Progesterone receptor modulators and uterine leiomyomas - implications and mode of action

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Abstract

Uterine leiomyomas are hormone-dependent benign tumors arising in the uterine muscle (myometrium). The tumors lead to gynecological disorders such as abnormal uterine bleeding, significant morbidity, and infertility. They affect at least 25% of women in reproductive age and typically disappear after the onset of menopause. At present, no effective long-term medication is available. The disease etiology of uterine leiomyomas and the underlying molecular mechanisms are poorly understood as reliable and predictive in vitro and in vivo models are lacking. In consequence, research in this field and the development of new treatment approaches are extremely challenging.

Progesterone receptor (PR) modulators are the only compound class for which beneficial effects on tumor size and symptoms have been demonstrated clinically, without induction of hypoestrogenism. Despite the first description of PR modulators in the late 80s, their molecular mechanism of action remains enigmatic. Classification of the multifarious spectrum of PR ligands has been based on in vivo models, distinguishing them as agonists (progesterone-like effects), antagonists (progesterone-inhibiting effects) and SPRMs (selective progesterone receptor modulators, a tissue-specific mosaic of agonistic and antagonistic effects). However, this present classification system demonstrates discrepancies as e.g. antagonists have been shown to exhibit species-specific partial agonistic effects.

The aim of this thesis was to provide a new classification system for PR modulators (PRMs) based on in vitro analyses, in particular of their effects in the absence of progesterone. The classification system should be used to select the most suitable PRM subclass for the treatment of uterine leiomyomas. Furthermore, analyses of the PR modulators' mechanism of action in an appropriate model system should elucidate the underlying hormone-mediated signaling cascades in uterine leiomyomas.

To refine the existing PR modulator classification system, protein-protein interaction studies as well as global gene expression profiling studies were conducted with a representative selection of PR ligands, revealing profound differences between the various subtypes. The progesterone-independent gene expression inhibition was unique for each PR modulator. These classification differences directly translated into distinct antiproliferative effects in in vitro analyses. Moreover, the modulators varied in functional inhibitory effects on estradiol action. Lonaprisan showed the strongest antiproliferative activity and was selected for subsequent analyses under disease-relevant conditions. For that purpose, a novel in vitro / in vivo model system for uterine leiomyomas was established using the Eker rat tumor-derived (ELT-3) cell line. Gene expression profiling identified downstream genes of hormone and antihormone action. In particular, transforming growth factor (TGF) α and inhibin β subunit B were demonstrated to influence ELT-3 cell proliferation using gene silencing. Both were regulated by lonaprisan.

In summary, PR modulators with high antiproliferative effects can be distinguished from other subclasses of PRMs using this new classification system. Lonaprisan displayed the highest antiproliferative efficacy amongst the PR modulators analyzed. This antiproliferative potential can be explained by its modulation of the downstream genes TGFα and inhibin β B and subsequent effects on the cell cycle, providing novel insights into the molecular pathophysiology of uterine leiomyomas.

Key words

Classification system, gene expression profile, uterine leiomyoma model system

Zusammenfassung

Uterine Myome sind hormonabhängige, gutartige Tumore in der glatten Muskulatur des Uterus (Myometrium). Sie rufen gynäkologische Dysfunktionen wie unregelmäßige und starke Blutungen, signifikante Morbidität und Infertilität hervor. Etwa 25% aller Frauen im reproduktiven Alter sind von Myomen betroffen, die nach dem Eintreten der Menopause häufig verschwinden. Zur Zeit gibt es keine geeignete medikamentöse Langzeittherapie. Die Etiologie sowie die molekularen Mechanismen der Tumore sind bisher unzureichend verstanden, da es an zuverlässigen und aussagekräftigen in vitro und in vivo Modell-Systemen mangelt. Folglich ist die Forschung in dieser Indikation sowie die Entwicklung neuer Therapieansätze sehr herausfordernd.

Progesteronrezeptor (PR)-Modulatoren sind die einzige Substanzklasse, für die klinisch ein Rückgang der Tumorgröße sowie eine Besserung der Symptome gezeigt werden konnte, ohne dass ein hypoestrogener Status induziert wird. Obwohl die ersten PR-Modulatoren bereits in den 80er beschrieben wurden, ist ihr molekularer Mechanismus bisher weitgehend unverstanden. Eine Klassifizierung des vielfältigen Spektrums an PR-Liganden basierte auf Erkenntnissen aus in vivo-Modellen, die zu der Einteilung in Agonisten (Progesteron ähnliche Effekte), Antagonisten (Progesteron inhibierende Effekte) und SPRMs (selektive Progesteronrezeptor-Modulatoren, ein gewebespezifisches Mosaik an agonistischen und antagonistischen Effekten) führten. Dieses Klassifizierungssystem weist jedoch Diskrepanzen auf, da gezeigt werden konnte, dass z.B. auch Antagonisten gewebespezifische partial-agonistische Effekte induzieren können.

Das Ziel dieser Arbeit war es, ein neues Klassifizierungssystem auf der Basis von in vitro-Analysen, insbesondere zu Progesteron-unabhängigen Effkten der PR-Modulatoren, zu generieren. Das Klassifizierungssystem sollte geignet sein, um eine Subgruppe an Modulatoren zu selektieren, welche für die Myomtherapie am geeignetsten erscheint. Zudem sollten Untersuchungen zu den molekularen Mechanismen dieser PR-Modulatoren in einem zweckmäßigen Modell-System dazu beitragen, die zugrunde liegenden, hormonabhängigen Signalwege in uterinen Myomen besser zu verstehen.

