were proellex, CDB-4124 and asoprisnil. In contrast to the antagonists described before, asoprisnil exhibits a partial agonistic/antagonistic profile in some in vivo models and is therefore referred to as a SPRM. Asoprisnil was tested in late

clinical trials for the treatment of uterine fibroids and endometriosis by TAP Pharmaceuticals (Elger et al., 2000). Furthermore, as result of an intensive optimisation process at Bayer Schering Pharma AG, Lonaprisan (ZK-230211), a compound with PR antagonistic activity and low unwanted hormonal activities was discovered (Fuhrmann et al., 2000). A more detailed description of Lonaprisan, whose molecular mode of action has been studied in this thesis, will be given in the next paragraph.

1.3.2.3.1 Lonaprisan

According to the classification of the PR antagonists described by Afhüppe in 2010 (Afhüppe et al, 2010), Lonaprisan is a type III PR antagonist (**Fig. 8**). Altogether, three types of PR antagonists have been defined on the basis of their in vitro properties. Type I antagonists (e.g. onapristone) do not lead to binding of the PR to DNA and exhibit pure antagonism, type II antagonists (e.g. mifepristone and asoprisinil) lead to DNA binding and possess a partial, tissue-specific, agonistic activity due to protein kinase A stimulation, while type III antagonists (e.g. Lonaprisan) induce strong PR binding to their cognate DNA response elements while showing pure antagonistic activity linked to strong corepressor recruitment.

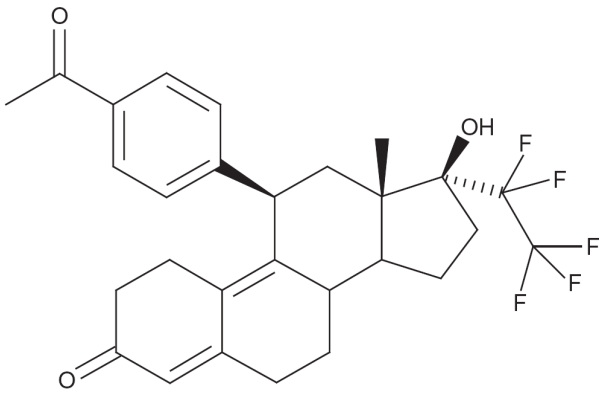


Figure 8. Lonaprisan chemical structure.
A characteristic structural feature of Lonaprisan is the pentafluoroethyl group in the D-ring of the steroid skeleton (Fuhrmann et al., 2000).

The high antiprogestagenic activity exhibited by Lonaprisan, associated with little or no other endocrinological effects, is the result of the introduction of a 17α-pentafluorethyl side chain in the D-ring of the steroid skeleton (Fuhrmann et al., 2000) (**Fig. 8**). In contrast to many other antiprogestins, Lonaprisan does not convert into an agonist in the

presence of protein kinase A (PKA) activators and shows high antiprogestagenic activity on both PR isoforms PR-A and PR-B. Several in vivo models have proved the high antiprogestagenic activity of Lonaprisan. Furthermore, this compound displays only marginal antiglucocorticoid effects. The antitumour activity of Lonaprisan was analyzed in rats with DMBA-induced mammary tumours. In control animals, progressive tumour growth was observed, while ovariectomy caused a complete tumour regression in 90% of the animals. A statistically significant growth inhibition was achieved with doses ≥ 2 mg/kg (**Fig. 9**), while the growth inhibitory effect of Onapristone was not statistically significant. Altogether, these data showed how Lonaprisan completely suppressed tumour growth in intact animals and was more potent than onapristone. The pharmacological properties of Lonaprisan make this compound a potential tool in the treatment of endometriosis, leiomyomas, breast cancer, and in hormone replacement therapy.

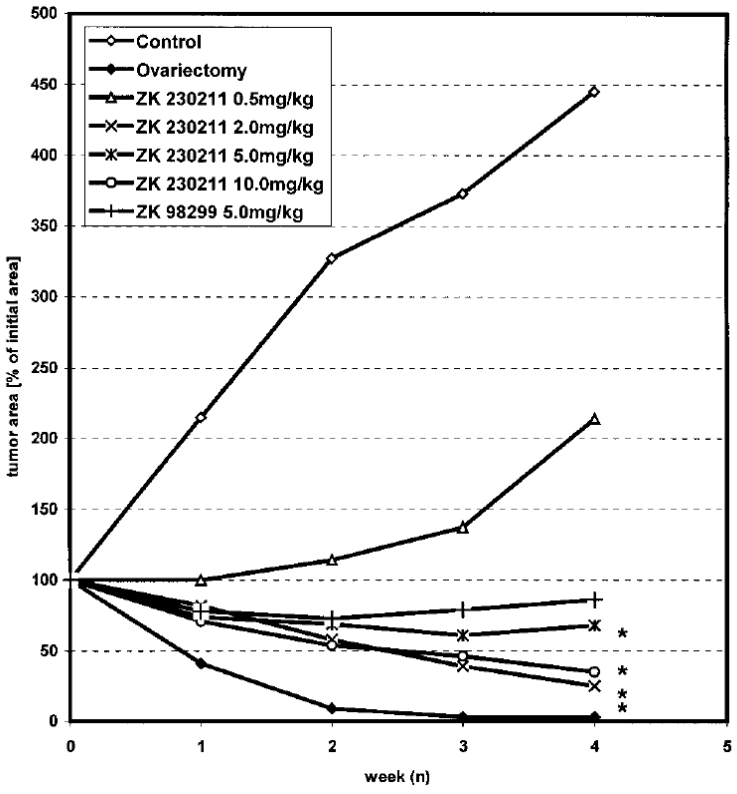


Figure 9. Antitumour effect of Lonaprisan (ZK230211) and onapristone (ZK98299) in the DMBA-induced mammary tumour model in the rat.

Mammary tumours were induced by a single oral administration of 10 mg of DMBA. Rats were treated for 4 weeks, and the tumour growth was measured (Fuhrmann et al., 2000).

1.4 Aim of the work

The aim of this thesis was to examine the role of the PR in breast cancer by using the potent, selective PR antagonist Lonaprisan (ZK230211) as tool compound. Novel insights for alternative therapy options in the treatment of mammary carcinoma may arise from a deeper understanding of the molecular mode of action of this drug. Also, identifying stratification biomarkers should help in screening for patients who may respond better to therapy.

After analysing the antagonistic effects of Lonaprisan on the PR, studying its effects on the PR itself and on PR-regulated genes, the Lonaprisan activity on breast cancer cells will be studied. Moreover an analysis of the relationship between Lonaprisan and the cell cycle regulatory protein p21 will be performed.

2 Materials and Methods

2.1 Materials

2.1.1 Materials, cells, sequences, devices

This section describes the materials, cells, sequences and devices used in this work sorted in alphabetical order.

2.1.1.1 Antibodies

BRCA1 (Ab1; MS110)	mouse	CalBiochem, Darmstadt, Germany
GAPDH (Mab 6C5)	mouse	Advanced ImmunoChemical Inc., Berlin, Germany
IgG	rabbit	Millipore, Schwalbach, Germany
p21 (C-19)	rabbit	Santa Cruz Biotechnology, Heidelberg, Germany
p21 (SX118)	mouse	BD Pharmigen, Erembodegem, Belgium
PR (Ab-8)	mouse	Neomarkers, Epsom, United Kingdom
PR (H-190)	rabbit	Santa Cruz Biotechnology
p-S345 PR	rabbit	gift of Carol A. Lange (University of Minnesota Cancer Center, USA)
Sp1 (PEP-2)	rabbit	Santa Cruz Biotechnology
Anti-mouse IgG - HRP		Amersham Biosciences, Freiburg, Germany
Anti-rabbit IgG - HRP		Amersham Biosciences
Anti-rabbit Alexa 568 fluorescent dye		Molecular probes, Invitrogen, Karlsruhe, Germany

2.1.1.2 Buffers

Elution buffer	0.1 M NaHCO3 and 1 % SDS
Lysis buffer	5 mM PIPES pH 8, 85 mM KCl, 0.5 % Nonidet P40 and protease inhibitors
RIPA buffer	10 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mM EGTA, 1 % Triton X-100, 0.1 % SDS, 0.1 % Na-deoxycholate, 140 mM NaCl and protease inhibitors
TE pH 8.0	10 mM Tris-HCl pH 8 and 1 mM EDTA
Wash Buffer I	20 mM Tris-HCl pH 8, 2 mM EDTA, 150 mM NaCl, 1 %

	Triton X-100 and 0.1% SDS
Wash Buffer II	20 mM Tris-HCl pH 8, 2 mM EDTA, 500 mM NaCl, 1 % Triton X-100 and 0.1 % SDS
Wash Buffer III	10 mM Tris-HCl pH 8, 1 mM EDTA, 250 mM LiCl, 1 % Nonidet P40 and 1 % Sodium deoxycholate

2.1.1.3 Cell culture

Alamar Blue	Invitrogen, Karlsruhe, Germany
Cell culture flasks	Corning, Berlin, Germany
Centrifuge tubes	Corning
cFBS	BIOCHROM AG, Berlin, Germany
DMEM/Ham's F-12 medium	BIOCHROM AG
FBS	BIOCHROM AG
Insulin	Sigma-Aldrich, Steinheim, Germany
L-Glutamine	GIBCO, Karlsruhe, Germany
PBS w/o Ca2+, Mg2+	BIOCHROM AG
RPMI 1640 w/o phenol red medium	BIOCHROM AG
Stripettes	Costar, Corning
Test plates 96/24/6 wells	TPP, Trasadingen, Switzerland
Trypsin-EDTA	BIOCHROM AG

2.1.1.4 Cell lines

Name	Description	Origin
HeLa	Human epithelial cervical cancer cell line	ATCC[a]
MCF7	Human breast cancer cell line derived from pleural effusion. ERα positive	ATCC[a]
T47D	Human breast cancer cell line derived from pleural effusion. PR and ERα positive	ATCC[a]

a American Tissue Culture Collection, Manassas, VA, USA

2.1.1.5 Chemicals

17β-estradiol	synthesised in-house at Bayer Schering Pharma, Berlin, Germany

Asoprisnil	synthesised in-house at Bayer Schering Pharma
Doxorubicin	Sigma-Aldrich
Tamoxifen	synthesised in-house at Bayer Schering Pharma
Lonaprisan	synthesised in-house at Bayer Schering Pharma
Mifepristone (RU486)	synthesised in-house at Bayer Schering Pharma
Onapristone	synthesised in-house at Bayer Schering Pharma
PR agonist (R5020)	synthesised in-house at Bayer Schering Pharma
Saponin	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	BIO-RAD, Herts, United Kingdom
Taxol	synthesised in-house at Bayer Schering Pharma
Others	SIGMA and Merck

2.1.1.6 Immunoprecipitation

Blue MaxTM Conical Tube 15 ml	Falcon, Becton Dickinson, Meylan Cedex, France
Cell culture 150 mm dishes	Corning
Disuccinimidyl glutarate	AppliChem, Darmstadt Germany
DNeasy Blood and Tissue kit	QIAGEN, Hilden, Germany
Dynabeads® Protein A	Invitrogen
Dynabeads® Protein G	Invitrogen
DynaMagTM-2	Invitrogen
Proteinase K	QIAGEN
RNase A	QIAGEN

2.1.1.7 Primers for quantitative PCR

Name	Sequence (5’ – 3’)
p21 Sp1 promoter region (promoter target) for	TCAGCTGCATTGGGTAAATCC
p21 Sp1 promoter region (promoter target) rev	ACTGTTAGAATGAGCCCCCTTTC
p21 Coding exon (irrelevant target) for	CTGGAGACTCTCAGGGTCGAA
p21 Coding exon (irrelevant target) rev	CCTTGGACCATGGATTCTGA
PR-B promoter for	TAACGGGTGGAAATGCCAACT
PR-B promoter rev	TCTGCTGGCTCCGTACTGCGG
PR-A promoter for	GCCGTCGCAGCCGCAGCCACT

PR-A promoter rev	ATCTCCACCTCCTGGGTCGGG

2.1.1.8 Primers for site-directed mutagenesis

Name	Sequence (5' – 3')
S345A mutant for	GCCCCGCCGCGGgcTgCACCCTGTGCCTCG
mPro mutant for	CGTTGGGGCCAgCGgCCCCGCTGgCGCCGCGAGCG
mDBD mutant for	CCTTACCTGTGGGAGCgcTAAGGTCTTCTTTAAGAGG

2.1.1.9 Protein extraction, PAGE and Western blot

10x PBS	GIBCO
Benzonase	Merck KGaA, Darmstadt, Germany
Bradford protein assay	BIO-RAD
Complete Mini EDTA-free tablets	Roche Diagnostics, Mannheim, Germany
Hyperfilm cassette	Amersham Biosciences
Hyperfilm ECL	Amersham Biosciences
Milk powder	ROTH, Karlsruhe, Germany
M-Per	Pierce Biotechnology, Dreieich, Germany
NuPAGE 4-12 % Bis-Tris gels	Invitrogen
NuPAGE LDS Sample Buffer	Invitrogen
NuPAGE MOPS SDS Running Buffer	Invitrogen
NuPAGE Transfer Buffer	Invitrogen
Precision Plus Protein Standards	BIO-RAD
PVDF Membrane	Invitrogen
Re-blot Plus Strong Solution	Millipore
Western Lightning Reagent	Amersham Biosciences, Freiburg, Germany

2.1.1.10 Quantitative PCR

QuantiFastTMSYBR®Green PCR Kit	QIAGEN
Primers	MWG, Ebersberg Deutschland
TaqMan® Fast Universal PCR Mix	Applied Biosystems, Darmstadt, Germany
TaqMan® Gene Expression Assays	Applied Biosystems
Fast-Optical 96-Well Reaction Plates	Applied Biosystems
Optical Adhesive Film	Applied Biosystems

2.1.1.11 RNA extraction and reverse transcription

QIAshredder	QIAGEN
RNeasy Mini Kit	QIAGEN
RNase-Free DNase Set	QIAGEN
SuperScriptTM III First Strand	Invitrogen

2.1.1.12 siRNA sequences

Name	Sequence (5' – 3')	
Human Sp1_1	GCCAAUAGCUACUCAACUA	Ambion, Applied Biosystems
Human Sp1_2	GAAGGGAGGCCCAGGGUA	Ambion, Applied Biosystems
Human Sp1_3	GGGCAGACCUUUACAACUC	Ambion, Applied Biosystems
Human p21_1	GGCGGUUAUGAAAUUCACCtt	Thermo Scientific Dharmacon, Epsom, United Kingdom
Human p21_2	GGAGUCAGACAUUUUAAGAtt	Thermo Scientific Dharmacon
Human p21_3	CCUAGACUGUAAACCUCUCtt	Thermo Scientific Dharmacon
Human BRCA1_1	UGCCAAAGUAGCUAAUGUAUU	Ambion, Applied Biosystems
Human BRCA1_2	GGAAACUUGAAACCUGGGCtt	Ambion, Applied Biosystems
ON-TARGETplus Non-targeting Pool		Thermo Scientific Dharmacon

2.1.1.13 TaqMan® gene expression assays for quantitative PCR

Name	Number
Cx43	Hs00748445_s1
FKBP54	Hs00188025_m1
GAPDH	4326317E

HSD11β2	Hs00388669_m1
p21	Hs00355782_m1
PR	Hs00172183_m1
Sp1	Hs00412720_m1
STAT5A	Hs00559643_m1
VIL2	Hs00931646_m1

2.1.1.14 Technical devices

7500 Fast Real-Time PCR System	Applied Biosystems
BBD 6220 incubator	Thermo Fisher Scientific, Waltham, USA
BiorupterTM	Diagenode, Liege, Belgium
Cell counter CASY®	Innovatis, Bielefeld, Germany
Centrifuge 5415R	Eppendorf, Hamburg Deutschland
Developing machine CURIX 60	AGFA, Mortsel, Belgium
FACS Calibur	BD Biosciences, Erembodegem, Belgium
HERA safe cleanbench	Heraeus, Hanau, Germany
Heraeus Multifuge 3S+	Thermo Fisher Scientific
Heraeus Multifuge 3SR+	Thermo Fisher Scientific
Heraeus Pico 21 Centrifuge	Thermo Fisher Scientific
Incubator	Heraeus
Incubator for shaking	INFORS, Bottmingen-Basel, Switzerland
Microscope Axiovert 40C	Zeiss, Oberkochen, Germany
ModFit LT software	Verity Software House, Topsham, ME
NanoDropTM 2000	Thermo Fisher Scientific
PCR Cycler Tetrad 2	BIO-RAD, Munich, Germany
pH Meter 761	Calimatic Knick, Berlin, Germany
Power Pack P25	Biometra, Goettingen Germany
Shaker Titramax 100	Heidolph, Schwabach, Germany
Sub-Cell GT Gel electrophoresis system	BIO-RAD
VictorTM X3	PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany
Water bath	Julabo 5B, Burladingen, Germany

2.1.1.15 Transfections

CulturPlate-96	PerkinElmer Life Sciences
FuGENE® HD transfection reagent	Roche
LumiCount Luminescence Microplate Reader	Packard, Meriden, CT, USA
Opti-MEM®	GIBCO
siPORT Amine transfection agent	Ambion, Applied Biosystems
SteadyLite Plus Reagent	PerkinElmer Life Sciences
TopSeal-A film	PerkinElmer Life Sciences

2.2 Methods

2.2.1 Cell culture methods

2.2.1.1 Cultivation of adherent eukaryotic cell lines

The T47D and MCF-7 human breast cancer cell lines, and the HeLa cervical carcinoma cell line were grown at 37°C in a 5% CO2 humidified environment in T75 flasks. T47D cells were grown routinely in phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped hormone-free fetal bovine serum, 4 mmol/l L-glutamine, 200 mU/ml insulin and 0.1 nM estradiol. MCF-7 cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% fetal bovine serum and 4 mmol/l L-glutamine. HeLa cells were grown in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum. For chromatin immunoprecipitation (ChIP) experiments, transcription assays and for the analysis of PR phosphorylation, cells were grown for 48 hours with starvation media consisting of phenol red-free RPMI 1640 medium supplemented with 5% charcoal-stripped hormone-free fetal bovine serum. For passaging, cells were washed with sterile 1 x PBS once, and trypsinised with 1.5 ml of Trypsin-EDTA for 2-5 mins at 37°C. After the addition of fresh growth medium, the cells were resuspended and plated in new flasks.

2.2.1.2 Freezing and thawing of eukaryotic cell lines

Cells at 70-80% confluency were harvested for freezing. Cells were trypsinised as described and transferred into a centrifuge tube. The cells were centrifuged at 900 rpm for 5 mins, resuspended in 1 ml freezing medium, consisting of complete medium plus 15% DMSO, and transferred into cryovials (Nunc, Langenselbold, Germany). The vials were incubated in a cryobox (Nunc) containing isopropanol at -80°C, and then transferred into a liquid nitrogen tank for long-term storage.

Cells frozen in liquid nitrogen were quickly thawed at 37°C in a water bath. Cells were then transferred into a tube with 40 ml fresh growth medium and centrifuged at 900 rpm for 5 min at room temperature. The cell pellet was resuspended in 10 ml fresh medium and plated in a new 75 cm^2 cell culture flask and further cultured as described.