Um das existierende Klassifizierungssystem für PR-Modulatoren zu verbessern, wurden Protein-Protein-Interaktions- und globale Genexpressionsstudien mit einer representativen Auswahl an PR-Liganden durchgeführt, die profunde Unterschiede zwischen den verschiedenen Subtypen deutlich machten. Die Progestron-unabhängige Inhibition der Genexpression war spezifisch für jeden einzelnen PR-Modulator. Die identifizierten Unterschiede in der Klassifizierung spiegelten sich direkt in verschiedenen antiproliferativen Effekten in in vitro-Analysen wider. Zudem unterschieden sich die Modulatoren in funktionell inhibitorischen Effekten auf die Estradiol-Wirkung. Lonaprisan wies insgesamt die stärksten antiproliferativen Eigenschaften auf und wurde für weitergehende Analysen in einem Krankheits-relevanten Modell gewählt. Zu diesem Zweck wurde ein neues in vitro / in vivo Modell-System für uterine Myome unter Verwendung der aus Eker-Ratten-Tumor abstammenden ELT-3 Zelllinie etabliert. Eine Genexpressionsanalyse identifizierte Gene, die durch Hormone und Antihormone reguliert werden. Insbesondere der transformierende Wachstumsfaktor TGFα und die

Inhibin β Untereinheit B wurden innerhalb des neuen Modell-Systems als Effektoren der ELT-3 Zellproliferation verifiziert. TGFα und Inhibin β B wurden beide durch Lonaprisan reguliert.

Zusammenfassend ist festzustellen, dass PR-Modulatoren mit starken antiproliferativen Effekten von anderen Subgruppen an PR-Modulatoren auf der Basis des neuen Klassifierungssystems herausgefiltert werden können. Lonaprisan zeigte die stärksten antiproliferativen Eigenschaften unter den analysierten PR-Modulatoren. Das antiproliferative Potential von Lonaprisan kann teilweise durch die Regulation der nachgeordneten Gene TGFα und Inhibin β B sowie Effekte auf den Zellzyklus erklärt werden. Dies bietet weitere Erkenntnisse für die molekulare Pathophysiologie von uterinen Myomen.

Schlagworte

Klassifizierungssystem, Genexpressionsprofil, Myom-Modell-System

Table of contents

Abbreviations

4-OHT	4-hydroxytamoxifen
AF	Transactivation function
AP	Activator protein
AUC	Area under the curve
bFGF	Basic fibroblast growth factor
BHD	Birt-Hogg-Dubé
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CI	Confidence interval
CT	Cycle threshold
DBD	DNA binding domain
DI	Dimerization domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
E2	Estradiol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELT	Eker rat leiomyoma tumor-derived
ER	Estrogen receptor
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GDD	Global drug discovery
GnRH	Gonadotropin-releasing hormone
HIF	Hypoxia-inducible factor
HLRCC	Hereditary leiomyomatosis and renal cell carcinoma
HMGA	High mobility group AT-hook
HRE	Hormone responsive element
Hsp	Heat shock protein
ID	Interaction domain
IGF	Insulin-like growth factor
LBD	Ligand binding domain
LH	Luteinizing hormone
LSMC	Leiomyoma smooth muscle cells
LTR	Long-terminal repeat
MEM	Minimum essential medium
MMTV	Mammalian mammary tumor virus

MSMC	Myometrial smooth muscle cells
NCoR	Nuclear receptor corepressor
NR	Nuclear receptor
NR-D	Nuclear receptor interacting domain
PA	Progesterone receptor antagonist
PAA	Polyacrylamide
PDGF	Platelet-derived growth factor
PI3K	Phoshatidylinositol 3 kinase
PR	Progesterone receptor
PRE	Progesterone responsive elements
PRM	Progesterone receptor modulator
RCC	Renal cell carcinoma
sc	subcutaneous
SCID	Severe combined immunodeficient
SERM	Selective estrogen receptor modulator
SGK	Serum glucocorticoid-regulated kinase
SH	Src homology
shRNA	Short hairpin ribonucleic acid
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
SOM	Self-organizing maps
SP	Specificity protein
SPF	Specific pathogen free
SPRM	Selective progesterone receptor modulator
SRC	Steroid receptor coactivator
SRF	Serum response factor
Stat	Signal transducer and activator of transcription
TGF	Transforming growth factor
TRG	Therapeutic research group
TSC	Tuberous sclerosis complex
UAE	Uterine artery embolization

1. Introduction

1.1 Uterine leiomyomas

Uterine leiomyomas (myoma, uterine fibroids) are benign neoplasms arising from the myometrial compartment of the uterus. They constitute the most common pelvic tumors in women. About 25% of women in reproductive age are thought to be affected by symptoms evoked through uterine leiomyomas. The diameters of symptomatic tumors can achieve 10 mm or exceed 20 cm. Moreover, the localization of the tumors varies (Figure 1). Typical symptoms include abnormal uterine bleeding, pelvic pressure and pain, dysmenorrhea, and reproductive dysfunction [1]. Currently, uterine leiomyomas are responsible for 200,000 hysterectomies annually in the United States (US), making them the leading cause of hysterectomy for premenopausal women in the US [2]. However, the exact prevalence of uterine leiomyomas as observed in pathological examinations of hysterectomized uteri has been reported to be even higher than 25% as a lot of these tumors are asymptomatic and grow slowly [3].

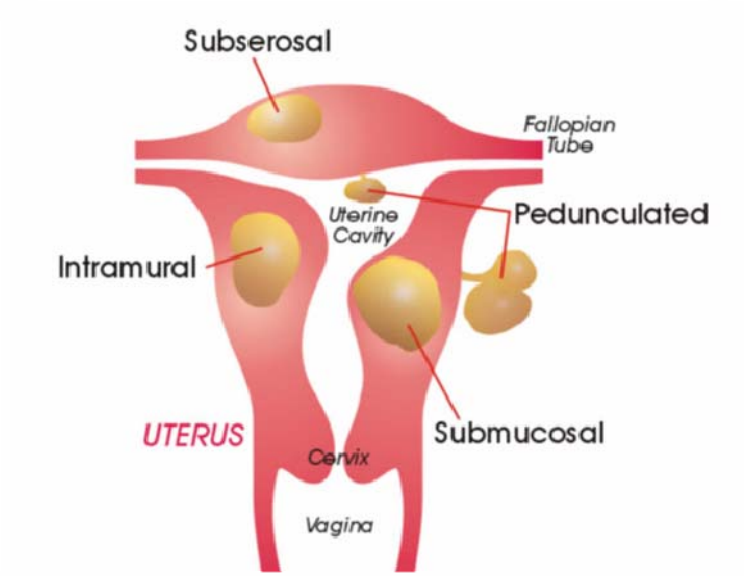

Figure 1. Types of uterine leiomyomas. Tumors can develop in the outer portion of the uterus (subserosal), within the uterine wall (intramural), under the lining of the uterine cavity (submucosal) or grow on small stalks that connect them to the inner or outer wall of the uterus, named pedunculated tumors (from www.uterine-leiomyoma.org).