2.2.2 Cell-based assays

2.2.2.1 Apoptosis assay-caspase glow 3/7 assay

For detection of caspase-3 and caspase-7 activation, T47D cells were plated in replicates of five in 96-well plates, treated with the compounds and analyzed using the Caspase Glow 3/7 Assay (Promega, Mannheim, Germany) following the manufacturer's instructions. Samples were read after 1 hour of incubation with the caspase substrate on a fluorescent plate reader using wavelengths of 480 and 535 nm for excitation and emission, respectively.

2.2.2.2 Cell cycle analysis

To perform cell cycle analysis, 200,000 cells per well were seeded in six-well plates in their growth medium and allowed to attach overnight. 10 nM Lonaprisan or vehicle (EtOH) were then added. After the indicated timepoints, cells were harvested by trypsinisation, pelleted by centrifugation and washed once with PBS. Media, washes and the pelleted cells were kept together. The cells were resuspended by pipetting several times in 1 ml ethanol (70%) in order to get a homogenous single-cell suspension. They were stored at −20 °C until the day of analysis when they were centrifuged and washed once with PBS. Cell pellets were then resuspended in 0.2 ml PBS containing 1.25 mg/ml ribonuclease A and 50 µg/ml propidium iodide. After careful resuspension, cells were transferred into a filter cap fitted polystyrene tube and

incubated at 4 °C in the dark for 4 h. Cells were then analyzed in a fluorescence-activated cell sorting Caliber flow cytometer. The results were processed using the ModFit LT software.

2.2.2.3 Cell proliferation

Cells were seeded at 10,000 cells/well in 96-well plates. After 24 hours, compounds were added in fresh medium. At the desired timepoint Alamar Blue was added to the plates at 10% of the culture medium. After two hours of incubation, proliferation was monitored by measuring the fluorescence emitted by a REDOX indicator using a microtiter well plate reader.

2.2.2.4 Senescence assay

After treatment with Lonaprisan, cells were stained using a Senescence β-Galactosidase Staining kit following the manufacturer´s instructions (Cell Signaling, New England Biolabs GmbH, Frankfurt am Main, Germany).

2.2.2.5 Viability assay

Cells were cultured in sterile, eight-well chamber slides at a concentration of 10,000 per well and treated with vehicle or Lonaprisan. After 72 hours of incubation, cells were analyzed by a Live/Dead viability/cytotoxicity assay according to the instructions of the manufacturer (Invitrogen Molecular Probes, Eugene, Oregon, USA). Cells were washed with PBS and incubated for 45 min at room temperature in the presence of appropriate concentrations of cell-permeant calcein AM, a substrate for ubiquitous intracellular esterases, and ethidium homodimer (Eth-1), which enters the cells with damaged plasma membranes and stains their DNA. To permeabilize the membrane, cells were exposed to 0.1% saponin for 5 min. At the end of the incubation the slides were mounted and observed under a fluorescence microscope.

2.2.3 Cloning and site-directed mutagenesis

2.2.3.1 Cloning of PR-B into pSD-MAM5

The PR-B isoform was cloned into the pSD-MAM5 expression plasmid using the Gateway® recombination cloning technology (Invitrogen).

2.2.3.2 Site directed-mutagenesis

Mutagenesis of the PR-B isoform was performed using the QuickChange II-XL mutagenesis kit (Stratagene, Amsterdam, The Netherlands) following the manufacturer´s instructions. Briefly, the PR-B expression plasmid was amplified using primers containing the desired mutation with an extension time of 1 min per kb. The parental DNA template was eliminated by DpnI endonuclease digestion and the vector DNA incorporating the desired mutations was then transformed into XL10-Gold ultracompetent cells. The plasmids were sequenced to confirm the presence of the mutations at the desired sites.

2.2.4 Promoter analysis methods

2.2.4.1 Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay is a powerful method for analyzing genomic DNA sequences bound to specific regulatory proteins. Briefly, in ChIP protein-DNA complexes are crosslinked, immunoprecipitated, purified, and amplified for gene- and promoter-specific analysis of known targets using real-time PCR. For ChIP experiments, MCF-7 cells were grown in 150 mm dishes, starved for 48 h and transfected with the PR-B expression plasmid. After treatment with Lonaprisan (10 nM) and R5020 (10 nM) for 1 h, cells were dual cross-linked at room temperature using 2 mM disuccinimidyl glutarate for 45 min and 1 % formaldehyde for 10 min. The reaction was stopped by adding glycine to a final concentration of 125 nM for 5 min at room temperature. Cells were washed twice in cold PBS and then pelleted by centrifugation at 4 °C for 5 min at 1,000 rpm and collected in lysis buffer. This was followed by nuclear lysis using RIPA-buffer. The samples were sonicated on ice for 2 x 10 min with a 30 s interval at high level using the BiorupterTM instrument resulting in chromatin fragmented to an average length of about 200 bp. The efficiency of the sonication was checked by agarose gel electrophoresis. After centrifugation at 13,000 rpm for 10 min and at 4 °C, the chromatin solution was cleared with 60 µl of Dynabeads® Protein A/G. The beads were then separated on a magnetic rack. A sample of the supernatant was collected for input measurements. For immunoprecipitation, 4 µg of appropriate antibody [anti-PR (H-190), Sp1 (Pep-2) and normal rabbit IgG] were added to the supernatant and incubated overnight on a rotatory shaker at 4°C. Then the samples were

mixed with 40 ul of Dynabeads® Protein A/G and incubated for 6 h at 4°C. The beads were then washed with buffer I, buffer II and buffer III which contained increasing salt concentrations, followed by two washes with TE buffer. Complexes were eluted in 2 x 250 µl elution buffer at 65 °C, under shaking for 10 min. The cross-link was reverted by adding NaCl to a final concentration of 160 mM followed by incubation at 65 °C overnight. The eluates were treated for 1 h with RNase H and then with proteinase K. The DNA was then purified using the DNeasy Blood and Tissue kit. The immunoprecipitated DNA was analysed by quantitative PCR using QuantiFastTMSYBR®Green PCR Kit.

2.2.4.2 Luciferase assay

The purpose of a reporter gene assay is to measure the regulatory potential of a certain DNA-sequence. This can be achieved by linking a promoter sequence to an easily detectable reporter gene such as that encoding for the firefly luciferase. Herein, transactivation assays based on the luciferase reporter gene have been performed. HeLa cells were seeded into 96-well plates at a concentration of 10,000 cells/per well. PR-B expression plasmid was cotransfected together with the p21 Luc-based reporter plasmids using FuGENE® HD Transfection Reagent in Opti-MEM®. Hormone induction was performed 6 h later and measurement of Luc activity was carried out after 24 h in a Victor multilabel reader, after addition of 100 µl of SteadyLite Plus Reagent. For all points the average value of six wells treated in parallel was taken.

2.2.5 Protein methods

2.2.5.1 ELISA

To monitor the expression levels of the PR in T47D and MCF7 cells, the NR Sandwich PR ELISA Kit has been used according to the manufacturer's instructions (Active Motif, Rixensart, Belgium). 10 µg of whole cell lysate were measured in this assay. This method uses two antibodies recognizing each a distinct epitope on the PR. The second antibody, called the Detecting Antibody, is used to detect the protein bound by the Capture Antibody. An HRP-conjugated Secondary Antibody is then used to quantitate the amount of bound Detecting Antibody. Subsequent incubation with developing solution provides a quantified colorimetric readout.

2.2.5.2 Immunocytochemistry

40 x 10^3 T47D cells and 50 x 10^3 HeLa cells were plated in each well of an 8-well Chamber Slide (Lab-Tek® II Chamber SlideTM System) and incubated under normal cell culture conditions for 24 hrs. One day later, T47D cells were incubated with Lonaprisan and R5020, while the HeLa cells were transfected with the plasmids expressing respectively PR-B, PR-A or with the empty vector. After 24 hours the cells were incubated with the PR ligand. At defined incubation timepoints, the cells were fixed with 4% paraformaldehyde in 1x PBS for 20 min. After washing twice with cold PBS, cells were permeabilised in 1x PBS + 0.1 % Triton X-100 for 10 min and washed again three times with PBS. The cells were then blocked with 10% fetal bovine serum in 1 x PBS for 30 min, which was followed by a brief washing step with 1x PBS + 0,5% BSA. Primary antibody was diluted in 1 x PBS + 1% BSA at 1:200 dilution for 1.5 hours at RT. Phalloidin (green), at 1:200 dilution, was also applied to visualize the cytoplasm. This was followed by three washing steps with 1x PBS + 0.5% BSA. Cells were further incubated with the appropriate secondary antibody coupled with Alexa 568 fluorescent dye (Molecular probes) and Hoechst 33342 for 30 min at room temperature in the dark. Cells were then washed 3x with 1x PBS + 0,5% BSA. The media chambers were removed by using the black slide separator provided with the kit and a coverslip was fixed over the slides with a mounting medium glue. Embedded cells were stored shielded from light until examination under a confocal microscope.

2.2.5.3 Western blot analysis

Whole-cell lysates were collected on ice by scraping in M-PER Mammalian protein extraction reagent, supplemented with complete protease cocktails inhibitor tablets, 0.5 mM DTT and benzonase. They were then incubated for 30 min on ice with periodic vortexing. Lysates were clarified by centrifugation for 10 min at 14,000 rpm and at 4°C. Soluble proteins were quantified by the Bradford method using the BCA protein assay, and equal amounts of protein were resolved by SDS-PAGE. The proteins were electrotransferred to a polyvinylidene difluoride membrane, immunoblotted with a specific antibody, and developed using AmershamTM ECLTM Western Blotting detection reagents according to the manufacturer´s protocol.

2.2.6 RNA Methods

2.2.6.1 RNA preparation from cultured cells

Total RNA was extracted using the Qiashredder-Kit, RNeasy Mini kit and the RNase-free DNase set. Breast cancer cells were grown in 6-well plates until they reached 70% confluence. Cells were washed twice with ice-cold PBS. Then cells were lysed with 350 µl of the Buffer RLT per well containing β-mercaptoethanol. After 5 mins, lysed cells were scraped off and put on Qiashredder spin columns placed in a 2 ml collection tube and centrifuge for 2 min at maximum speed. The flow-through was mixed with an equal volume of 70% ethanol and put onto RNeasy Mini-columns. The columns were centrifuged for 5 mins at room temperature at ≥8000 x g in a swinging-bucket centrifuge. The flow through was discarded away. 350 µl Buffer RW1 were pipetted into the RNeasy mini column, and centrifuged for 15 s at ≥8000 x g to wash. The flow through was discarded away. 10 µl of DNAase stock solution were added to 70 µl Buffer RDD. The DNase I incubation mix (80 µl) was added directly onto the RNeasy silica-gel membrane and place on the benchtop (20-30°C) for 15 min. 350 µl Buffer RW1 were added into the RNeasy mini column, and centrifuged for 15 s at ≥8000 x g, discarding the flow through. The RNeasy columns were transferred into a new 2 ml collection tube and 500 µl Buffer RPE were added onto the RNeasy columns. The columns were centrifuged for 15 s at ≥8000 x g to wash and the flow-through was discarded away. Another 500 µl Buffer RPE were added to the RNeasy column. Followed a centrifuge for 2 min at ≥8000 x g to dry the RNeasy silica-gel membrane. To elute, the RNeasy columns were transferred in a new 1,5 ml collection tube. 30-50 µl RNase-free water were pipetted directly onto the RNeasy silica-gel membrane and centrifuged for 1 min at ≥8000 x g to elute. The eluted total RNA was immediately stored at -80 °C.

2.2.6.1.1 Quality control and quantitation of total RNA

Total RNA concentration was determined using the NANO DROP 2000 spectrophotometer and the ND-100 program. The absorbance of the solution containing the RNA was measured at wavelengths between 260/280 and 320 nm. To ensure significance, readings at 260 nm should be between 0.1 and 0.9. The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA

with respect to contaminants that absorb in the UV, such as protein and phenol. Pure RNA has an A260/A280 ratio of 1.9–2.1.

2.2.6.2 TaqMan® real-time PCR

The real-time PCR was performed as a two-step reaction. At first, total RNA was converted into cDNA. Subsequently as a separate step, the cDNA was amplified and measured by a 7500 Fast-Real Time PCR System. The special design of TaqMan probes combined with 5'- 3' nuclease activity of the polymerase, allows direct detection of PCR products by the release of a fluorescent reporter during the PCR on the ABI PRISM Sequencing Detector. The reverse transcription step was carried out following standard procedures. One to two micrograms of total RNA were reverse-transcribed with random hexamer primers and SuperScriptTM III reverse transcriptase according to the manufacturer´s protocol. Real-time PCR quantification was performed using a Fast Real-Time PCR instrument according to the standard cycling program (95 °C for 20 s, 40 cycles of 95 °C for 3 s followed by 60 °C for 30 s). The PCR Mix and the TaqMan gene expression assays were purchased from Applied Biosystems. As template 50 ng of cDNA per reaction were added. For the analysis of the results, the cycle threshold (Ct) value of the gene of interest obtained for each sample was normalised to the Ct value of human GAPDH (ΔCt) and then to the control sample (ΔΔCt). Results are expressed as percentage ($2^{-\Delta\Delta Ct}$) in comparison to the control. For quantification of the DNA obtained by Chromatin Immunoprecipitation, the SYBR® Green (Qiagen) method was used, following the manufacturer´s instructions (95 °C for 5 min, 40 cycles of 95 °C for 10 s followed by 60 °C for 30 s). For the analysis of the results, the Ct value of the region of interest obtained for each antibody approach was normalised to the input Ct of this region. Results are expressed as percentage ($2^{-\Delta Ct}$ x 100) compared to the input.

2.2.7 Transfection

2.2.7.1 RNA interference (siRNAs)

Long double-stranded RNAs (dsRNAs; typically >200 nucleotides, nt) can be used to silence the expression of target genes in a variety of organisms and cell types (e.g., C. elegans, Drosophila and Arabidopsis; Hannon, 2002). Upon introduction, the long dsRNAs is processed by the RNA interference (RNAi) pathway. The dsRNAs are cut into 20-25 nucleotide (nt)-long small interfering RNAs (siRNAs) by an RNase III-like

enzyme called Dicer (initiation step). Alternatively siRNAs can be directly administered to the cells. The siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effecter step). In this thesis double-stranded siRNA (21 nucleotides) were used (Tuschl et al., 1999, Fire 1998). SiPORT™ Amine Transfection Agent (Ambion) was used to transfect the T47D breast cancer cells. This transfection solution consists of polyamines that deliver siRNA into mammalian cells with minimal cytotoxicity. The volumes and the amounts in the following protocol were used for a transfection in one well of a 6-well plate. Approximately 24 hrs before the transfection, cells were plated in normal growth medium so that they could be 50% confluent after 24 hours. The cells were incubated overnight under normal cell culture conditions. 5 µl of transfection agent, siPORT Amine, were diluted into OPTI-MEM I without serum for a final volume of 100 µl. The solution was well mixed and incubated at room temperature for 10 minutes. 10 µl of 20 µM siRNA (for a final concentration of 100 nM) were diluted into OPTI-MEM I for a final volume of 100 µl. The solution was incubated for 15 minutes at room temperature. The diluted siRNA was added to the diluted transfection agent and mixed by gently flicking the tube. The dilution was incubated for 15 minutes at room temperature. The transfection agent/siRNA complex in OPTI-MEM was added drop by drop to the cells. Without swirling, the dish was gently rocked back and forth to evenly distribute the complexes. The cells were incubated under normal cell culture conditions for 24 hrs. After 24 hrs, the medium containing the transfection agent/siRNA complex was substituted with normal growth medium and the cells were incubated for further treatments. The total RNA and proteins were extracted from the cells at different time points after the transfection start and the knock-down of the gene of interest was assessed by real-time PCR and Western blots.

2.2.7.2 Transient transfection

Transfections with the various plasmids were carried out using FuGENE HD (Roche Diagnostics) for all the cell lines used. The transfection method followed the manufacturer´s suggested protocol.

3 Results

The aim of this thesis was to better understand the role of the PR in breast cancer using the potent, selective PR antagonist Lonaprisan (ZK230211) as tool compound. A deeper understanding of the molecular mode of action of this drug may offer novel insights for the improved treatment of the disease. In particular, the identification of stratification biomarkers could help in the screening of patients who are expected to better respond to therapy.

3.1 Regulation of PR target genes by PR ligands

The first step in the characterisation of the mode of action of the PR antagonist Lonaprisan consisted in the determination of its antagonistic effect on PR-regulated genes. Since T47D breast cancer cells express constitutively high levels of PR, they were chosen as model to study the actions of progesterone and synthetic progestins in this thesis.

A fundamental prerequisite for the activity of all PR ligands is indeed the presence of the PR. For this reason, first the endogenous expression levels of the PR in T47D and MCF7 cell lines were compared by ELISA. In addition PR levels were evaluated in MCF7 cells by Western blot analysis, after transfection with plasmids coding for the PR isoforms PR-A and PR-B (**Fig. 10-11**). A strong PR expression was seen by ELISA in the T47D cells and a weaker expression in the MCF7 cells, although much lower than in the T47D cell. In the Western blot experiment however no endogenous PR expression was visible.

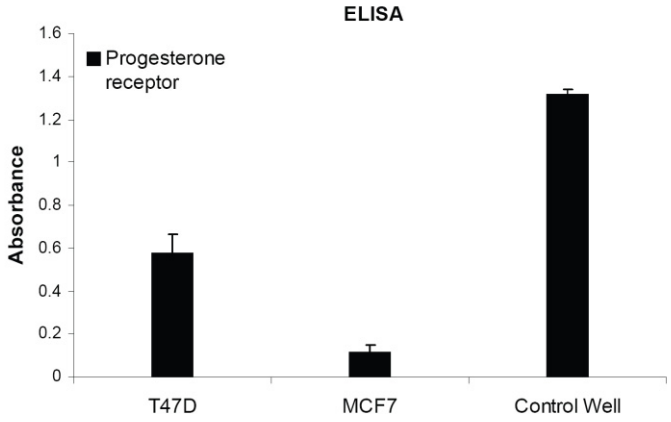


Figure 10. Quantitative detection of PR levels by ELISA.
A PR sandwich ELISA kit was used to detect the total PR amount in the T47D and MCF7 cell lines. In the control well a T47D nuclear extract provided with the kit was used as a positive control.

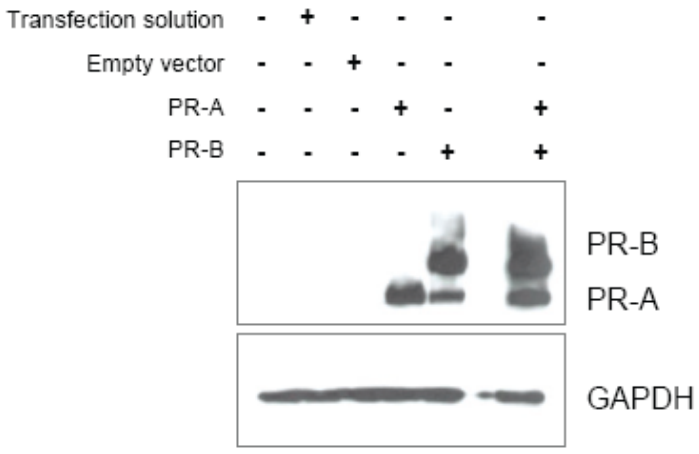


Figure 11. PR expression in MCF7 cells after PR isoforms over-expression.
MCF7 cells were transiently transfected with empty vector, PR-A, or PR-B expression plasmids, alone or in combination. After 24 hours, whole-cell lysates were prepared and subjected to immunoblotting using antibodies directed against PR or GAPDH.