Uterine leiomyomas have been demonstrated be of clonal origin [4]. Approximately 40% of these tumors display non-random cytogenetic alterations which allow the classification into well-defined subgroups (partial deletion of chromosome 7q, trisomy 12 or rearrangements like 12q14, 6p21 or 10q22). Identification of candidate genes for uterine leiomyoma predisposition include the HMGA2 gene, a member of the non-histone chromosomal high mobility group gene family, which is located on chromosome 12 [5]. Hereditary cancer syndromes such as the hereditary leiomyomatosis and renal cell

carcinoma (HLRCC), tuberous sclerosis complex (TSC), and Birt-Hogg-Dubé (BHD) syndromes often emerge prior to the development of uterine leiomyomas and suggest a shared genetic origin [6].

Several predisposing factors for uterine leiomyomas have been identified [7, 8]. Obesitiy and early age at menarche, which increases a woman's overall lifetime exposure to estrogen, are known risk factors. Women with African-American ethnicity are at higher risk to develop symptomatic uterine leiomyomas than Caucasian women, and African-American women often develop a more severe disease. Parity is also a significant risk factor, i.e. the age at the birth of the last child is inversely associated with the risk for developing uterine leiomyomas.

Epidemiological and experimental evidence points towards an essential role for ovarian hormones in the pathogenesis of uterine leiomyomas: The tumors are rarely observed before puberty, appear during the reproductive years, often change dramatically in volume during pregnancy, and typically regress or become asymptomatic after the onset of menopause [9, 10]. However, the exact role of estradiol and progesterone in the etiology and pathogenesis of this disease remains unclear. There is significant evidence that estradiol is a major mediator of myometrial and leiomyoma cell proliferation. Leiomyomas are hyperresponsive to estradiol and exhibit elevated levels of estrogen receptor (ER) [11-13]. Leiomyomas also exhibit alterations in estrogen metabolism, including elevated aromatase levels [14]. An alternative hypothesis posits that progesterone predominantly influences leiomyoma growth [15]. The progesterone hypothesis is supported by the ability of progestins to reverse the uterine leiomyoma shrinkage induced by GnRH agonist therapy when applied as add-back treatment [16, 17]. In addition, mitotic rates in leiomyomas are increased during the secretory phase of the menstrual cycle when progesterone levels are maximal [18]. Increased proliferation is also observed upon treatment with progesterone in smooth muscle cells derived from uterine leiomyomas, but not in cells derived from the myometrium [19]. Furthermore, progesterone receptor (PR) expression levels are higher in uterine leiomyomas compared to normal myometrium [13, 20] and increased PR expression has been demonstrated to be positively correlated with tumor growth [21].

Several cytokines and growth factors may also foster leiomyoma growth through paracrine and autocrine mechanisms. These factors include transforming growth factor β (TGFβ), epidermal growth factor (EGF), insulin-like growth factor I and II (IGF-I/II), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) [22]. In particular, TGFβ probably contributes to myoma growth by stimulating the deposition of the extracellular matrix (ECM) [23]. Parathyroid hormone-related protein, bFGF and prolactin are vasoactive themselves, promote angiogenesis and could contribute to the profuse menstrual bleeding seen in women with uterine leiomyomas [24].

1.2 Therapy of uterine leiomyomas

The standard treatment of uterine leiomyomas is surgical excision of the myomas (myomectomy) or surgical removal of the entire uterus (hysterectomy). Hysterectomy eliminates both the symptoms and

the risk of recurrence. Myomectomy successfully relieves the symptoms but it does not affect the underlying process. Thus, there is a high risk of recurrent myomas, and approximately 50% of women develop uterine leiomyomas again within five years after myomectomy [25]. Moreover, the vast majority of uterine leiomyomas lies within the uterine wall (intramural; Figure 1). These tumors are difficult to treat in a minimally invasive procedure. In the past decade, intramural myoma treatment is provided by uterine artery embolization (UAE) [26, 27]. The induction of global uterine ischemia is symptomatically effective and less invasive as myomectomy, but there are ongoing objections regarding the implication of UAE on fertility and pregnancy [28].

Currently, there is no effective long-term medication for the treatment of uterine leiomyomas available. Non-surgical treatment has primarily involved the application of gonadotropin-releasing hormone (GnRH) agonists which suppress ovarian hormone production, leading to a reduction of leiomyoma size. However, uterine leiomyomas return to pretreatment volume after discontinuation of GnRH agonists, because the tumor cellularity is not affected [29, 30]. Because this compound class has specific detrimental side effects including significant loss of bone mass and hot flushes due to the hypoestrogenic status, this regimen is only used as preparatory treatment prior to surgical excision.

Selective estrogen receptor modulators (SERMs) are another class of compounds which have been investigated as potential medication for uterine leiomyomas. SERMs bind to the estrogen receptor and exhibit agonistic or antagonistic activities in a tissue-specific manner. Ideally, a SERM would retain the positive effects of estrogen on bone, brain and the cardiovascular system, but would act as an antagonist in the breast and in the uterus. Unlike their success in preclinical studies [31], to date, SERMs have yielded no significant reduction of tumor size or symptoms in clinical trials [32], most likely due to compound-related drawbacks like ovarian stimulation or partial agonism.

Progesterone receptor modulators appear to possess good therapeutic potential for the treatment of uterine leiomyomas. The most conclusive evidence that PR antagonists and selective progesterone receptor modulators (SPRMs) have beneficial effects on tumor size and symptoms comes from clinical observations. Several prospective clinical trials investigating the effect of the PR antagonist mifepristone on uterine leiomyomas have been published [33]. Women with symptomatic uterine leiomyomas were treated with doses between 5 mg and 50 mg per day, for a period of 3 to 12 months. Overall, the application of the PR antagonist mifepristone leads to a shrinkage of uterine leiomyomas that depends on dose and duration of treatment. Furthermore, mifepristone reduces the prevalence and severity of dysmenorrhea, menorrhagia, and pelvic pressure. Similar improvements of symptoms have been observed in a randomized, double blind, placebo-controlled study in uterine leiomyoma patients with the SPRM asoprisnil [34]. Treatment with 5, 10 and 25 mg asoprisnil, for a period of 3 months, is associated with dose- and time-dependent beneficial effects on leiomyoma volume, uterine bleeding, bloating and pelvic pressure. Furthermore, asoprisnil is associated with follicular-phase estrogen concentration and minimal hypoestrogenic symptoms.

1.3 Model systems for uterine leiomyomas

Besides humans, reports on spontaneous development of uterine leiomyomas in other species are rather anecdotic. German shepherd dogs have been reported to develop myomas due to a mutation in the canine BHD gene [35], and guinea pigs can be forced to develop uterine leiomyomas when primed with high doses of estradiol for a long period [36, 37].

The best characterized model system for uterine leiomyomas is the Eker rat. The Eker mutation has been identified as an endogenous retroviral insertion between exons 30 and 31 of the tuberous sclerosis complex (TSC) 2 tumor suppressor gene, which leads to an inactivation of this gene [38]. The TSC1 and the TSC2 genes encode the proteins hamartin and tuberin, respectively, both of which form a complex that functions as a negative regulator of the phoshatidylinositol 3 kinase (PI3K) pathway, downstream of Akt kinase [39]. Cells lacking tuberin or hamartin display increased activity of the Ras homologue Rheb, resulting in constitutive expression of mTOR. mTOR belongs to the family of phosphatidylinositol kinase-related kinases and regulates the activity of the hypoxia-inducible transcription factor (HIF) α [40]. Dysregulation of this pathway leads to increased protein translation and cell proliferation [41-43].

Rats homozygous for the TSC2 mutation ($TSC2^{Ek/Ek}$) die between embryonic days 11-13 and have characteristic brain defects [44]. Non-carriers ($TSC2^{+/+}$) are phenotypically normal. Eker rats are heterozygous for the germline transmission of the TSC2 gene ($TSC2^{Ek/+}$) and have a predisposition towards spontaneous development of tumors in the kidney (renal cell carcinoma, RCC), in the spleen (hemangiosarcoma), and female rats also in the uterus (leiomyomas) [45]. Uterine leiomyomas arise by 12-16 months of age with a frequency of 60-70%. The tumors share phenotypic, biochemical, and genetic characteristics with the cognate human disease, including ER and PR expression, responsiveness to steroid hormones, and aberrant HMGA2 expression [46, 47].

Several cell lines have been established from the Eker rat uterine leiomyomas. They are designated as ELT cells, for Eker rat leiomyoma tumor-derived cells. Five of these cell lines (ELT-3, -4, -6, -9 and -10) display expression of smooth muscle actins (α, γ) and desmin, confirming their smooth muscle origin [48]. In particular, the ELT-3 cell line offers suitable characteristics like ER and PR expression as well as tumorigenicity in nude mice [49, 50].

1.4 Ovarian hormones

The ovarian hormones estradiol and progesterone both are synthesized from the cholesterol derivative pregnenolone. Their production is regulated by the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) both of which are released from the anterior pituitary gland in response to the hypothalamic gonadotropin-releasing hormone (GnRH).

Estradiol is the primary female sex steroid which is produced from androgens through enzymatic conversion by aromatase. It is primarily secreted from the developing follicles in the ovaries, the corpus luteum, and the placenta. Estradiol belongs to the group of estrogens, which are pleiotropic hormones with multiple actions in reproductive tissues such as mammary gland, uterus and ovary, and in many non-reproductive tissues including bone, fat tissue, the central nervous system, and the cardiovascular system [51]. The effects of estradiol are mediated by the estrogen receptor (ER).

Progesterone belongs to a class of hormones called progestogens and is the major naturally occurring human progestogen. Progesterone is produced in the corpus luteum, the adrenal glands, the brain, and, during pregnancy, in the placenta. The C-21 steroid hormone is a key regulator of normal female reproductive functions. It is involved in the regulation of uterine and mammary gland development and differentiation, ovulation, ovum implantation, and maintenance of pregnancy [52, 53]. The diverse effects of progesterone on female reproductive target tissues are mediated via the progesterone receptor (PR).

1.5 Estrogen and progesterone receptors

The progesterone receptor (PR) as well as the estrogen receptor (ER) are class I members of the nuclear receptor (NR) family of ligand-dependent transcription factors [54, 55]. Nuclear receptors are characterized by common structural motifs: the amino terminal A/B region, the DNA-binding domain (DBD), a hinge region and the carboxy terminal ligand binding domain (LBD). The DBD is composed of two highly conserved zinc fingers that distinguish nuclear receptors from other DNA-binding proteins [56].

The estrogen receptor (ER) exists as two different forms, usually referred to as estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2), each encoded by a separate gene [57-60]. ESR1 and ESR2 have similar domain structures and very high amino acid identity in their DNA-binding domains (97%). The N-terminal A/B domains (17% identity) and the ligand-binding domains (60% identity) are more divergent [61], especially regarding the transactivation function (AF) domains AF-1 and AF-2. Studies of the receptors' tissue distribution indicate that ESR1 has a broad expression pattern and is predominantly expressed in the uterus, mammary gland, testis, pituitary, liver, kidney, heart and skeletal muscle. ESR2 has a more focused pattern with high expression levels in the ovary, prostate, epididymis, and hypothalamus [62, 63]. In cells in which both receptors are present, ESR2 functions as an efficient dominant inhibitor of ESR1 transcriptional activity [64]. The predominant role of ESR1 in mediating estrogen responses has been confirmed by knockout mice [65-67]. Mice with deletion of both, ESR1 and ESR2, have a phenotype similar to mice lacking only ESR1, but exhibit a unique ovarian pathology [68].