Then, the ability of Lonaprisan to inhibit the expression of various endogenous progesterone-inducible genes was evaluated in the two cell lines above analysed for PR content. Previous studies have identified a number of progesterone-regulated endogenous cellular genes, including genes regulated similarly by both PR isoforms, and genes differentially regulated by PR-A vs. PR-B (Richer et al., 2002). In this experiment, the expression of four known progesterone-responsive genes in T47D cells was measured using real-time PCR to determine the respective mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the expression of which was not affected by progestin, was used as a control gene. T47D cells were treated for 24 hours with two different concentrations of the synthetic progestin R5020 (50 and 100nM), and tested for the expression of the following progesterone-regulated genes: FKBP54 (54-kDa PR-associated immunophilin), STAT5A (signal transducer and activator of transcription 5A), VIL2 (villin 2, also called ezrin), and HSD11β2 (hydroxysteroid 11- β dehydrogenase 2) (**Fig. 12 A-D**). For FKBP54, STAT5A and HSD11β2 a significant progesterone-stimulated increase in mRNA levels was shown already at the concentration of 50 nM. Furthermore, at the higher concentration of 100 nM a stronger increase in the mRNA level compared to the vehicle treated control was observed in all four tested genes. The combination treatment of R5020 at 50 nM with two concentrations of Lonaprisan (10 and 50 nM) reversed the progesterone stimulation of these genes. No conclusion can be drawn for VIL2 since the 50 nM R5020 concentration used was not sufficient to observe a significant stimulation. As expected, the treatment with 10 nM Lonaprisan did not result in any stimulatory effects. These findings confirm that Lonaprisan, as a pure antagonist, is able to antagonize the stimulatory activity of the synthetic progestin on a series of endogenous progesterone-responsive genes.

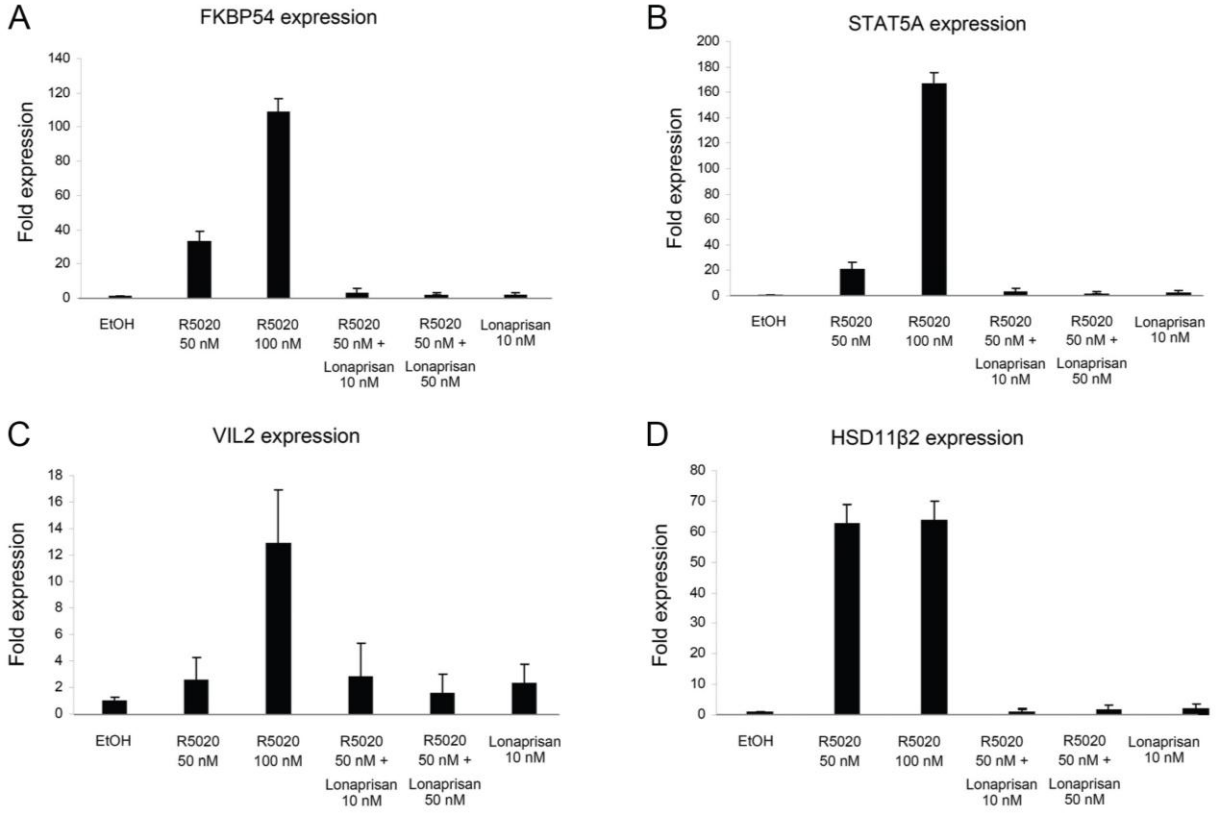


Figure 12. Regulation of PR-dependent genes by PR-ligands in T47D cells.

Sub-confluent proliferating T47D cells were incubated for 24 hours with the indicated concentrations of the synthetic progestin R5020, the PR antagonist Lonaprisan and EtOH as vehicle control, alone or in combination. The cells were then harvested and the mRNA analyzed by real-time PCR to test the expression levels of the following progesterone-regulated genes: FKBP54 (54-kDa PR-associated immunophilin) (A), STAT5A (signal transducer and activator of transcription 5A) (B), VIL2 (villin 2, also called ezrin) (C) and HSD11β2 (hydroxysteroid 11- β dehydrogenase 2) (D).

Moreover, in line with the results of the ELISA and the Western blot assays, a weaker progestin induction was observed when the expression levels of FKBP54, STAT5A and HSD11β2 was tested by real-time PCR in MCF7 cells, where very low endogenous PR levels had been detected (**Fig. 13**).

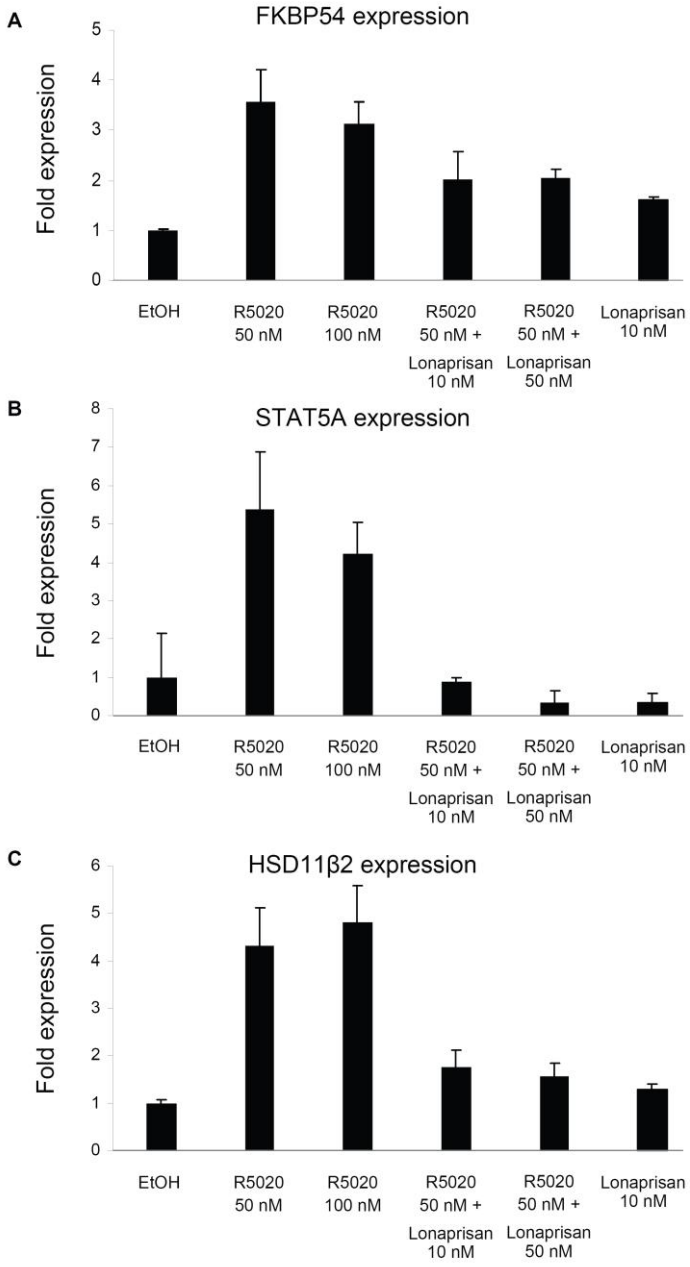


Figure 13. Regulation of PR-dependent genes by PR ligands in MCF7 cells.

Sub-confluent proliferating MCF7 cells were incubated for 24 hours with the indicated concentrations of the synthetic progestin R5020, the PR antagonist Lonaprisan and EtOH as vehicle control, alone or in combination. The cells were then harvested and the mRNA analyzed by real-time PCR to test the expression levels of the following progesterone-regulated genes: FKBP54 (54-kDa PR-associated immunophilin) (A), STAT5A (signal transducer and activator of transcription 5A) (B), and HSD11β2 (hydroxysteroid 11- β dehydrogenase 2) (C).

3.2 Lonaprisan causes nuclear translocation of PR

It has been previously shown that Lonaprisan belongs to the III class of PR antagonists, characterised by strong PR binding to its cognate response elements on the DNA (Afhueppe et al., 2010). In order to confirm that Lonaprisan induces nuclear translocation of the PR, I performed confocal microscopy on T47D cells treated with Lonaprisan, R5020 or EtOH as vehicle control at three different time points (24, 48 and 72 hours). The cells were treated with a PR antibody, and also with phalloidin (red) and Hoechst 33342 (blue) to visualize the cytoplasm and nuclei, respectively. As shown in **Fig.14**, unliganded PR resided almost exclusively in the cytosol and nuclear staining was hardly visible. Following R5020 treatment, PR localised almost exclusively to the nuclei of T47D cells. The situation was similar for Lonaprisan, which also led to nuclear translocation . It was furthermore interesting to find out if the effects of Lonaprisan differed between the PR isoforms. For that, HeLa cells which do not express PR were transiently transfected with expression vectors for either PR isoform or the empty vector as a control (**Fig.15**). After 24 hours, the cells were incubated for further 24 hours with Lonaprisan or R5020, and then stained as previously described for the T47D cells. Again, before the treatment PR resided almost exclusively in the cytosol, while no nuclear staining was visible. After treatment however, the PR-A and PR-B isoforms localised to the nuclei whereas no staining was detectable in the cells transfected with the empty vector. The results show that similarly to R5020, Lonaprisan induces nuclear translocation of both PR isoforms.

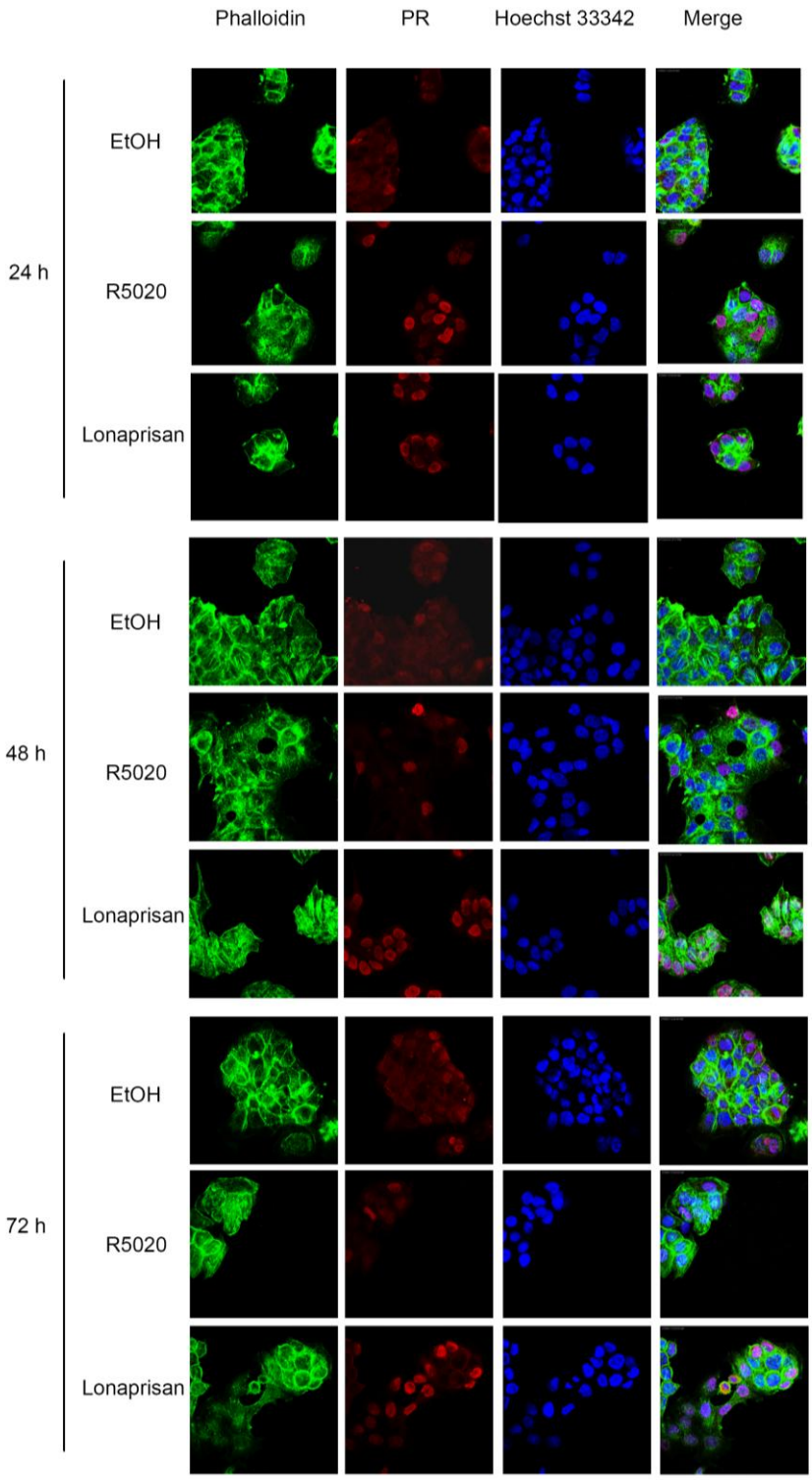


Figure 14. Lonaprisan causes PR translocation into the nucleus of T47D cells.

T47D cells were cultured on 8-well chamber slides and treated for 24, 48 and 72 hours with R5020 and Lonaprisan, before being fixed in 4% paraformaldehyde. PR was visualised with a rabbit polyclonal antibody followed by the addition of Alexa 568 (red) labeled anti-rabbit antisera. Phalloidin (green) and Hoechst 33342 (blue) were also applied to visualize the cytoplasm and nuclei, respectively.

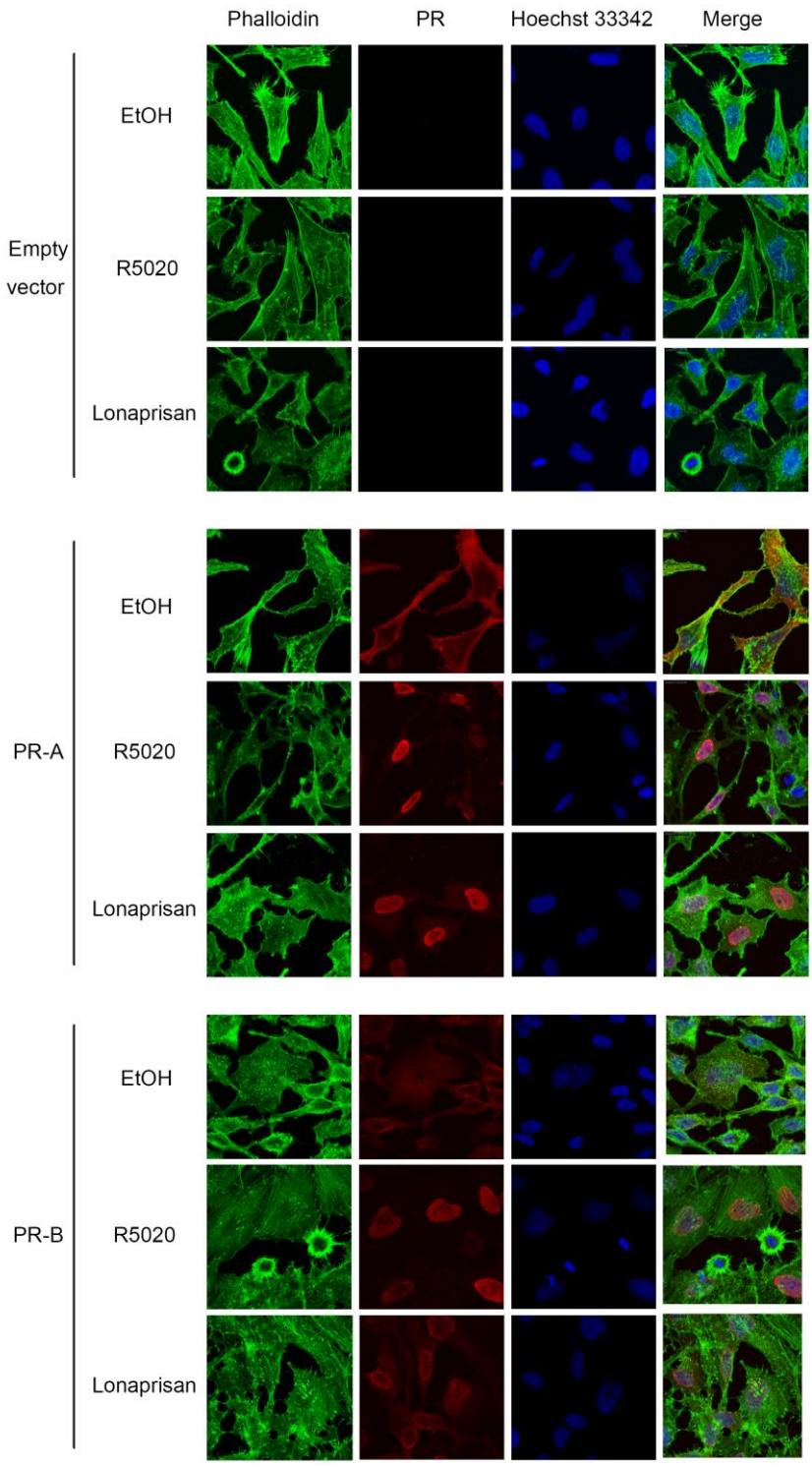


Figure 15. Lonaprisan causes PR translocation into the nucleus of PR over-expressing HeLa cells. HeLa cells were cultured on 8-well chamber slides and after transient transfection with empty vector, PR-A and PR-B expression plasmids, were treated for 24 hours with R5020 and Lonaprisan. PR was visualised with a rabbit polyclonal antibody followed by the addition of Alexa 568 (red) labeled anti-rabbit antisera. Phalloidin (green) and Hoechst 33342 (blue) were also applied to visualize the cytoplasm and nuclei, respectively.