The progesterone receptor (PR) exists as two different receptor isoforms, PR-A and PR-B [69]. Both isoforms are transcribed from the same gene and arise through transcription from two alternative promoters [70] or by alternative initiation of translation from a unique mRNA [71]. PR-A and PR-B

isoforms contain two transactivation function (AF) domains, AF-1 within the N-terminus and AF-2 within the ligand-binding domain. PR-B differs from PR-A only by an additional stretch of 165 amino acids at the N-terminus of the protein [72-74]. The PR-B specific domain encodes a third transactivation function (AF-3) which is absent from PR-A. Both PR isoforms are expressed in female reproductive tissues. The ratio of PR-A and PR-B expression varies and is dependent on the hormonal status of the cell [75, 76]. To delineate the individual roles of the receptor subtypes in vivo, PR isoform specific knockout mice were generated more recently [77-79]: The PR-A isoform is both necessary and sufficient to elicit the progesterone-dependent reproductive responses required for female fertility whereas PR-B is required for normal proliferative responses to progesterone in the mammary gland.

The endogenous ligands estradiol and progesterone normally up-regulate gene expression when bound to their cognate nuclear receptors. This stimulation of target gene expression is referred to as an agonistic response. The agonistic effects of endogenous hormones can be mimicked by certain synthetic ligands (agonists). Other synthetic nuclear receptor ligands have no apparent effect on gene transcription in the absence of endogenous ligand. However, they block the agonist effects through competitive binding to the same binding site in the nuclear receptor. These ligands are referred to as antagonists (see 1.6).

The general pathway of estrogen and progesterone inducible NR-mediated gene transcription has been well characterized: In the absence of ligand, the NR is associated with cytoplasmic heat shock proteins and fixed in a transcriptionally inactive conformation. Ligand binding induces a conformational change of the NR protein which dissociates from the heat shock protein complex and migrates into the nucleus where it binds as dimer to hormone response elements (HREs) in promotors of steroid hormone target genes (Figure 2) [80]. Depending on the type of ligand (agonist or antagonist), the transcription of NR target genes is either activated or repressed [81]. Studies with the ER [82, 83] and more recent studies with the PR [84] have demonstrated that the position of the helix 12 within the transactivation function 2 (AF-2) of the ligand binding domain (LBD) is variable and shifts significantly upon binding of a ligand. In agonist-bound receptors, helix 12 is packed tightly against the LBD. The induced binding surface is affine for NR boxes of the general sequence LxxLL (L = leucine and x = any amino acid) which are present in many coactivators [85, 86]. In antagonist-bound receptors, helix 12 is dislocalized from the LxxLL-binding surface. The turn facilitates preferential binding of proteins with another leucine-rich motif, the LxxxI/HIxxxI/L motif, which is present in CoRNR boxes in the carboxy-terminal interaction domains (IDs) of corepressors [87]. Once recruited to the promotor, coactivators enhance transcriptional activity through self-possessed enzymatic activities or they recruit secondary cofactors such as acetyltransferase proteins, methyltransferases and ubiquitin ligases [88-90]. Corepressors inhibit the transcriptional activity of the receptor-ligand complex by inherent enzymatic activities or by recruiting secondary cofactors such as histone deacetylases [91-93].

However, some of the genes that are regulated by steroid hormone receptors do not contain HRE-like sequences [94]. Thus, ligand-bound nuclear receptors can also modulate gene expression without binding directly to DNA (Figure 2). The transcription of non-HRE target genes is facilitated through

nuclear protein-protein interactions with other DNA-binding transcription factors such as SP1 and AP-1 [95, 96].

In addition to their direct effects on transcription, steroid hormone receptor ligands have been identified to influence the activity of many other pathways by so-called “non-genomic mechanisms” in the cytoplasma [97-100]. In contrast to genomic effects which have an onset in the range of minutes to hours, rapid non-genomic effects exhibit an onset within seconds to minutes and are insensitive to inhibitors of transcription and translation [101]. Rapid non-genomic effects involve the activation of cytoplasmic signal transduction cascades. In particular, interaction with c-Src kinase and activation of mitogen-activated kinases such as the mitogen-activated protein kinase 1 (ERK), the phosphoinositide-3-kinase (PI3K) and the serin/threonine protein kinase Akt have been described for the ER and the PR [100, 102-104]. Kinase cascades activate or inhibit downstream target proteins like Elk-1 and serum response factor (SRF) transcription factors by phosphorylation and subsequently modulate gene expression of cell proliferative factors like cyclin D1 and the signal transducer and activator of transcription (Stat) 3 (Figure 2) [100, 105-108].

Recently, the distinction between the rapid kinase activation and genomic actions has been converged [109, 110]. For the PR, activated kinases have been shown to be recruited along with the phosphorylated nuclear receptor to an integrated PRE-containing promotor [111]. The kinases lead to histone modifications as well as recruitment of coactivators and general transcription factors. In this model, rapid signaling is not only an alternative pathway, but a concurrent pathway integrated into the activation of direct gene induction by nuclear PR [110, 111].