3.3 Lonaprisan inhibits proliferation of the breast cancer cell line T47D

After having shown that Lonaprisan was able to induce nuclear translocation of the PR and inhibit the expression of endogenous progesterone regulated genes, the optimal concentration needed for Lonaprisan inhibition of proliferation was determined. T47D cells were cultured under conditions leading to optimal growth rate, i.e. in medium supplemented with insulin and with 10 nM estradiol, and in charcoal-stripped serum. Proliferation was monitored by measuring the fluorescence emitted by a REDOX indicator, which reflects the metabolic activity of the cells. Lonaprisan application led to a concentration-dependent decrease in cell proliferation after 144 hours of treatment, with an IC50 in the low nanomolar range (**Fig. 16A**). On the basis of these data, a 10 nM Lonaprisan concentration was selected as the optimal concentration for full inhibition of T47D cell proliferation. A time course study at this concentration was then performed and a significant inhibition of T47D cell proliferation for up to 6 days was found, when compared to cells treated with vehicle only (**Fig. 16B**).

3.3.1 Antiproliferative activity of Lonaprisan in T47D with BRCA1 knock-down

Several lines of evidence suggest that mutations in the breast cancer susceptibility gene 1 (BRCA1) are linked to an increased incidence of breast cancer. It has been shown that mutation in BRCA1/p53 leads to increased mammary ductal branching, alveologenesis, and proliferation and BRCA1 depletion leads to a 3.5-fold increase in PR-A and in PR-B proteins, due to impaired PR degradation by the proteasome pathway. Furthermore, treatment with the PR antagonist mifepristone inhibits mammary tumourigenesis by decreasing ductal branching and alveolar proliferation in BRCA1/p53-deficient mice (Poole et al., 2006). Since Lonaprisan shows a strong anti-proliferative activity in T47D cells, it was interesting to investigate whether Lonaprisan was still able to inhibit the proliferation after BRCA1 silencing as demonstrated for mifepristone *in vivo*. First, I demonstrated that depleting BRCA1 with small interfering RNA (siRNA) in T47D cells resulted in increased levels of both PR isoforms (**Fig. 17**). Using Western blot analysis, I could show that the expression of BRCA1 was already silenced 24 hours after siRNA transfection, and that the knock-down was maintained for at least 96 hours. The antiproliferative activity of Lonaprisan after BRCA1 knock-down was then evaluated.

As shown in **Fig. 18**, Lonaprisan showed a stronger anti-proliferative effect in T47D cells lacking BRCA1 expression, compared to the wild-type cell lines. In the proliferation assay two different siRNAs were used to inhibit the expression of BRCA1. The BRCA1 silencing with the two BRCA1 siRNAs at the latest time point examined in the proliferation assay was further confirmed by Western blot analysis (**Fig. 19**).

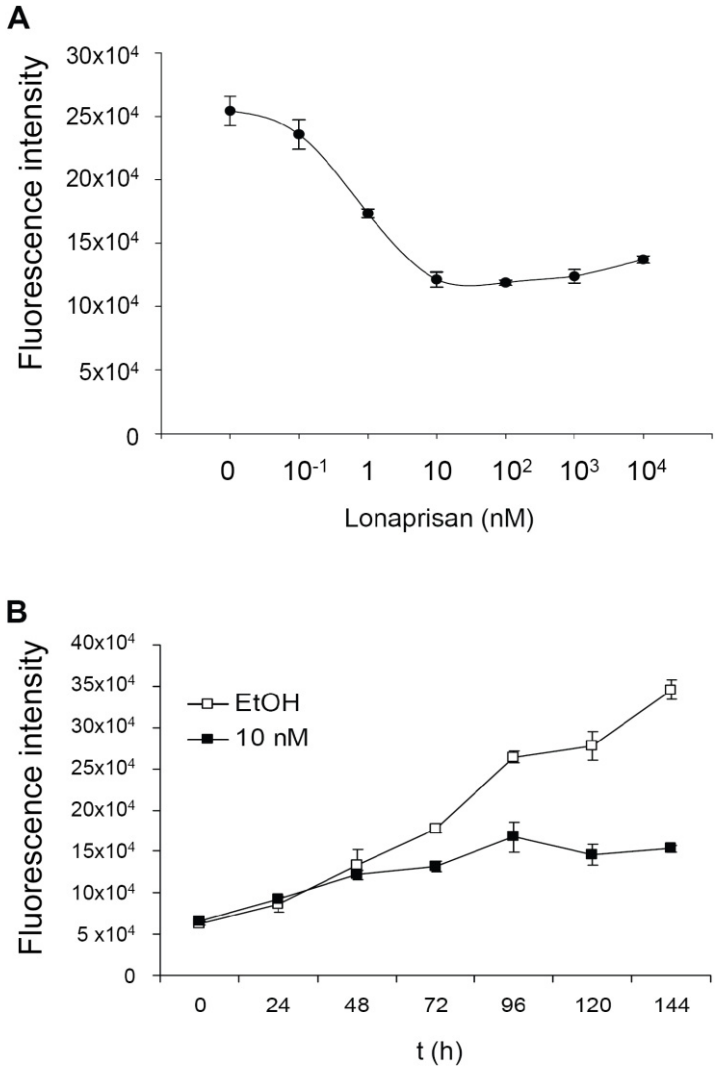


Figure 16. Regulation of T47D cell growth by Lonaprisan.

A. Determination of optimal Lonaprisan concentration. T47D cells were grown in presence of increasing concentrations of the compound for 144 hours. Alamar Blue was then added to the plates at 10% of the culture medium. After two hours of incubation, proliferation was monitored by measuring the fluorescence emitted by a REDOX indicator using a microtiter well plate reader. **B.** Determination of the optimal timepoint. T47D cells were incubated with 10 nM Lonaprisan for up to 144 hours. Cell proliferation was measured as above.

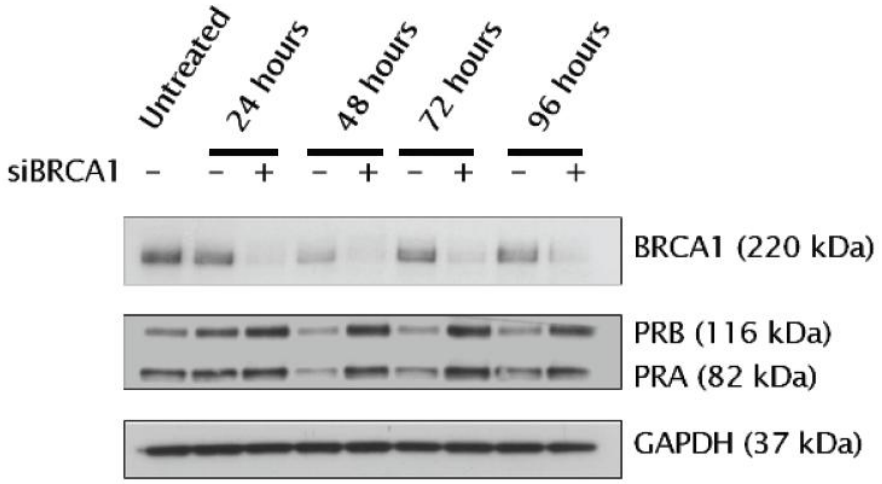


Figure 17. Increase in PR amount after BRCA1 knock-down.

T47D cells were transfected with 100 ng scrambled siRNA, with a specific siRNA targeting BRCA1, or left untreated. At four timepoints (24, 48. 72 and 96 hours), whole-cell lysates were prepared and subjected to immunoblotting using antibodies directed against BRCA1, PR or GAPDH as a loading control.

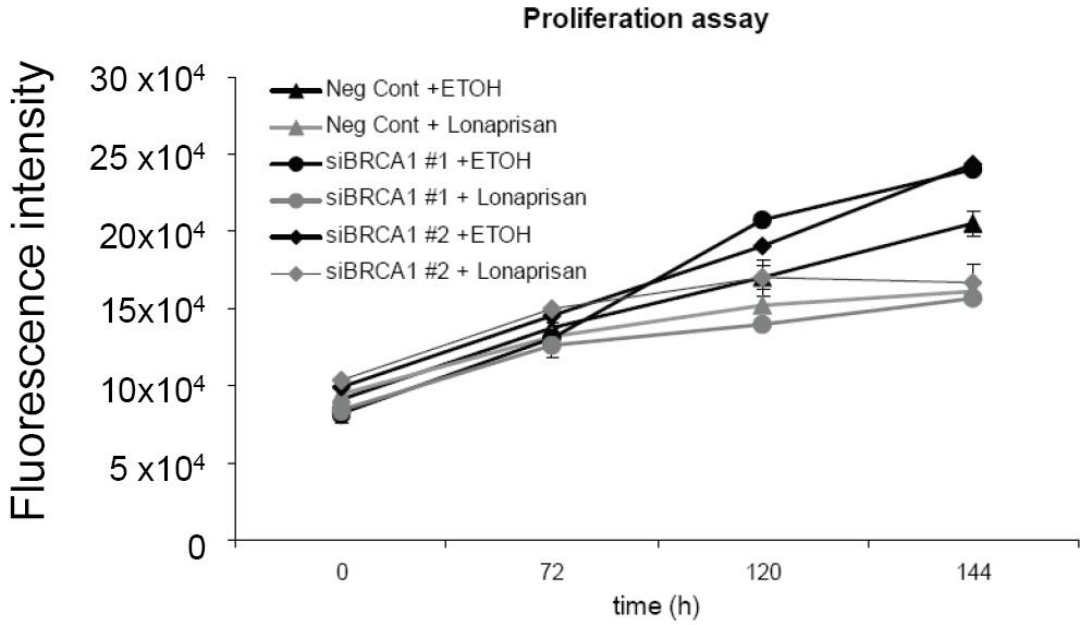


Figure 18. Anti-proliferative effects of Lonaprisan in BRCA1-silenced T47D cells.

T47D cells were transfected with 100 ng scrambled siRNA, a negative control (Neg Cont) or with two specific siRNAs targeting BRCA1, siBRCA1 #1 and siBRCA1 #2. They were then treated with either vehicle, EtOH or 10 nM Lonaprisan for 72, 120 and 144 h. Alamar Blue was then added to the plates at 10% of the culture medium. After two hours of incubation, proliferation was monitored by measuring the fluorescence emitted by a REDOX indicator.

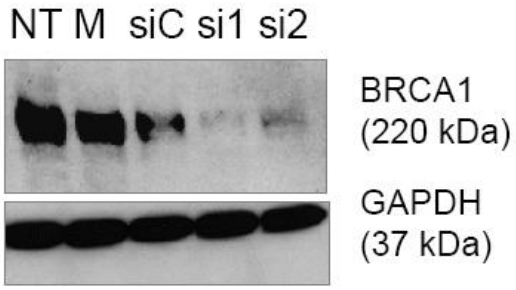


Figure 19. BRCA1 knock down validation.
T47D cells were treated with siRNAs specific for BRCA1 (si1 and si2), with scrambled siRNA control (siC), with transfection reagent only (M) or were left untreated (NT). Cell extracts were prepared and tested for BRCA1 and GAPDH protein levels by immunoblotting.

3.4 Lonaprisan inhibits DNA synthesis and induces G0/G1 arrest

To analyse the influence of Lonaprisan on the cell cycle distribution of T47D cells, FACS analysis was then performed on three consecutive days on these cells treated with 10 nM estradiol and 10 nM Lonaprisan. The cells were analyzed for DNA content using propidium iodide. The inhibition of cell proliferation was associated with a strong reduction of the number of cells in the S phase which was visible already after one day of treatment (**Fig. 20**). Also, an arrest in the G0/G1 phase of the cell cycle was observed after Lonaprisan treatment, and this effect was more pronounced over time.

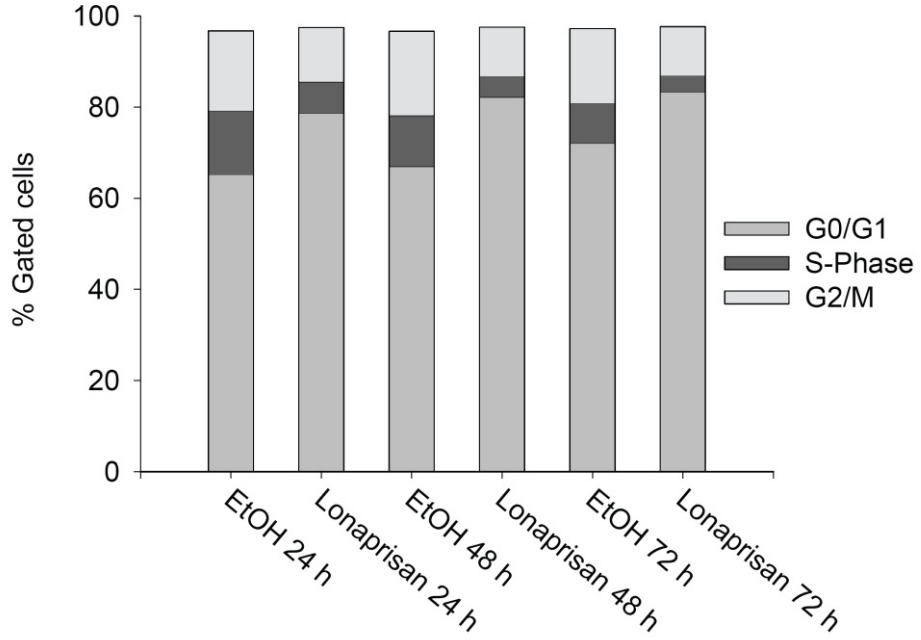


Figure 20. Cell phase distribution after Lonaprisan treatment.
Cell cycle analysis. T47D cells were fixed with EtOH and stained with propidium iodide following treatment with 10 nM Lonaprisan for up to 72 hours.

To find out whether the inhibitory action of Lonaprisan was associated with apoptotic cell death, a Caspase-Glo 3/7 Assay was performed (**Fig. 21**). T47D cells were grown for three days and treated with different compounds. Treatment with Lonaprisan, with the progestin R5020 or with both compounds simultaneously had no apoptotic effect. Treatment with the antiestrogen tamoxifen led to an induction of apoptosis, but only in the absence of estradiol. A combined treatment with tamoxifen and Lonaprisan gave similar apoptotic signals. As a positive control, T47D cells were incubated with the cytotoxic agent Taxol. Here, a strong induction of the apoptotic pathway was observed in absence and more so in presence of estradiol. The intensity of the apoptotic signals was however reduced following a combination treatment of Taxol with Lonaprisan.

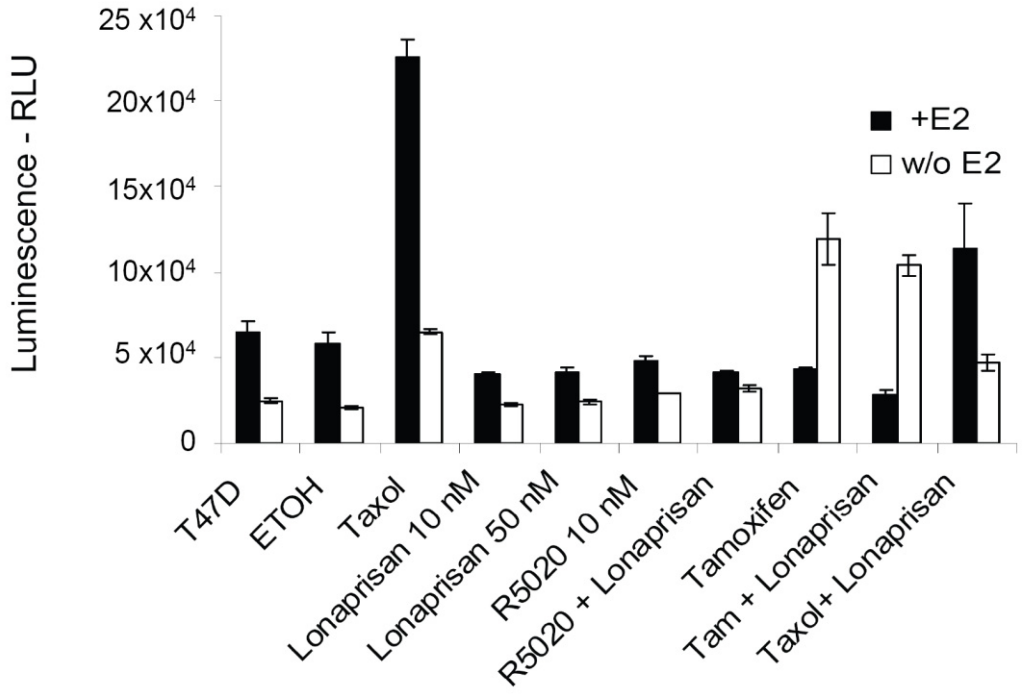


Figure 21. Caspase 3/7 activation by PR ligands.

Caspase-3/7 enzymatic activity. T47D cell lysates containing equal cell numbers were assayed in triplicate for protease activity which was monitored by DEVD-AFC cleavage. The error bars represent the standard deviations.

3.5 The cytostatic activity of Lonaprisan is reflected in a senescence-like phenotype

Lonaprisan-treated T47D cells underwent a permanent cell cycle arrest and had a different aspect, mainly a larger size and flat morphology, characteristic of a senescence-like phenotype. In order to confirm this, a senescence assay was performed. First, the viability of the cells was examined. T47D cells treated with 10 nM Lonaprisan for three days were exposed to the fluorochromes calcein AM and EthD-1. Calcein AM is a cell-permeable compound that is converted by cytoplasmic esterases into a green fluorescent product. EthD-1 binds to DNA and emits red fluorescence only if the plasma membrane is damaged and allows penetration of the substance. Viability of the cells treated with Lonaprisan was evidenced by the conversion of calcein AM into a green fluorescence product and the lack of red fluorescence in the nuclei (**Fig. 22A-B**). As a positive control, T47D cells were treated with 0.1% saponin to permeabilize the

plasma membrane and exposed to calcein AM and EthD-1, as before. **Fig. 22C** shows that this allowed EthD-1 to enter into the cells and to bind to DNA, as evidenced by the red fluorescence.

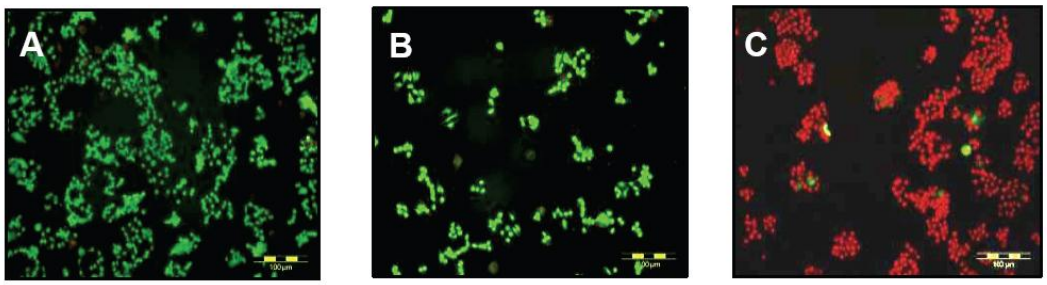


Figure 22. Effect of Lonaprisan on the viability of T47D cells.
Viability/cytotoxicity assay. **A.** Exposure to EtOH, vehicle control. **B.** Treatment with 10 nM Lonaprisan. **C.** Treatment with 0.1 % saponin. Compound treatment was for 72 h and followed by exposition to calcein AM and EthD-1. Magnification: x100.