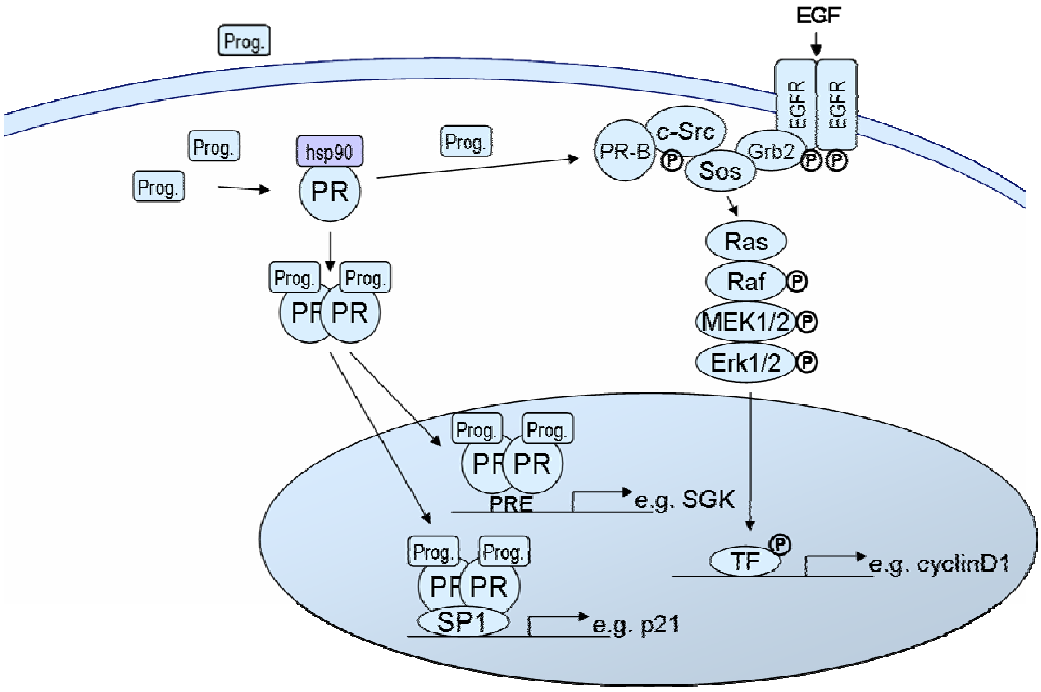

Figure 2. Integration of rapid PR signaling and nuclear transcription activity. Classically, activated PR dimers bind to PREs in the promotor regions of target genes such as SGK to initiate transcription. Ligand binding to PR-B also mediates non-classical gene transcription through rapid extra-nuclear activation of the EGFR, c-Src kinase, and Erk1/2 MAPK cascade which regulates cyclin D1 transcription independently of PR transcriptional activity. Additionally, PR tethers to transcription factors like SP1 to regulate transcription of genes like p21.

1.6 Synthetic progesterone receptor ligands

Because of its pivotal function in female reproduction, the progesterone receptor has become an important drug target for women's healthcare. Since 1938, many synthetic PR ligands have been generated to modulate PR activity. Currently, the PR ligands used in the clinic are all based on a steroidal scaffold. They are characterized as agonists, antagonists, and ligands with a mix of both PR agonist- and antagonist-like properties. In a recent approach, non-steroidal PR ligands have been generated to avoid potential issues with steroid receptor selectivity and modulation of common metabolic pathways employed by steroidal compounds. These compounds, such as the tanaproget derivative PRA-910, often exhibit species- and context-specific activities [112-114]. The nomenclature of steroidal and non-steroidal PR ligands is somehow arbitrary, in particular for ligands with antagonistic properties. In the following sections, PR antagonists and partial agonists are summarized up as progesterone receptor modulators (PRMs) as this nomenclature is engaged with PR ligand classification systems [115, 116].

Synthetic PR agonists are the major component of oral contraceptives and are widely used for the postmenopausal hormone replacement therapy. In addition, they provide new treatment options for several gynecological disorders including endometriosis and abnormal uterine bleeding [117]. One of the most widely used synthetic progesterone analogue for studying progestin distribution and biological functions is promegestone (R5020).

The starting point of drug discovery in the area of progesterone receptor antagonists (PAs) was the synthesis of mifepristone (RU486) in 1981 [118, 119]. Mifepristone is a well characterized antagonist of PR function and has been used clinically as contraceptive [120, 121]. However, it also displays anti-glucocorticoid activity. More recently, it has become evident that mifepristone is an incomplete progesterone receptor antagonist which exhibits partial agonistic activity in a species- and tissue-selective manner [122], leading to the designation 'mixed antagonist'. In an effort to optimize PR antagonists' structures with regard to steroid receptor selectivity and PR activity profile, several modifications of the steroid nucleus were generated [123, 124]. To date, numerous PAs are available, exhibiting a broad spectrum ranging from pure antagonists to mixed antagonists. Based on in vivo experiments and in vitro characteristics such as DNA binding activity and transcriptional behavior of the antagonist-occupied PR in the presence of protein kinase A activators like cAMP, three types of PR antagonists have been described exemplified by onapristone (type I), mifepristone (type II) and lonaprisan (type III) [115, 116]. [125-127]. Type I antagonists prevent the binding of the PR to DNA. Type II antagonists promote DNA binding of the PR and act as PR antagonists in the transactivation assays procedure under most circumstances, but in the presence of protein kinase A activators (cAMP) they behave like PR agonists. Type III antagonists promote a strong binding to DNA, but do not display any PR agonistic activity in the presence of cAMP. In general, PR antagonists have been shown to provide new treatment options in several indications of women's health. They are effective in hormone replacement therapy, in the treatment of breast cancer [128, 129] and in the treatment of gynecological disorders such as endometriosis [130, 131] and uterine leiomyomas [132, 133].

Similar to PR antagonists, selective progesterone receptor modulators (SPRMs) have also been shown to be efficacious in the treatment of gynecological disorders [34, 134, 135]. The term SPRM is assigned to compounds with tissue-specific mixed agonistic/antagonistic properties [136, 137]. The most conclusive evidence for a partial agonistic activity of SPRMs like J1042, asoprisnil or J912 comes from studies in the Mc Phail test. This test assesses the endometrial proliferation and transformation in immature rabbits [138]. In contrast to PR antagonists like mifepristone or onapristone which behave as pure antagonists in this assay, SPRMs display partial agonistic properties [116, 139]. Other in vivo models like uterine and vaginal morphology in guinea pigs show a mosaic of progesterone agonist and antagonist effects for SPRMs [116, 136, 139]. The molecular mechanism of SPRMs gene regulation through PR is poorly characterized. However, their ability to change into transcriptional agonists in the presence of cAMP has led to the classification of SPRMs as type II PR antagonists [115]. Therefore, in vitro, SPRMs can not be distinguished from mixed antagonists like mifepristone so far.

1.7 Aim of the thesis

The ovarian hormones estradiol and progesterone have received attention as mediators of uterine leiomyoma development and growth. Hence, modulation of these steroid hormone signaling pathways is thought to provide new treatment options for gynecological disorders like uterine leiomyomas. In particular, progesterone receptor (PR) modulators with antagonistic properties provide new approaches as their long-term use is not associated with loss of bone mass and hypoestrogenism. Clinical observations confirm that the application of the PR antagonist mifepristone and the selective progesterone receptor modulator (SPRM) asoprisnil yields in a reduction of leiomyoma tumor size.

Clearly, the PR modulators developed so far exhibit a broad range of biological activities and the one major goal in PR modulator research is the desire to obtain compounds with highly potent properties and reduced endocrine side effects. The prevalent challenge in this investigation is the insufficient correlation of classification in vivo and activities in classical in vitro experiments, in particular for PR antagonists and SPRMs.

The primary aim of this thesis was to reassess and to further refine previous classification systems for PR modulators. In particular, progesterone-independent effects of PR modulators should be identified on a cellular level. The analyses considered specific types of ligands described so far, focusing on the pure agonist R5020, the non-steroidal PR modulator PRA-910, SPRMs (J1042, asoprisnil, J912), the mixed antagonist mifepristone, classical antagonists (onapristone, ZK 137316) and the pure antagonist lonaprisan (Figure 3). Interaction studies were conducted to analyze relevant biological processes of PR transcriptional and non-genomic functions induced by different PR modulators. In addition, a global gene expression profiling study was performed in T47D cells, a breast cancer cell line which provides a suitable model system for PR signaling.

The second aim of this thesis was to elucidate the mode of action of PR modulators in the treatment of uterine leiomyomas. The analyses should include the investigation of inhibitory effects on estradiol action as progesterone action is directly related to estradiol action in organisms. A suitable model system should be established based on Eker rat leiomyoma tumor-derived (ELT-3) cells to facilitate analyses under disease relevant conditions. The model system included the development of a transduction system for gene silencing and therefore allowed for functional analyses of specific genes of interest. Ovarian hormone-regulated genes which might contribute to uterine leiomyoma growth were identified in a global gene expression profiling study in ELT-3 cells. The impact of PR modulator downstream genes on ELT-3 cell proliferation were confirmed using the new model system for gene silencing.

2. Materials and methods

2.1 Cell culture and cellular assays

2.1.1 Cell culture

Stably transfected steroid receptor-free human neuroblastoma (SK-NM-C; clone C23.43 and VIII-1.1) cells expressing either the human PR-A or PR-B and the mammalian mammary tumor virus promoter linked to the LUC reporter gene [140, 141] were maintained in minimum essential medium (MEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 4 mM L-glutamine, 0.1 mM non essential amino acids and 1mM sodium pyruvate (PAA Laboratories, Cölbe, Germany).

Human cervix carcinoma (HeLa) and human osteosarcoma (U2OS) cells were obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and were maintained in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Human breast carcinoma (T47D) cells were obtained from the American Type Culture Collection (ATCC; LGC Promochem, Wesel, Germany) and were maintained in phenol red-free RPMI 1640 (PAA Laboratories) supplemented with 10% FBS and 100 U/ml penicillin, 0.1 mg/ml streptomycin and 4 mmol/L L-glutamine (all from Invitrogen).

Rat uterine leiomyoma (ELT-3) cells were kindly provided by C. Walker (University of Texas MD Anderson Cancer Center, Smithville, TX, USA) and were maintained in a medium (maintenance medium) consisting of DMEM and Ham's F12 medium (Invitrogen) in equal amounts supplemented with 10% FBS, 1.6 x 10^{-6} M ferrous sulfate, 5.0 x 10^{-8} M sodium selenite, 1.2 x 10^{-8} M vasopressin, 1.0 x 10^{-9} M T_3, 0.025 mg/ml insulin, 1.0 x 10^{-8} M cholesterol, 2.0 x 10^{-7} M hydrocortisone and 1.0 x 10^{-9} M transferrin (all from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). In experiments that require hormonal starvation, the medium was changed into medium consisting of phenol red-free DMEM/F12 medium supplemented with 1% bovine serum albumin (BSA), 1.6 x 10^{-6} M ferrous sulfate, 5.0 x 10^{-8} M sodium selenite, 1.2 x 10^{-8} M vasopressin, 1.0 x 10^{-9} M T_3, 1.0 x 10^{-8} M cholesterol, 2.0 x 10^{-7} M hydrocortisone, 1.0 x 10^{-9} M transferrin (all from Sigma) and MEM sodium pyruvate (Invitrogen), called basal medium. For proliferation assays, phenol red-free DMEM/F12 medium supplemented with only 1% BSA was used.

293FT cells were used for optimal lentivirus production [142]. 293FT cells were purchased from Invitrogen and maintained in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 0.1 mM non essential amino acids (all from Invitrogen).

Fetal bovine serum (FBS) was purchased from BioWhittaker, Inc. (Walkersville, MD, USA). All cell lines were cultured at 37°C with 5% CO_2.

2.1.2 Chemicals

The PR antagonists lonaprisan (ZK 230211), onapristone, mifepristone (RU486) and ZK 137316, the SPRMs asoprisnil, J1042, and J912, the non-steroidal PR modulator PRA-910, the ER agonist estradiol (E2), the ER modulator 4-hydroxytamoxifen (4-OHT) and the ER antagonist ZK 191703 were synthesized at Bayer Schering Pharma AG Research (Berlin, Germany). The standard synthetic progesterone analogue promegestone (R5020) and the vehicle dimethyl sulfoxide (DMSO) were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The vehicles ethanol and arachis oil were obtained from Roth (Carl Roth GmbH, Karlsruhe, Germany).