Then senescence was determined using a senescence-associated β-galactosidase assay. After three days of treatment, positive β-gal staining of Lonaprisan-treated T47D cells was observed (**Fig. 23B**). This was not seen for the vehicle-treated cells (**Fig. 23A**). As a positive control, the cells were incubated for three days with 350 nM doxorubicin, a DNA-damaging agent known to induce a cell cycle arrest resembling replicative senescence (**Fig. 23C**).

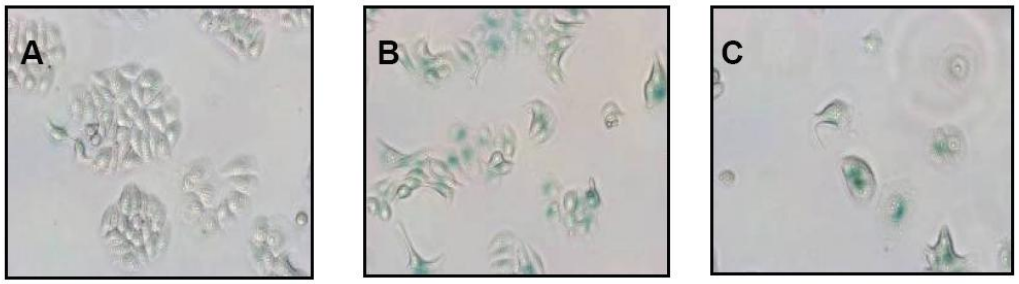


Figure 23. The cytostatic activity of Lonaprisan is reflected in a senescence-like phenotype.
Senescence assay. **A.** Cells grown in normal medium. **B.** Treatment with 10 nM Lonaprisan. **C.** Treatment with 350 nM doxorubicin. Compound treatment was for 72 h and followed by staining with SA-β-gal which leads to blue precipitation in the cytoplasm of senescent cells. Magnification: x200.

3.5.1 Lonaprisan inhibition of Connexin43 mRNA expression

To further support the observation that Lonaprisan induced senescence, the level of Connexin43 (Cx43) expression was determined. Statuto et al. (Statuto et al., 2002) previously reported a progressive decrease of Connexin43 and cell-cell communication during replicative senescence of cultured HEL-299 fibroblasts, and termed Cx43 as a biomarker of cell senescence. Data from gene expression profiling experiments previously performed in our laboratory already showed Connexin43 to be one of the most strongly down-regulated gene after Lonaprisan treatment. To confirm this result, real-time PCR was performed in T47D cells after incubation with 10 nM Lonaprisan for up to 144 hours. Indeed a strong reduction of Connexin43 expression was observed as early as 6 h post Lonaprisan treatment. This effect was more pronounced up to 96 h, after which a small increase was seen, probably due to metabolisation of Lonaprisan (**Fig. 24**).

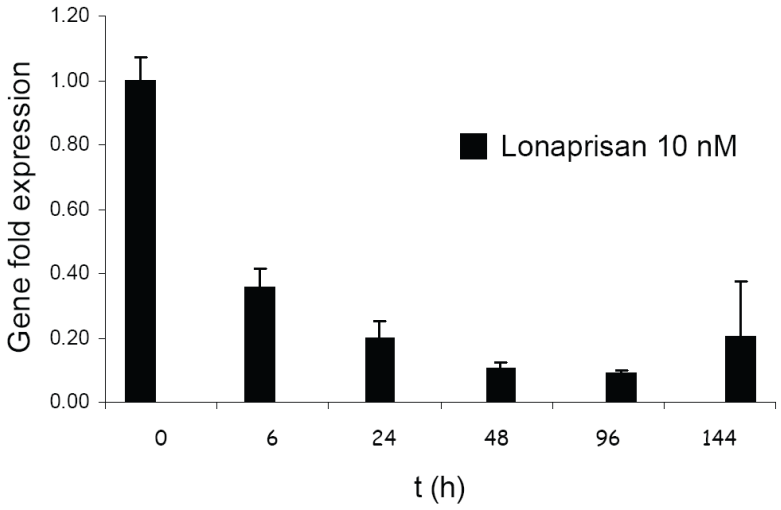


Figure 24. Reduced expression of connexin43 after Lonaprisan treatment.
T47D cells were incubated for up to 140 h with 10 nM Lonaprisan. At the indicated timepoints, the cells were harvested and total RNA was extracted. The RNA was subjected to RT-PCR to determine the mRNA levels for Connexin43 or GAPDH.

3.6 Lonaprisan and R5020 induce the expression of p21 mRNA and protein

In order to further understand the molecular mechanisms involved in the G0/G1 cell cycle arrest induced by Lonaprisan, I analysed the induction of the cyclin-dependent kinase inhibitor p21, an established senescence marker (Collado and Serrano, 2006) and a previously described late progestin-responsive gene (Groshong et al., 1997). The regulation of p21 expression by PR ligands has been reported in several studies. Early work showed induction of p21 by progesterone (Groshong et al., 1997); (Owen et al., 1998) and by the PR antagonists RU486 and ORG 31710 (Musgrove et al., 1997). Later work suggested the progestin R5020 and the PR antagonist RU486 to enhance the transcription of p21 and other Sp1-dependent genes by an indirect mechanism (Shatnawi et al., 2007). More recently however, a direct p21 induction by progestin has been reported (Faivre et al., 2008). Recruitment of agonist-bound PR to the p21 promoter was shown, and this was prevented by RU486 cotreatment. To clarify the action of Lonaprisan in the regulation of p21 expression, I looked at p21 expression in T47D cells exposed to 10 nM Lonaprisan for 0 to 120 hours (**Fig. 25**).

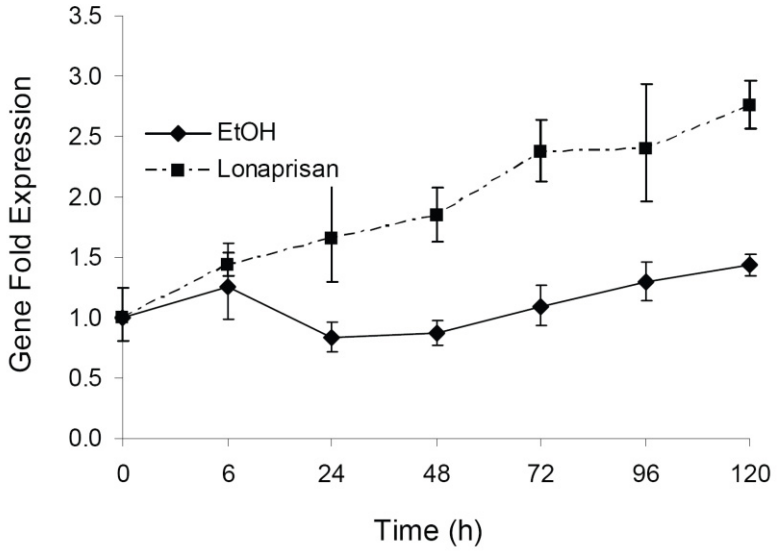


Figure 25. Induction of endogenous p21 mRNA and protein by PR ligands.
T47D cells were incubated for up to 120 h with 10 nM Lonaprisan. At the indicated timepoints, the cells were harvested and total RNA extracted. The RNA was subjected to RT-PCR to measure mRNA levels for p21 or GAPDH.

The expression of the p21 gene was steadily up-regulated between 6 and 120 h post-treatment, in comparison to the ethanol-treated cells. The effects of Lonaprisan were then compared with those of R5020 by real-time PCR and Western blot analysis. T47D cells were treated for one to three days with either ligand and analyzed. This revealed that after one day of treatment Lonaprisan and R5020 showed similar stimulation of p21 expression (**Fig. 26A**). At later timepoints R5020 showed little induction whereas the effects of Lonaprisan remained at the same level for up to 72 hours. Western blot analysis showed that Lonaprisan and R5020 had differential effects on p21 protein levels as well (**Fig. 26B**).

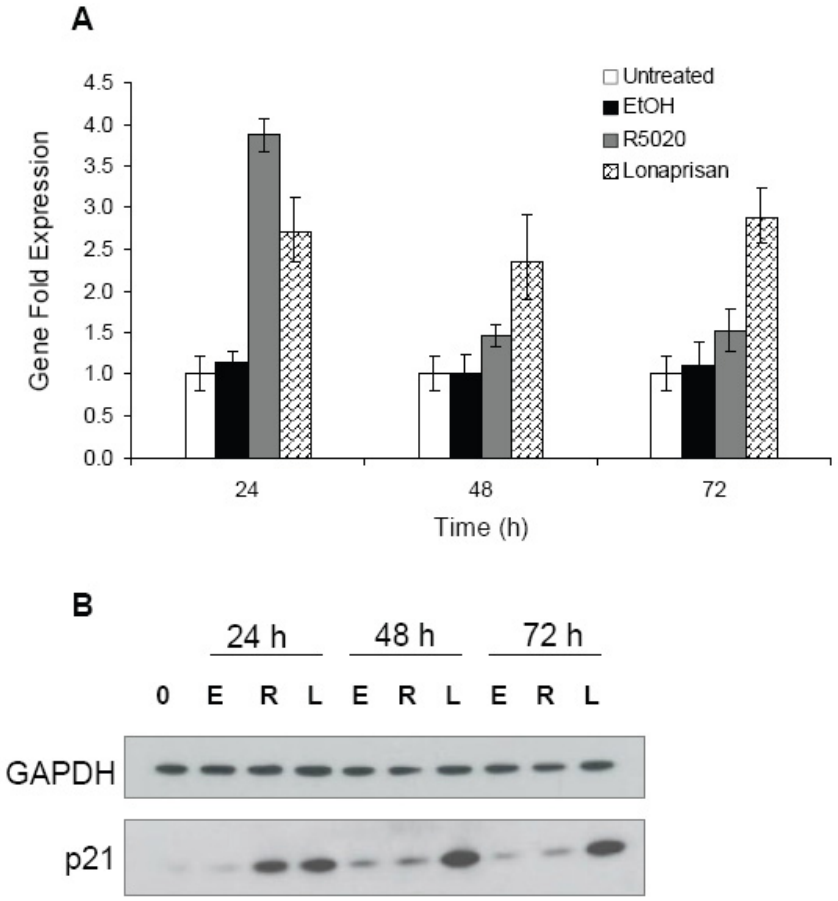


Figure 26. Stimulation of p21 expression by Lonaprisan and R5020 in T47D cells.

A. T47D cells were incubated for the indicated times with vehicle, R5020 and Lonaprisan before determining p21 transcript levels by RT-PCR. **B.** Western blot of total lysates from T47D cells treated with EtOH, E, R5020, R and Lonaprisan, L for the indicated timepoints and probed with p21- or GAPDH-specific antibodies.

An increase in p21 protein was only observed after 24 hours of R5020 treatment, but not at later timepoints. Conversely, Lonaprisan addition was followed by an increase in p21 protein up to 72 hours post-treatment. Finally, the stimulatory effects of PR ligands were confirmed in another cellular background by using HeLa cells. Since these cells do not express endogenous PR, a transient transfection with a PR-B expression vector was performed before treatment with the two compounds (**Fig. 27**). Here also, an induction of p21 expression by Lonaprisan and by R5020 was observed, but only when PR-B was overexpressed.

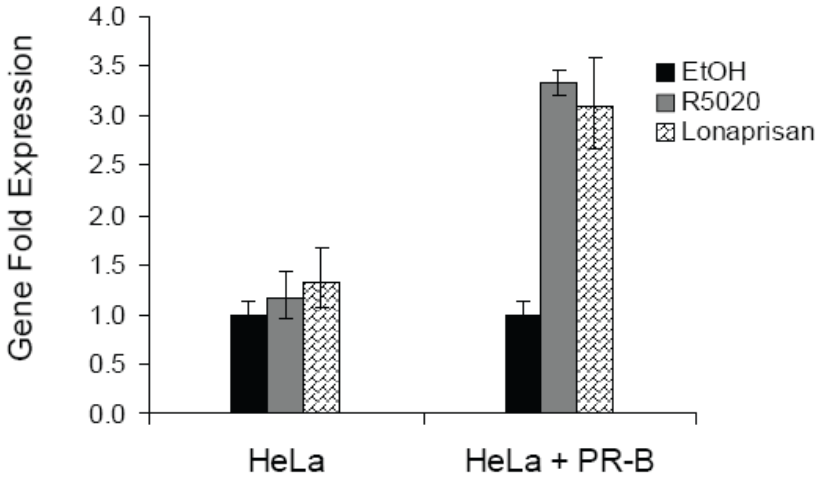


Figure 27. Stimulation of p21 expression by Lonaprisan and R5020 in HeLa cells.
HeLa cells were transfected with an expression vector for the PR-B isoform and treated with Lonaprisan or the progestin R5020 for 24 hours. The levels of p21 mRNA were quantified by RT-PCR.

3.7 p21 silencing reduces the inhibitory potential of Lonaprisan

As p21 induction upon exposure to Lonaprisan correlated with the inhibition of T47D cells proliferation, it was interesting to know whether p21 was a key player in the observed cell cycle arrest. To address this, an siRNA-mediated knock-down experiment in T47D cells was performed. Three p21-specific siRNAs were transfected using the siPORT Amine transfection solution. A scrambled siRNA was used as control. Cell lysates were tested for p21 and GAPDH protein expression to ascertain that significant reduction had taken place. All three siRNAs were equally effective in decreasing p21 levels compared to the controls, and the protein levels of GAPDH were not affected by

any of these siRNAs (**Fig. 28A**). Then, the Lonaprisan-directed cell cycle arrest was monitored by measuring cell proliferation after p21 silencing. After overnight treatment with the specific siRNAs, fresh medium supplemented with a range of Lonaprisan concentrations (0-100 nM) was added to the cells which were then incubated for another five days. Decreasing p21 expression strongly reduced the inhibitory potential of Lonaprisan, in comparison to the controls (**Fig. 28B**). Thus, endogenous p21 is required for maximal Lonaprisan inhibition of T47D cell proliferation.

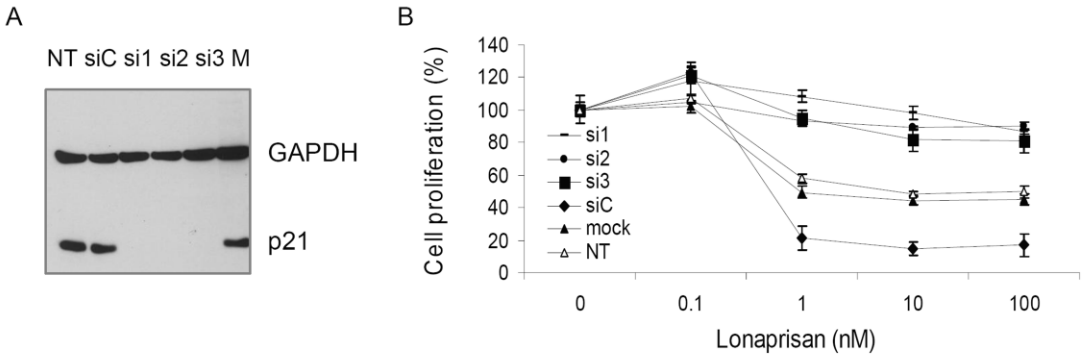


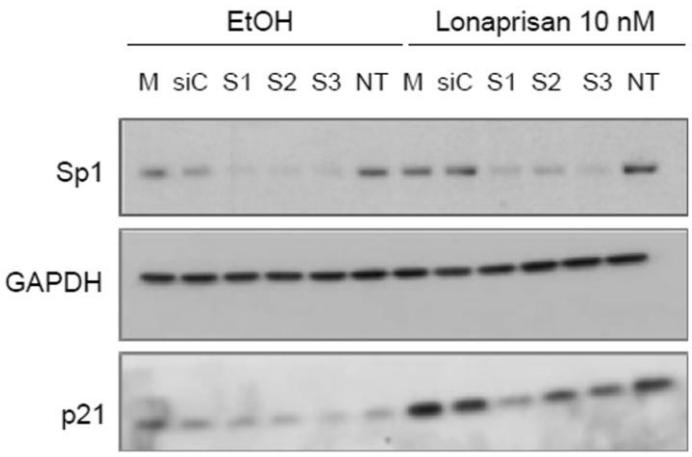
Figure 28. Effect of p21 knock-down on Lonaprisan-induced T47D cell growth arrest.
A. T47D cells were treated with siRNAs specific for p21, si1, si2, si3, with scrambled siRNA control, siC, with transfection reagent only, M or were left untreated, NT. Cell extracts were prepared and tested for p21 and GAPDH protein levels by immunoblotting. **B.** T47D cells were transfected as above before treatment with the indicated Lonaprisan concentrations. Cell proliferation was measured 5 days later by Alamar Blue staining.

3.8 Sp1 transcription factor supports the p21 induction by Lonaprisan

The mechanisms responsible for p21 regulation by PR are not clearly understood. It has been proposed that PR is recruited to the promoter of p21 through interaction with the Sp1 transcription factor (Faivre et al., 2008); (Owen et al., 1998). To verify if the stimulation in p21 expression by Lonaprisan necessitated the presence of Sp1, I examined the consequence of Sp1 silencing on the p21 induction. Three siRNAs targeting the Sp1 gene were designed and transfected into T47D cells. The efficacy of the knock-down was determined at the protein level (**Fig. 29 A**). A dramatic reduction was observed in comparison to the controls and the extent of Sp1 knock-down was similar in presence and in absence of 10 nM Lonaprisan. No changes were noted in the

GAPDH protein levels. In ethanol-treated cells, there was little difference, regardless of the Sp1 status, at the p21 level. Strikingly, in Lonaprisan-treated cells, Sp1 knock-down dramatically reduced the extent of p21 stimulation evoked by the PR antagonist, at both protein (**Fig. 29 A**) and mRNA (**Fig. 29 B**) levels.

A



B

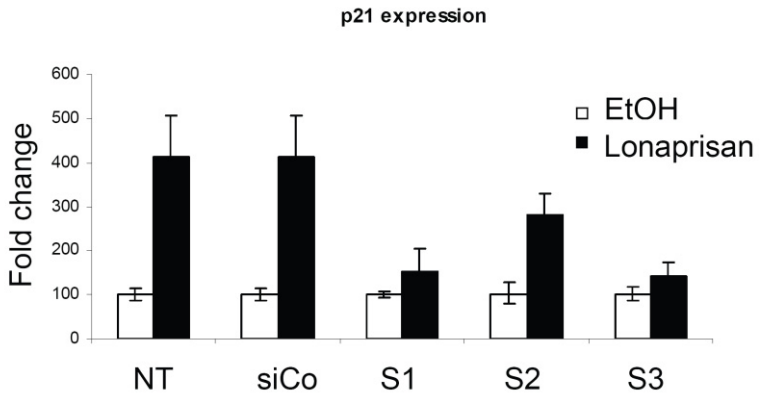


Figure 29. Sp1 is essential for p21 induction by Lonaprisan.

A. T47D cells were transfected with 100 ng scrambled siRNA, siC, with specific siRNAs targeting Sp1, S1; S2; S3, with transfection reagent only, M or left untreated, NT. They were then treated with either vehicle, EtOH or 10 nM Lonaprisan for 48 h. Whole-cell lysates were prepared and subjected to immunoblotting using antibodies directed against Sp1, p21 or GAPDH as a loading control. **B.** T47D cells were treated as above and the levels of p21 mRNA were quantified by RT-PCR.

3.9 PR-B binds to the p21 promoter upon exposure to Lonaprisan and R5020

Rapid recruitment of PR-B and Sp1 to the GC-rich p21 proximal promoter following treatment with the PR agonist R5020 has been shown (Faivre et al., 2008). To find out whether Lonaprisan-bound PR was also recruited to the p21 promoter, ChIP assays were performed (**Fig. 30**). These experiments were carried out first in MCF-7 cells for which I had an optimised protocol. As these cells express low PR amounts only, they were transfected with a PR-B-expressing vector prior to compound treatment. For ChIP, PR or Sp1 were immunoprecipitated from cross-linked chromatin preparations by using specific antibodies. After reversion of cross-linking and isolation of DNA, real-time PCR was performed using p21 promoter-specific primers flanking the proximal GC-rich Sp1 sites. Primers for amplification of a sequence distal to the promoter of the p21 gene were used as control. These results show that in response to R5020 or to Lonaprisan treatment, PR-B was rapidly recruited to the p21 promoter region in the vicinity of the Sp1-binding site (**Fig. 30A**). Sp1 was also present in this regulatory region, albeit in a ligand-independent fashion (**Fig. 30B**). Furthermore, the direct induction of p21 by Lonaprisan in T47D cells was also demonstrated. ChIP experiments were performed in T47D cells after 24 hours Lonaprisan and R5020 treatment (**Fig. 31 A,B**). The results show a much stronger recruitment of PR to the p21 promoter after Lonaprisan treatment compared to R5020 and to the vehicle control. Also in this case Sp1 was already present in the p21 promoter mainly in a hormone-independent fashion.

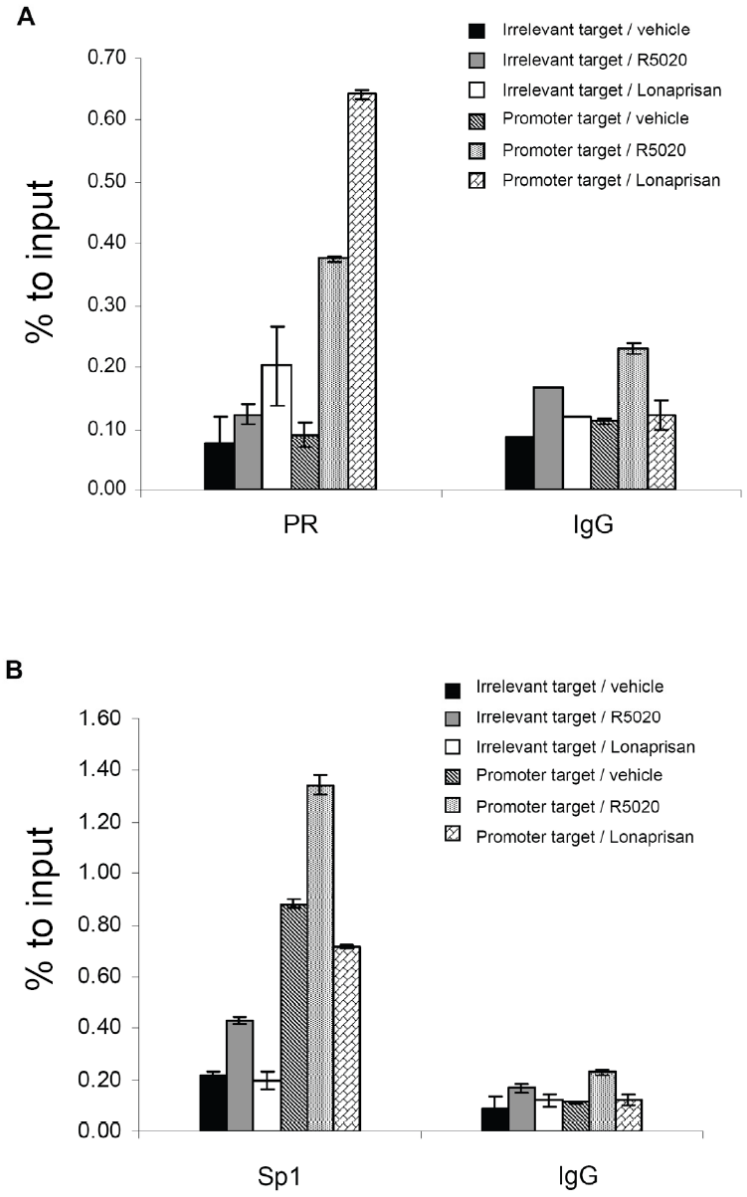


Figure 30. PR-B is recruited to the p21 promoter after treatment with PR ligands.

MCF-7 cells were transiently transfected with a PR-B expression vector and treated for 60 min with vehicle, R5020 or Lonaprisan. ChIP was then performed with antibodies directed against PR (panel **A**) or Sp1 (panel **B**), or rabbit IgG for a specificity control. Primers specific for the Sp1 elements located in the basal p21 promoter were used to measure enrichment at the target sequences by real-time PCR. Primers for an irrelevant target sequence distal to the p21 promoter were used in the control. Signal intensities are depicted as fold differences in relation to the irrelevant targets in the vehicle-treated controls.

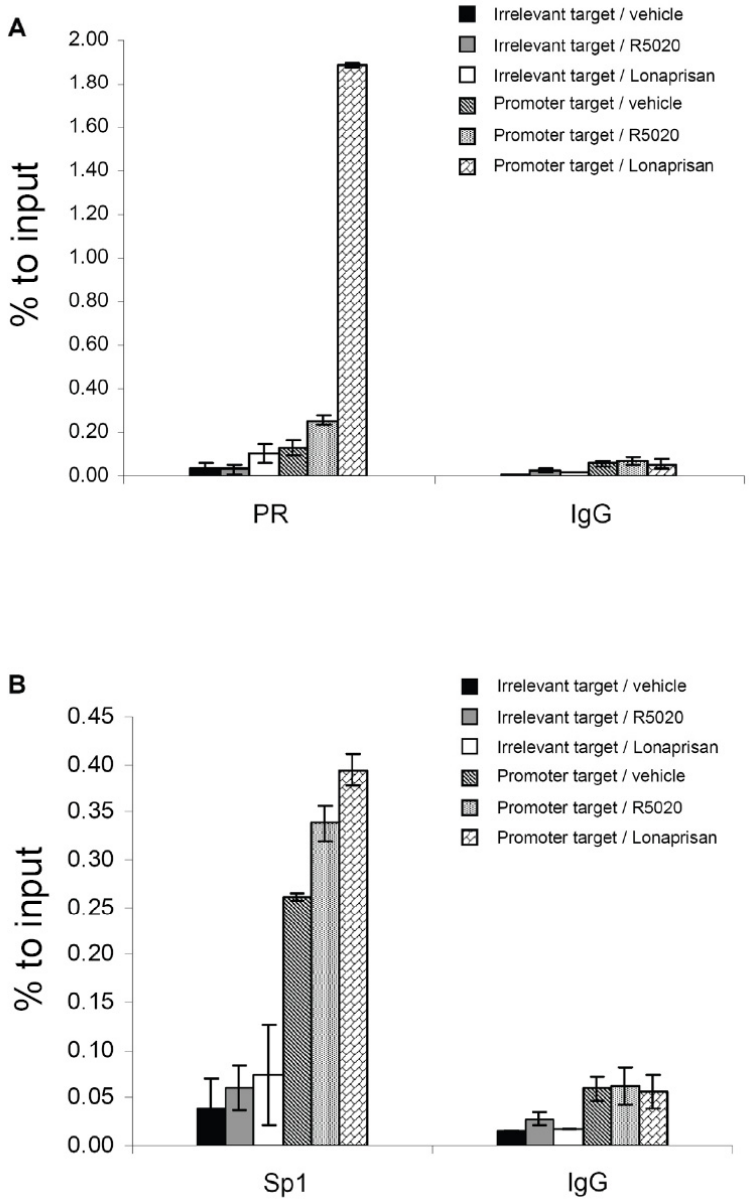


Figure 31. PR-B is recruited to the p21 promoter after treatment with PR ligands.

T47D cells were treated for 24 hours with vehicle, R5020 or Lonaprisan. ChIP was then performed with antibodies directed against PR (panel **A**) or Sp1 (panel **B**), or rabbit IgG for a specificity control. Primers specific for the Sp1 elements located in the basal p21 promoter were used to measure enrichment at the target sequences by real-time PCR. Primers for an irrelevant target sequence distal to the p21 promoter were used in the control. Signal intensities are depicted as fold differences in relation to the irrelevant targets in the vehicle-treated controls.

3.10 The p21 promoter is activated by PR in a ligand-dependent manner

To confirm the ligand-dependent regulation of the p21 promoter by PR, a luciferase reporter assay was used. Two different vectors containing either the 2400 bp-long p21 large promoter or only a 93 bp-long short region linked to a luciferase reporter gene were used (**Fig. 32A**).

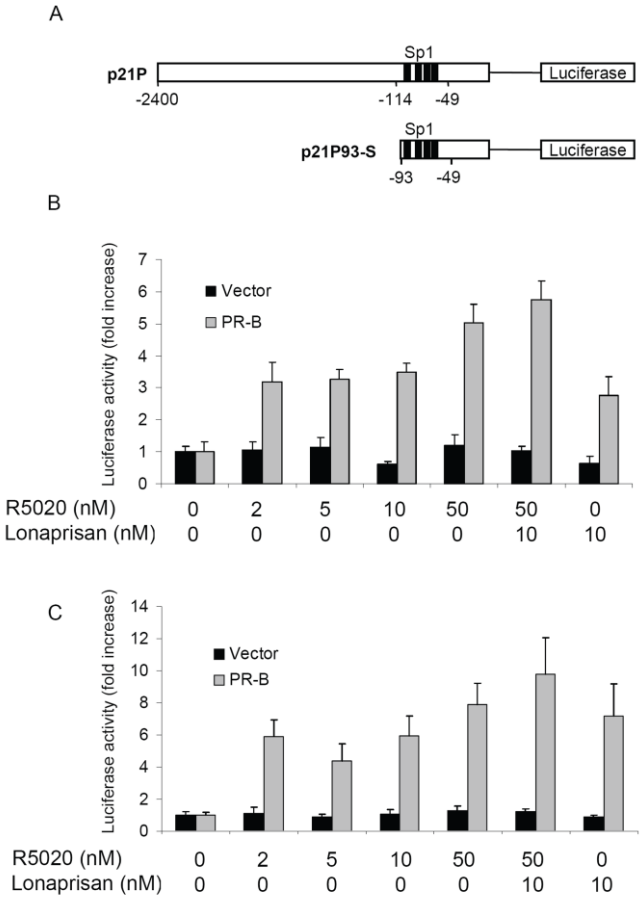


Figure 32. Regulation of the p21 promoter by PR ligands.

A. Schematic representation of the full-length p21 promoter-luc, p21P and the p21 minimal promoter-luc, p21P93-S reporter constructs. **B.** HeLa cells were transfected with the full-length p21-promoter-luc plasmid and an expression plasmid for either PR-B or empty vector. The cells were then treated with R5020 and/or Lonaprisan, and harvested 24 h later to measure luciferase activity. **C.** HeLa cells transfected with the p21 minimal promoter luc reporter plasmid were treated as above. The values represent the fold increase relative to the vehicle-treated control. All the determinations were made in triplicate, and the standard deviation is indicated.

Both promoter regions contain four Sp1-binding sites previously shown to be responsible for regulation by progesterone. HeLa cells were transiently transfected with a reporter construct and a PR-B expression vector. Following Lonaprisan treatment, a strong increase of reporter activity was observed both for the long and the short promoter forms (**Fig. 32B and 32C**). This was also seen after treatment with R5020 or when both compounds were given simultaneously (**Fig. 32B and 32C**).

3.11 PR ligands induce Ser 345 PR phosphorylation

Next it was investigated how Lonaprisan-bound PR regulated p21 expression. Non-genomic effects subsequent to PR phosphorylation have been associated with p21 expression control (Faivre et al., 2008). Following agonist binding, rapid phosphorylation at Ser 345 takes place, thus leading to binding of PR to the Sp1 transcription factor and regulation of p21 gene expression. It was therefore investigated whether Lonaprisan treatment was also followed by phosphorylation of the PR at this position. Following 48 hours of starvation, T47D cells were treated with Lonaprisan or R5020. The cell lysates were prepared and subjected to Western blotting using a phospho-specific Ser 345 antibody. A robust phosphorylation of PR-B and of the shorter PR-A form at this position was observed after 30 and 60 min of treatment with either ligand, or when both were given in combination (**Fig. 33A**). Three additional PR ligands with antagonistic properties were also tested. RU486 stimulated Ser 345 phosphorylation at 30 and 60 min, albeit to a lesser extent than Lonaprisan. Contrarily, onapristone and asoprisnil showed no, or only a slight effect on Ser 345 phosphorylation (**Fig. 33B-C**).

To confirm these results in a different cell system, HeLa cells were treated similarly. As previously, a transient transfection with a PR-B-expressing plasmid was performed (**Fig. 33D**). Two different concentrations were used for transfection and equal levels of expression were achieved. Upon exposure to R5020 or Lonaprisan for 30 and 60 min, an intense Ser345 phosphorylation was detected in the treated samples compared to the vehicle controls. This was not observed in the non-transfected or in the untreated PR-B-transfected HeLa cells.

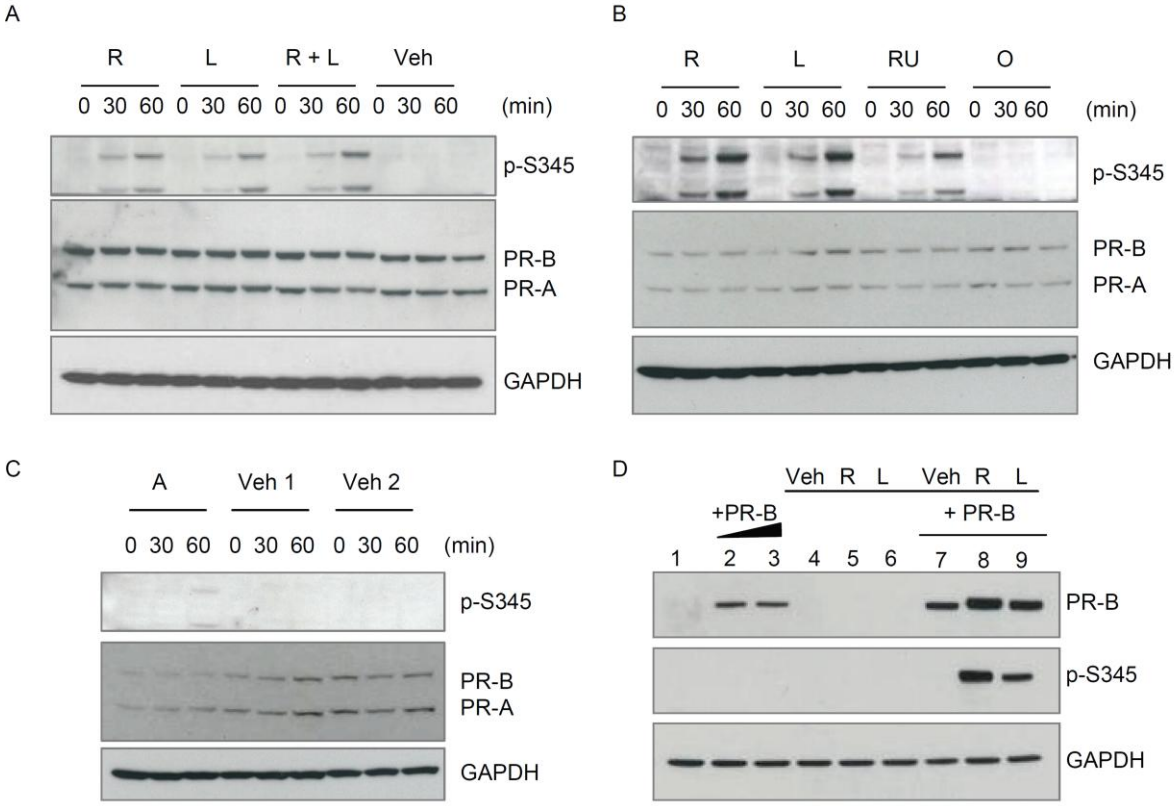


Figure 33. PR ligands induce Ser 345 PR phosphorylation.

A. T47D cells were treated with R5020, R, Lonaprisan, L or R5020 + Lonaprisan, R+L at the indicated timepoints. Cell lysates were prepared and subjected to immunoblotting using an antibody specific for phospho-Ser 345. Antibodies against total PR or GAPDH were used as loading controls. **B.** T47D cells were treated at the indicated timepoints with the following PR ligands: R5020, R, Lonaprisan, L, mifepristone, RU, onapristone, O and **C.**, with asoprisnil, A. Whole-cell lysates were prepared and analysed by immunoblotting as above. **D.** HeLa cells were transfected with a PR-B expression vector and incubated for 24 hours with vehicle, EtOH, R5020, R or Lonaprisan, L. Whole-cell lysates were prepared and analysed by immunoblotting as above.

3.12 Ligand-dependent activation of the p21 promoter by PR mutants

Having established that PR bound to the p21 promoter and needed Sp1 for regulation, it was important to further define which signalling pathway was involved. Three different PR-B mutants were generated for that purpose. The Ser344/345Ala (S344/345A) PR-B mutant lacks the phosphorylation site previously shown to be activated by Lonaprisan.

As expected this mutant was not recognised by the phospho-Ser345 specific Ab (**Fig. 34 A**).

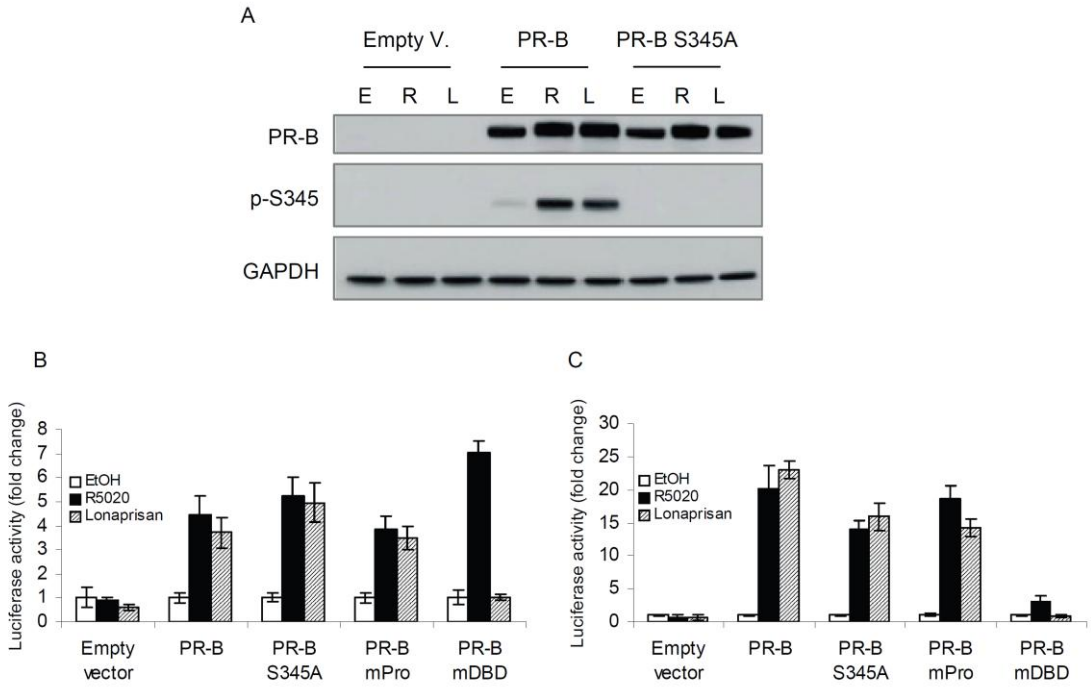


Figure 34. Ligand-dependent activation of the p21 promoter by PR mutants.

A. Western blot of total cell lysates from HeLa cells transfected with either PR-B wild-type or the Ser345A mutant, or with an empty vector. Treatment was for 24 hours with vehicle, E, R5020, R or Lonaprisan, L. Immunoblotting was performed with an antibody against the PR or against phospho-Ser345 PR. GAPDH levels were determined as loading control. **B.** and **C.** HeLa cells were co-transfected with either the p21 long promoter or the p21 P93-S small promoter constructs, and with expression plasmids for wild-type PR-B, or for the mutants S345A, mPro and mDBD. The cells were then treated as indicated with vehicle, 10 nM R5020 or 10 nM Lonaprisan. After 24 h treatment, the cells were lysed and luciferase activity was determined. The values for luciferase activity are expressed as fold increase compared to the corresponding vehicle-treated control.

When expressing this mutant in HeLa cells, it was surprisingly found it had the same stimulatory effect as the wild-type form on the p21 short and large promoter, after treatment with either Lonaprisan or R5020 (**Fig. 34B-C**). As the activation of c-Src following PR phosphorylation is critical for the interaction with Sp1, I tested a PR mutant where the Src interaction motif had been altered (mPro). This mutant was also able to stimulate both p21 promoter constructs, like the wild-type PR form did. Finally,

I tested a PR mutant with impaired DNA-binding properties. The mDBD mutant (C587A) cannot properly form the first zinc finger domain which is necessary for DNA binding. Upon transfection of the corresponding expression plasmid, a strong stimulation of the long p21 promoter was observed when treating the cells with R5020 but not with Lonaprisan (**Fig. 34B**). The situation was different for the short p21 promoter. Here, neither R5020 nor Lonaprisan treatment was followed by stimulation (**Fig. 34C**).

3.12.1 Progesterone receptor stability in the PR mutants

To further investigate the results of the luciferase assay performed with the PR mutants, the PR stability after transfection was studied using Western blot analysis (**Fig.35**). Although an increase in PR was visible after treating the cells with R5020 and Lonaprisan, no marked differences were noted among the different PR mutants.

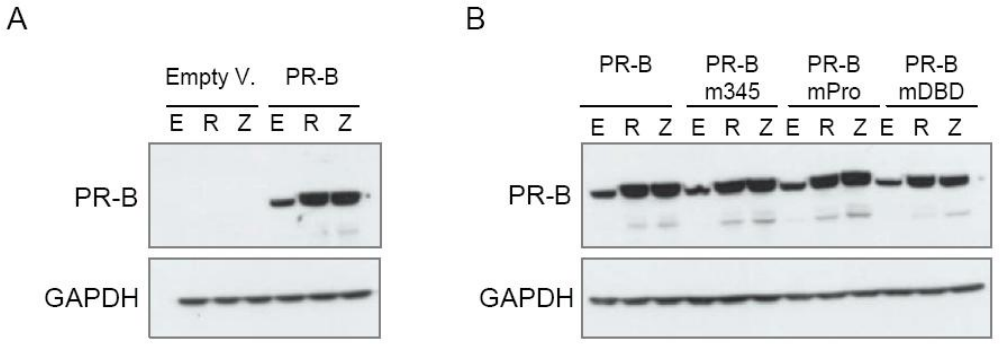


Figure 35. Western blot of PR mutants.
Western blot of total cell lysates from HeLa cells transfected with either PR-B wild-type or the S345A, mPro and mDBD mutants, or with an empty vector. Treatment was for 24 hours with vehicle, E, R5020, R or Lonaprisan, L. Immunoblotting was performed with an antibody against the PR. GAPDH levels were determined as loading control.

3.13 Endogenous p21 and Sp1 are required for PR increase by Lonaprisan treatment

Lonaprisan was able to induce p21 gene expression for a longer time than R5020 and this up-regulation correlated with cell proliferation arrest. Moreover an increased PR content, both at the mRNA and at the protein level, was observed after treatment with

Lonaprisan, in comparison to R5020 exposure (**Fig. 36 A-B**). Since p21 is recruited to the PR promoter and functions as a regulator of ER transcriptional activity (Fritah et al., 2005), it was interesting to investigate whether a positive feedback loop between PR and p21 existed. I therefore examined the consequence of Sp1 and p21 knock-down on PR increase after Lonaprisan treatment. After knocking-down the expression of Sp1 and p21, using in both cases three different siRNAs, a reduction of PR-A but not of PR-B was visible (**Fig. 37 A-B**).

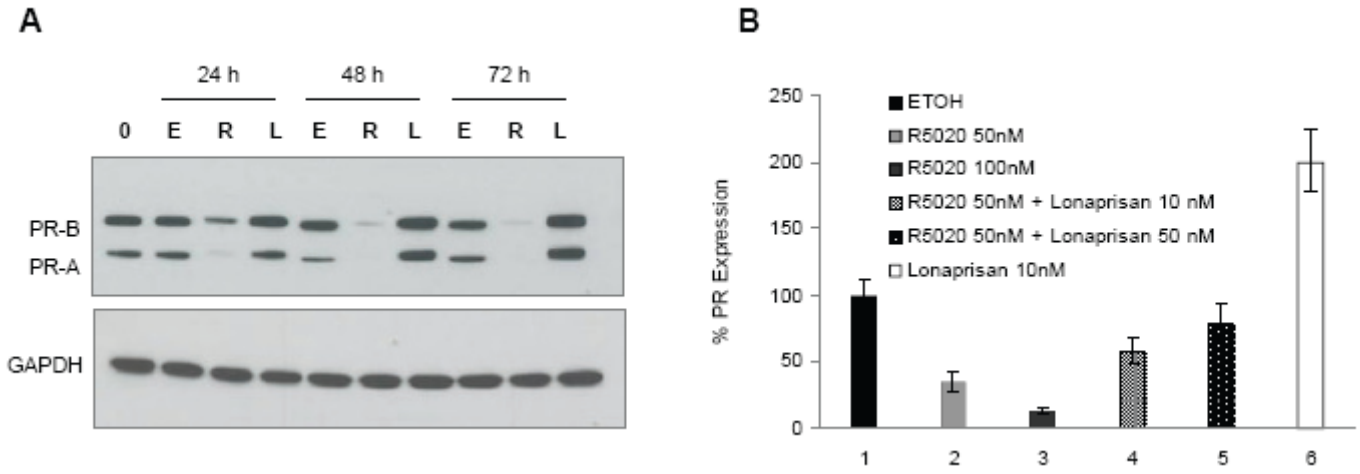


Figure 36. PR expression after treatment with cognate ligands.

A. T47D cells were exposed to vehicle (E), 10nM R5020 (R) or 10nM Lonaprisan (L) for the indicated time points. Whole-protein extracts were obtained and separated by electrophoresis, and the immunoblots were probed with PR and GAPDH antibodies. **B.** PR mRNA expression was measured by real-time PCR in T47D cells treated for 24 h with vehicle, R5020 and Lonaprisan, alone or in combination. The values represent percentage of induction of PR mRNA compared with vehicle-treated controls and normalised to GAPDH.

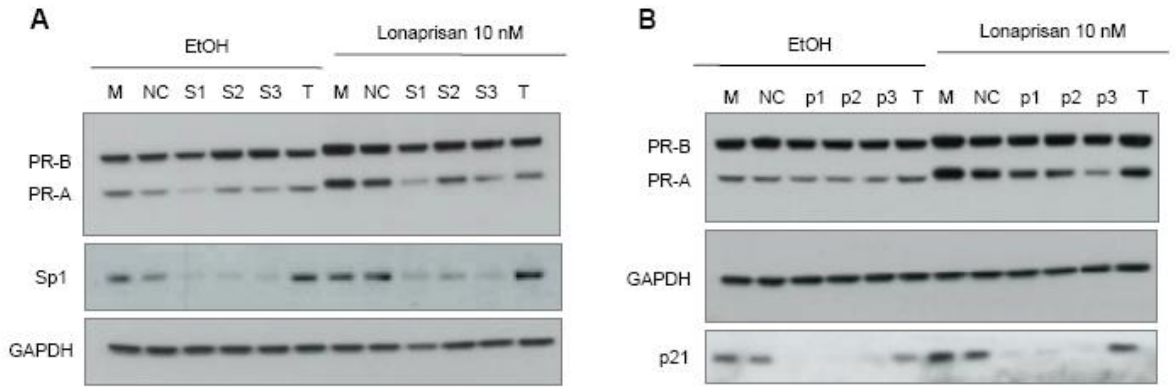


Figure 37. Endogenous p21 and Sp1 are required for PR increase by Lonaprisan treatment.
A., B. T47D cells were transfected with three different Sp1 siRNA (S1, S2, S3), three p21 siRNA (p1, p2, p3) or a scrambled siRNA control (NC), and then treated with 10 nM Lonaprisan. After 48 h of treatment, the cells were harvested and the total cell lysates were probed with antibodies against Sp1, p21, PR and GAPDH as a loading control. M (mock), T (T47D cells untreated).

To further investigate how p21 may control expression of the PR-A isoform, the association of p21 to the PR regulatory regions in T47D cells was examined using ChIP assay (**Fig. 38**). T47D cells were grown in normal culture medium and treated for 24 hours with 10 nM of Lonaprisan. This timepoint was chosen because this is when the induction of p21 expression by Lonaprisan reaches its maximum. A p21-specific antibody was used to immunoprecipitate the crosslinked protein DNA-complexes. The binding to the PR promoter was analyzed by real-time PCR with specific primers spanning the PR-A and PR-B promoter regions. Interestingly, as shown in **Fig. 38**, p21 was recruited to the PR-A but not to the PR-B promoter following Lonaprisan treatment. These results are in line with the existence of a positive feedback loop between the PR and p21 and suggest that the increased expression of p21 by Lonaprisan which is involved in cell cycle arrest may also be responsible for the maintenance of the high level of PR observed after treatment.

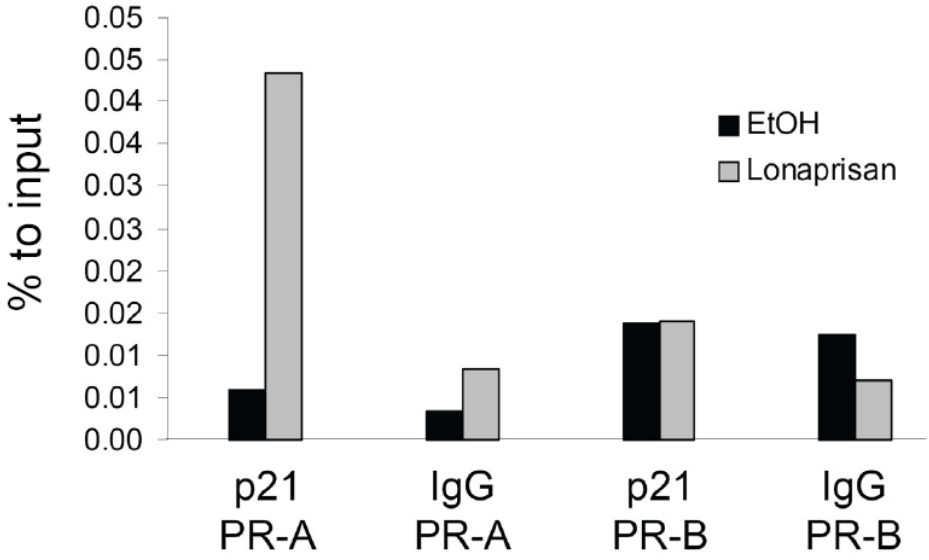


Figure 38. Recruitment of p21 to the PR-A promoter after Lonaprisan treatment.

Cross-linked, sheared chromatin from T47D cells incubated with or without Lonaprisan (EtOH, vehicle control), was immunoprecipitated with a p21 antibody or rabbit IgG as specificity control. Bound DNA was analyzed by real-time quantitative PCR. PR-B, PR promoter region -125 to +187; PR-A promoter region +352 to +616.

4 Discussion

In this work it was shown that the pure PR antagonist Lonaprisan potently inhibited the proliferation of T47D cells by blocking them in the G0/G1 phase and inducing a senescence-like phenotype. Furthermore it was demonstrated that p21 was a central mediator of the effects of Lonaprisan which directly stimulated the p21 promoter after PR binding in a Sp1-dependent fashion. Lonaprisan treatment led to PR phosphorylation at Ser345, but this post-translational modification was not needed for stimulation of the p21 promoter in a cell-based transactivation assay. On the other hand, intact DNA-binding properties of the PR were mandatory for p21 induction by Lonaprisan. It was furthermore shown that p21 was able to induce the expression of PR by direct binding to the PR-A promoter, suggesting the existence of a possible positive feedback loop between PR and p21.

Since the synthesis of the first antiprogestin mifepristone, several other PR antagonists with different pharmacological profiles have been identified (Spitz, 2006). Three types have been described based on their *in vitro* properties (Afhuppe et al., 2010). While Lonaprisan belongs to the class III of PR antagonists which is characterised by strong PR binding to its cognate response elements, type I antagonists (e.g. onapristone) do not lead to binding of the PR to DNA and exhibit pure antagonism whereas type II antagonists (e.g. mifepristone and asoprisinil) lead to DNA binding and possess a partial, tissue-specific, agonistic activity due to protein kinase A stimulation.

4.1 Antagonistic activity of Lonaprisan on PR

Several synthetic PR ligands have been developed which compete for binding with the natural hormone and are capable of inhibiting receptor activity. Mifepristone (RU486) was the first of these progesterone antagonists that exhibited antiprogesterone activity in humans. It has been used in numerous clinical studies in the gynecologic and obstetrical fields. In this thesis, a third generation PR antagonist was studied in detail. The activity of this compound, named Lonaprisan was demonstrated in the breast cancer cell lines T47D and MCF7 where it strongly inhibited the expression of selected genes known to be induced by progesterone. Antagonising progesterone action by competitive antagonism of PR binding with a synthetic inactive analogue is an effective way to

ablate the physiological action of this hormone (Leonhardt et al., 2003). In addition, prevention of p160 coactivator recruitment by ligands inducing an inactive LBD conformation has the potential to lead to strong antagonistic activity. Interestingly, Mifepristone is able to antagonize progesterone action at substoechiometric concentrations of progesterone. Three mechanisms contribute to this unusual potency. First, mifepristone promotes a higher affinity interaction of PR with the DNA than the agonist R5020 *in vitro* (Edwards et al. 1995) and the antagonist-bound PR can effectively compete with binding of agonist-bound PR to PREs *in vivo* (Leonhardt et al., 2003). Also, Afhueppe and colleagues have shown that Lonaprisan binding leads to PR binding to its response elements with higher affinity than in presence of mifepristone (Afhueppe et al. 2010). A second contributing mechanism is the ability of PR bound to antagonist to heterodimerize with PR bound to R5020. Using coimmunoprecipitation assay *in vitro* (DeMarzo et al. 1992) and by performing a mammalian two-hybrid assay with receptor ligand specificity mutants (Leonhardt et al. 1998), it was demonstrated that PR bound to mifepristone can heterodimerize with PR bound to R5020. Using electrophoretic mobility shift assay (EMSA), it was also observed that mixed R5020/mifepristone heterodimers had a significantly reduced ability to bind to PREs (DeMarzo et al. 1992). These results were similar to the results of Meyer et al. (Meyer et al. 1990) who found in EMSA that mixed R5020/mifepristone heterodimers could not bind to PREs. Heterodimerisation could potentially sequester a portion of cellular PR bound to agonist in an inactive form without requiring direct binding of mifepristone to PR. A third contributing factor to the antagonistic potency of mifepristone is the ability of PR to recruit corepressors to promoters in the presence of this antagonist. PR in the absence of ligand or in presence of agonist has weak affinity for corepressors. Nuclear receptor corepressor (NCoR) was identified by yeast two-hybrid assay as a factor that interacts with mifepristone-bound PR but not agonist-bound PR (Jackson et al. 1997). The recruitment of corepressors by Lonaprisan has also been recently demonstrated (Afhueppe et al. 2010). Using a mammalian two-hybrid system and a pulldown experiment, it was shown that Lonaprisan has higher affinity to the corepressor NCoR than PR bound to mifepristone or onapristone. Corepressors such as NCoR function as part of large protein complexes and may have enzymatic properties including histone deacetylase activity which modulate DNA accessibility and transcriptional activity.

The classical mechanism for PR signalling is the dimerisation of the steroid receptor upon ligand binding, followed by translocation into the nucleus where it binds to PREs. In this thesis it was demonstrated that following Lonaprisan treatment an increased nuclear localisation of the PR occurred. The confocal microscopy data presented in this thesis confirm the enhanced nuclear localisation of the Lonaprisan-bound PR shown by Afhueppe and colleagues who analysed the nuclear and cytosolic fractions of T47D cells (Afhueppe et al., 2010). In addition, by transfecting HeLa cells with the two different PR isoforms, it has been possible to show that Lonaprisan acted on both isoforms.

4.2 Inhibition of cell proliferation by Lonaprisan

It is known from the literature that progesterone can be a proliferative or a differentiating hormone. This paradox was clarified by Groshong and colleagues already in 1997 (Groshong et al. 1997). By using a breast cancer cell line (T47D-YB) that constitutively expresses the PR-B isoform and is resistant to the proliferative effects of epidermal growth factor (EGF), they were able to show that progesterone treatment accelerated the passage of T47D-YB cells through the first mitotic cell cycle, but arrested them in late G1 of the second cycle. This arrest was accompanied by decreased levels of cyclins D1, D3, and E, disappearance of cyclins A and B, and sequential induction of the cyclin-dependent kinase (cdk) inhibitors p21 and p27Kip1 (Groshong et al. 1997). They concluded that progesterone is neither inherently proliferative nor antiproliferative, but capable of stimulating or inhibiting cell growth depending on whether the treatment is transient or continuous. On the contrary, as shown here, the PR antagonist Lonaprisan inhibits breast cancer cell proliferation by immediately arresting the cells in the G1 phase. Moreover, I showed that the accumulation of T47D cells in the G0-G1 phase of the cell cycle was accompanied by a reduction in the number of cells in the G2/M and S phase, and that this cell cycle distribution was maintained over time. Afhueppe et al. have in addition demonstrated that compared to Lonaprisan, onapristone was not able to antagonise estrogen-induced distribution of cell cycle stages and that mifepristone had no effect on the estrogen-induced S phase entry (Afhueppe et al., 2010). It has been suggested that the ability of PR antagonists to reduce the number of cells in S phase may offer a clinical advantage. Indeed, it has been established that the S-phase fraction is a highly significant predictor of disease-free survival among

axillary node-negative patients with diploid mammary tumours (Bergers et al., 1997). In contrast, conventional endocrine therapies for breast cancer such as tamoxifen and ovariectomy do not alter the distribution of cells in the cell cycle phases (Michna et al., 1992).

4.2.1 BRCA1 and hormone dependent tumors

Another important characteristic of Lonaprisan was assessed in this work, namely the anti-proliferative activity in breast cancer cells where the breast cancer susceptibility gene 1 (BRCA1) is silenced. It is known that mutations of BRCA1 lead to an increased risk of breast cancer, ovarian cancer, and several other hormone-responsive tumour types (Thomopson and Easton, 2002). Increasing evidence suggests that BRCA1 functions as a "caretaker" to maintain genomic integrity (Rosen et al., 2003). This function however would not explain the predilection of BRCA1 carriers to develop hormone-dependent cancers. Interestingly, unlike for sporadic cancers where early pregnancy has a risk-reducing effect, in BRCA1 mutation carrier pregnancy increases the risk or accelerates breast cancer development, probably due to the high circulating levels of estrogen and progesterone (Narod, 2001). Previous studies indicate that BRCA1 is able to inhibit PR signalling in breast carcinoma cells (Ma et al., 2006) and knock-down of endogenous BRCA1 results in a four-fold increase in progesterone-stimulated PR activity (Rosen et al., 2003) and in PR protein level, due to a defect in PR degradation by the proteasome pathway (Poole et al., 2006). The results shown in the present work confirm the observed increased PR protein levels after BRCA1 knock-down and demonstrate that Lonaprisan is able to inhibit the proliferation of BRCA1-silenced breast cancer cells *in vitro*. Poole et al. also reported that treatment of BRCA1/p53-deficient mice with the PR antagonist mifepristone prevents mammary tumourigenesis. This finding suggests that an anti-progestin may be successfully used in the treatment of premenopausal breast cancers that are PR-positive and under-express BRCA1 or have a BRCA1 mutation.

4.3 Apoptosis and PR ligands

The results of the cell cycle analysis together with the measurement of the caspase 3/7 activation revealed that Lonaprisan did not induce apoptotic cell death in breast cancer cells and had a protective effect against apoptotic induction when the cells were treated

with the cytotoxic agent taxol. In addition, no induction of apoptosis was measured when combining R5020 with Lonaprisan or with R5020 alone. A similar protective effect from cell death was previously observed by Moore and colleagues when treating T47D cells with progestin (Moore et al., 2006). They showed that progestins protect from serum-starvation-induced apoptosis in T47D cells. However, in contrast to the results reported here, they observed anti-apoptotic effects only in presence of progestins and this was inhibited by the antiprogestin mifepristone. The use of a novel, type III PR antagonist in this work could explain the different result observed here. Moore et al. also found that progestin inhibits cell death caused by chemotherapeutic agents such as doxorubicin and 5-fluorouracil. A recent study has further confirmed the protective effect of progesterone against apoptosis. Morrissy et al. have shown that progesterone but not estrogen protects cardiomyocytes from apoptotic cell death induced by doxorubicin (Morrissy et al., 2010). Again, the cytoprotective effect of progesterone was ablated by mifepristone. Interestingly, the cytoprotective effect elicited by progesterone in cardiomyocytes was associated with induction of the Bcl-xL gene, a known anti-apoptotic protein. Moreover, induction of apoptosis by PR antagonists has also been reported, although in a different cell system. Friberg and colleagues have shown that PR antagonists have apoptotic effects on rat granulosa cells (Friberg et al. 2007). They hypothesised that PGR antagonists and statins induce apoptosis by a common mechanism. The inhibition of cholesterol synthesis reduces the availability of isoprenylation substrates, resulting in decreased functionality of one or several proteins and apoptosis.

4.4 Lonaprisan senescence induction

The antiproliferative effects of Lonaprisan were found to correlate with the induction of senescence. Originally the term “senescence” was defined as a series of cellular changes associated with aging. It now refers more generally to a signal transduction program leading to irreversible arrest of cell growth, accompanied by a distinct set of changes in the cellular phenotype (Shay and Roninson, 2004). It is believed that senescence is a potent cellular program preventing transformation and indeed tumourigenesis involves a series of events that allow cells to bypass senescence. Nevertheless, tumour cells have still retained the capacity to senesce. It has been shown that most conventional anticancer therapies activate DNA damage signalling pathways which usually induce

apoptotic cell death but in some cases lead to a senescence-like terminal growth arrest (Shay and Roninson, 2004). In addition to chemotherapy and radiation therapy, both senescence and apoptosis can be induced by manipulating the expression of essential growth regulatory genes. Overexpression of tumour suppressors or inhibition of oncogenes can indeed induce rapid senescence in tumour cells. In the work of Chang et al. an association between senescence and p21 induction has been reported (Chang et al., 2000). They demonstrated that induction of the cyclin-dependent kinase inhibitor p21Waf1/Cip1/Sdi1 triggers cell growth arrest associated with senescence and damage response, and that overexpression of p21 from an inducible promoter in a human cell line induces growth arrest and phenotypic features of senescence. Furthermore, Collado and Serrano in their review included p21 among the markers of senescence (Collado and Serrano, 2006). The induction of p21 observed upon Lonaprisan treatment correlates well with the senescent phenotype observed in the treated cells, although the link between senescence and the PR to date has only been reported in ovarian cancer cells and in response to cAMP (Takahashi et al., 2009). The rapid G1 growth arrest induced exogenously is however different from the replicative senescence caused by progressive telomere shortening (Sherr, 1998; Sherr and DePinho, 2000; Ramirez et al., 2001; Drayton and Peters, 2002). This growth arrest is similar to replicative senescence since the cell cannot divide even if stimulated by mitogens (e.g. estradiol). Also, as in the case of replicative senescence, these cells remain metabolically and synthetically active and show characteristic changes in morphology (Dimri et al., 1995; Serrano et al., 1997; Drayton and Peters, 2002). Even in the senescence-like phenotype observed after Lonaprisan treatment, the cells remain viable, as demonstrated by the viability-cytotoxicity assay. Both the telomere-based and non telomere-based growth arrest may be due in part to repression of genes required for cell cycle progression and upregulation of growth inhibitory genes. The term “STASIS” (stress or aberrant signalling-induced senescence) has been coined to refer to the common senescence-like arrest mechanism in response to diverse signals and to not confuse this type of growth arrest with replicative senescence (Drayton and Peters, 2002; Wright and Shay, 2002). It is important to note that STASIS may be an evolutionary conserved mechanism adopted by the cells to defend themselves from oncogenic insults. Since both the replicative senescence and STASIS are important anti-tumourigenesis mechanisms, every genetic

or epigenetic change allowing the cells to escape senescence has the potential to contribute to the process of neoplastic transformation.

Senescence, together with mitotic catastrophe and apoptosis, has major antiproliferative effects and is a determinant of the long-term success of cancer therapy. In contrast to cell death, however, senescence leaves tumour cells alive and physiologically active. Senescent cells within the tumour can produce secreted factors with both tumour-promoting and tumour-suppressing activities. More aggressive cancers may be associated not only with the lack of senescence but also with the presence of senescent cells that express tumour-promoting factors and CDK inhibitors that induce them. In contrast, tumours containing senescent cells that express high levels of secreted growth inhibitors but few tumour-promoting factors should have a more favourable prognosis. Although the breast cancer study of Poele et al. (2002) is the only report that directly addresses the induction of senescence during chemotherapy in patients' tumours, indirect observations in radiation therapy suggest that senescence may be a primary mode of treatment response. Gathering more knowledge about the pathways that lead to senescence and the gene expression patterns of senescent cells may help to identify more effective treatments for cancer.

4.4.1 Role of Cx43 in the induction of senescence

In line with the senescence-like phenotype observed after Lonaprisan treatment, a strong down-regulation of the mRNA levels of Cx43 by Lonaprisan has been shown. This gene is a member of the connexin gene family which plays an important role in gap junctions and also in parturition. Statuto et al. (Statuto et al., 2002) reported that decrease in Cx43 expression was associated with the replicative senescence of cultured HEL-299 fibroblasts. Moreover, it has also been shown that the expression and function of Cx43 in astrocytic cells and aortic endothelium were age-related, suggesting a role in the process of cell aging (Jones et al. 2004; Yeh et al. 2000; Cotrina et al. 2001; Dilley et al. 2003). Later, Zhang and colleagues demonstrated that down-regulation of Cx43 expression by high glucose promoted the senescence of glomerular mesangial cells (Zhang et al. 2006). Interestingly, it has been recently shown that the p54nrb transcriptional repressor interacts directly with PR independently of progesterone, leading to down-regulation of Cx43 transcription (Dong et al., 2009). It may therefore

be that the effect of Lonaprisan on the Cx43 gene occurs via formation of a repressive complex including p54nrb.

4.5 p21 induction by PR ligands

Stimulation of p21 and other Sp1-controlled genes has already been observed following PR agonist treatment in conditions leading to proliferation arrest (Groshong et al., 1997); (Gizard et al., 2006). These effects are not mediated by classical binding to PREs but rather by PR tethering to the Sp1 transcription factor and binding to G/C-rich promoter regions (Owen et al., 1998). This additionally involves the TReP-132 coactivator which directly interacts with Sp1 (Gizard et al., 2006). The PR antagonists mifepristone and ORG 31710 also induce p21 expression in sensitive breast cancer cells but no detailed promoter studies have been reported using these ligands (Musgrove et al., 1997). More recently, ChIP experiments showed binding of the PR to the p21 promoter following treatment with the agonist R5020, an effect blocked by mifepristone and necessitating intact Sp1-interacting properties (Faivre et al., 2008). On the other hand, the role of the PR/Sp1 complex in directly stimulating Sp1 target genes has also been questioned (Shatnawi et al., 2007). Both the delayed response and absence of PR association to target promoters following agonist or antagonist treatment were taken as evidence for an indirect mechanism of action involving an unidentified coactivator. In this thesis p21 induction is shown to be more sustained after Lonaprisan than after progestin treatment, in line with the strong antiproliferative properties of the antagonistic compound. I also found that PR bound to the p21 promoter in a ligand-dependent fashion at a region known to be essential for control by Sp1, and this interaction was stronger in presence of Lonaprisan than of R5020. Importantly, binding of Sp1 to this region was constitutive, in line with a PR-dependent activation of prebound Sp1. Stimulation of p21 promoter reporter plasmids by both ligands was comparable and slight addictive effects were observed upon cotreatment.

4.5.1 Role of Ser345 phosphorylation in p21 induction

The implication of Sp1 was demonstrated in this thesis by the knock-down experiments and more indirectly by examining PR Ser345 phosphorylation. Here I showed for the first time that a PR antagonist can lead to strong Ser345 phosphorylation, comparably to the R5020 effects. Cotreatment even showed additivity to take place. RU486 and

especially asoprisnil showed weaker, but still significant effects on PR Ser345 phosphorylation. Surprisingly however, abolishing the Ser345 phosphorylation site or mutating the PR region which is essential for interaction with c-Src and consequent PR phosphorylation was still compatible with full stimulation of the p21 promoter by Lonaprisan. Indeed, only a weak interaction between Lonaprisan-bound PR and c-Src has been found in a mammalian two-hybrid assay (Afhuppe et al., 2010). This and the strong PRE binding observed following Lonaprisan treatment (Afhuppe et al., 2010) suggest that genomic effects predominated here.

4.5.2 PR mutants

In line with this, Lonaprisan was not able to stimulate a PR mutant devoid of PRE-binding ability, as evidenced when using two different p21 promoter constructs. The situation was different for R5020 where a strong stimulation of the long, but not of the short p21 promoter was observed. These results suggest Ser345 phosphorylation and c-Src not to be essential players in the induction of the p21 gene by Lonaprisan. Genomic effects via direct PR binding to the p21 promoter, even though it lacks canonical PREs, are compatible with the results shown in this thesis. Previous work shows PRE half-sites to be sufficient for recognition by the PR (De Amicis et al., 2009), possibly in combination with the flanking DNA region (Roemer et al., 2006). Indeed a careful examination of the p21 promoter region allowed the identification of two PRE half-sites (not shown) and further studies are now needed to find out whether they are recognised by the PR. However it can not entirely be ruled out that gross conformational changes consecutive to mutation in the zinc finger region abolished the interaction of Lonaprisan-bound PR with Sp1. This is however unlikely as R5020 bound to the same mutant can still stimulate the long p21 promoter, which according to the prevailing model necessitates binding to Sp1 (Faivre et al., 2008). Also, binding enrichment of the mDBD PR mutant to the Sp1-binding region of the p21 promoter, as seen for PR wild-type form, was observed after Lonaprisan and R5020 treatment, suggesting that interaction with Sp1 could still take place (not shown). I therefore favour a model where Sp1 is constitutively present at the p21 promoter and activated by liganded PR. PR binding to a distant enhancer site and leading to DNA looping is additionally needed for long-range interacitions and gene activation to take place. The situation may be comparable to that of estrogen-bound ERα which stimulates p21 by direct binding at

half ERE/AP-1 sites. Sp1 is necessary for estrogen-induced p21 stimulation but not involved in ER recruitment (Mandal and Davie, 2010). Further promoter studies with other Lonaprisan-regulated genes (Afhuppe et al., 2009) should help to better understand the specificities of this regulation.

4.6 Increase in PR after Lonaprisan treatment

Often, advanced-stage breast cancers are steroid hormone receptors negative and/or are resistant to endocrine therapies. Positivity for the PR is a key marker of steroid hormone dependence and indicator of disease prognosis in breast cancer. It has been shown that the PR loss is associated with an aggressive tumour phenotype characterised by acquisition of enhanced sensitivity to growth factors (Horwitz et al., 1975; Elledge et al., 1992). Among the factors regulating PR levels, PR ligands play also a crucial role. It is known from the literature that within 6 to 8 h after occupancy by progestins, the receptors are extensively down-regulated. Lange et al. have demonstrated that PRs are targeted for down-regulation by phosphorylation (Lange et al., 2000). Indeed, liganded PRs are substrate for MAPK-induced phosphorylation, which targets the receptor for degradation by the 26S proteasome. The data presented in this thesis demonstrate in addition that PR is down-regulated by R5020 treatment but stabilised after Lonaprisan exposure. Moreover I could show that this increase in PR occurred not only at the protein level, but was also a result of increased transcriptional activation. In line with this Fritah et al. demonstrated that p21 itself is able to control the transcriptional activity of ERα in activating PR expression (Fritah et al., 2005). In particular, they showed that the estrogen-induced expression of the PR in MCF7 cells was enhanced by p21 and conversely reduced after p21 knock-down. In addition, they have shown that p21 facilitates ERα-dependent transcription through direct association with the transcription factor (ERα) bound to the PR-B promoter. The results reported here concord partially with those of Fritah et al. I demonstrated that Lonaprisan induced protein and mRNA levels of PR and this effect was reversed when both p21 and Sp1 were down-regulated. However, PR down-regulation seemed to apply specifically to the A isoform of PR. This result was further confirmed by ChIP analysis which showed an increased recruitment of p21 to the PR-A promoter rather than to the PR-B promoter. Taken together the increased Lonaprisan-dependent p21 induction and increased PR levels suggest a positive feedback loop to exist between steroid hormones and p21.

4.7 Conclusion and outlook

In conclusion, my work provides evidence that Lonaprisan exerts an inhibitory activity on cell proliferation, characterised by arrest of the cell cycle, induction of senescence while keeping the cells in a viable status, and induction of p21 expression. This is the result of a direct interaction of PR with the p21 promoter which furthermore implicates Sp1 (**Fig. 39**).

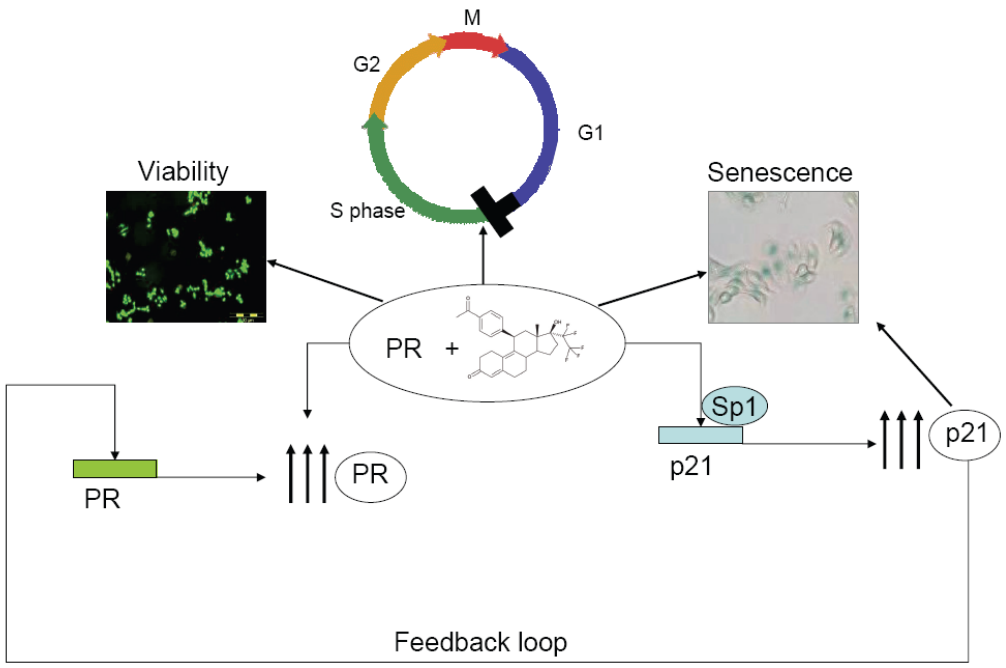


Figure 39 Proposed model

Lonaprisan arrests the cells in the G0/G1 phase of the cell cycle and leads to a senescence-like phenotype, while inducing the expression of the p21 gene. As a positive feedback mechanism, p21 can activate the expression of PR by direct binding to the PR promoter.

Here, the regulation of PR expression by p21 has also been shown, suggesting the existence of a positive feedback mechanism between PR and p21 after Lonaprisan treatment. A deeper understanding of the relationship between PR and cell cycle regulatory proteins such as p21 should bring insights for the development of novel therapy options in the treatment of hormone-dependent breast cancer and for the prevention of recurrence.

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7 List of original publications

Publication

Busia L, Faus H, Hoffmann J and Haendler B: The antiprogestin Lonaprisan inhibits breast cancer cell proliferation by inducing p21 expression. Mol. Cell. Endocrinol. (2011) 333:37-46.

Posters

Busia L, Kunde J, Korr D, Faus H, Sommer A, Weiss B and Hoffmann J: Preclinical studies demonstrate antiproliferative activity of the progesterone receptor antagonist Lonaprisan (ZK230211) in BRCA1 negative breast cancer cells and suggest potential predictive markers for response. 18th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology Seefeld, Tyrol, Austria, 2008.

Busia L, Haendler B and Hoffmann J: Senescence-like growth arrest induced by the progesterone receptor antagonist Lonaprisan in breast cancer cells. 17th ECDO Euroconference on Apoptosis, Institut Pasteur, Paris, 2009.

Two participations in the Young Scientist Poster Session. Berlin, Bayer Schering Pharma. October 2008 and October 2009.

Oral presentation

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Young Researcher´s Report, Mid-Term Review Meeting, Apoptrain Network, June 26, 2009 Carat Hotel Frankfurt Airport, Germany.

8 Curriculum vitae

For reasons of data protection, the Curriculum vitae is not published in the online version

Ehrenwörtliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig durchgeführt und verfasst habe. Dabei wurden keine anderen als die angegebenen Quellen und Hilfsmittel verwendet.

Berlin, 22.02.2011

Laura Busia