2.1.3 Transactivation assay

SK-NM-C VIII-1.1 cells (10,000 per well) were seeded onto 96-well dishes in MEM containing 5% charcoal-stripped FBS. After 48 hours PR ligands were added and incubation was continued for 24 hours. To determine agonistic activity, cells were cultured in the presence of increasing concentrations (10^{-11} to 10^{-6} M) of R5020, PRA-910, PR antagonists (lonaprisan, ZK 137316, onapristone, mifepristone) and SPRMs (J912, J1042, asoprisnil). As a negative control for reporter gene induction, cells were cultured in medium containing vehicle (0.1% DMSO). To determine antagonistic activity, cells were treated with increasing concentrations of PR modulator in combination with 100 pM R5020. Medium was removed and 160 µl of luminescence reporter gene assay system Steadylite HTS (Perkin Elmer, Inc., Waltham, MA, USA) was added to each well. Plates were incubated for 15 minutes at room temperature to ensure complete cell lysis and luciferase reaction, and were read in a TopCount NXT (Perkin Elmer, Inc.). LUC expression was given as a normalized response value relative to the maximal LUC expression produced by the reference agonist R5020. Data was analyzed to obtain the maximum efficacy, EC_{50} and IC_{50} values using Sigma Plot 8.0 software.

2.1.4 Mammalian two-hybrid assay

HeLa cells (10,000 per well) were seeded onto 96-well dishes in phenol red-free DMEM containing 5% charcoal-stripped FBS. After 24 hours cells were transfected with FuGENE 6 following the manufacturer's specifications (Roche, Mannheim, Germany). Transfection mix (10 µl) containing pCMV-GAL4/cofactor, pCMX-VP16/PR or pCMV-NFκB/PR (Table 1) and pFR-luc expression plasmid (Stratagene; La Jolla, CA, USA) in threefold volume of FuGene 6 filled up with OptiMEM (Invitrogen, Karlsruhe, Germany) was added to the medium and cells were permitted to recover overnight. Medium was removed and cells were treated with R5020, PRA-910, PR antagonists (lonaprisan, ZK 137316, onapristone, mifepristone) and SPRMs (J912, J1042, asoprisnil) in increasing concentrations (10^{-12} to 10^{-7} M) for 24 hours. Cells were then subjected to the transactivation assays procedure to obtain luciferase expression as described above (2.1.3).

Table 1. Plasmids utilized for mammalian two-hybrid assays.

Vector	cDNA	Fragment	Interrogated Sequence	Restriction sites
pCMV-GAL4	**hs SRC-1**	aa 1-1399	NM_147223	NotI / XhoI
pCMV-GAL4	**hs SRC-2**	aa 1-1464	NM_006540	BamHI / XbaI
pCMV-GAL4	**hs SRC-3**	aa 1-1422	NM_006534	NotI / XhoI
pCMV-GAL4	**hs SRC-1_NR-D**	aa 613-773	NM_003743	BamHI / HindIII
pCMV-GAL4	**hs SRC-3_NR-D**	aa 601-762	NM_181659	BamHI / HindIII
pCMV-GAL4	**hs NCoR**	aa 1792-2440[1, 2]	NM_006311	BamHI / EcoRI
pCMV-GAL4	**hs SMRT**	aa 1255-1495[1]	U37146	BamHI / EcoRI
pCMV-GAL4	**m c-Src kinase**	aa 1-250	NM_007783	EcoRI / XbaI
pCMV-NFkB	**hs PR-B**	aa 1-933	NM_000926	XbaI / EcoRI
pCMV-NFkB	**hs PR-A**	aa 165-933	NM_000926	XbaI / EcoRI

1) Cohen et al. 2001; 2) Webb et al. 2000

2.1.5 Cyclin D1 luciferase assay

To study the regulation of cyclin D1 transcription by PR modulators, U2OS cells (10,000 per well) were plated onto 96-well dishes and were allowed to attach overnight in phenom red-free DMEM supplemented with 3% charcoal stripped FBS. Cells were transfected with plasmids encoding firefly luciferase under the control of a 953 bp fragment of the human cyclin D1 promoter (pGL3-953CdLuc) and pSG5-hPR-B [70]. Plasmids were introduced with the FuGene 6 reagent following the manufacturer's specifications (Roche, Mannheim, Germany). One day after transfection, cells were starved in serum-free, phenol red-free medium overnight, and were then treated for 24 hours with R5020, PR antagonists (lonaprisan, ZK 137316, onapristone, mifepristone) and SPRMs (asoprisnil, J1042, J912) in increasing concentrations (10^{-12} to 10^{-7} M). Treatment with vehicle (0.1% DMSO) served as a negative control. To obtain luciferase expression, cells were subjected to the transactivation assays' procedure as described above (2.1.3).

2.1.6 Proliferation assay

T47D cells (5,000 per well) were seeded onto 96-well dishes in phenol red-free RPMI 1640 (PAA Laboratories, Cölbe, Germany) containing 5% charcoal-stripped FBS. After 24 hours cells were treated with E2, PR agonist R5020, PR antagonists (lonaprisan, ZK 137316, onapristone, mifepristone) and SPRMs (J1042, asoprisnil) in increasing concentrations (10^{-12}, 10^{-10} , 10^{-8} , 10^{-6} M) either with or without 100 pM E2 for 5 days. As a negative control, cells were cultured in medium containing vehicle (0.1% DMSO). Medium was removed and the CellTiter-Glo Luminescent Cell Viability Assay was performed according to the manufacturer's recommendations (Promega, Mannheim, Germany). Proliferation rate was given as percentage of the proliferation produced by the reference E2 that was termed as 100%. Statistical significance was assessed by the Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons vehicle vs. PR ligand/E2 (agonism) or E2 vs. PR ligand + E2 (antagonism).

ELT-3 cells (1,000 per well) were seeded onto 96-well dishes in maintenance medium (2.1.1). After 24 hours medium was changed into phenol red-free DMEM/F12 medium (Invitrogen, Karlsruhe, Germany) containing 1% BSA (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) to starve cells out of hormones overnight. To determine E2-induced proliferation, E2 was added in increasing concentrations (10^{-12} to 10^{-6} M) for 9 days. E2-containing media were refreshed after 3 and after 6 days and CellTiter-Glo Luminescent Cell Viability Assay was performed on days 0, 3, 6 and 9 as described above. To analyze the effects of ER modulators (4-OH-tamoxifen, ZK 191703) and PR modulators (R5020, lonaprisan, ZK 137316, onapristone, mifepristone, J1042, asoprisnil), compounds were added in increasing concentrations (10^{-12}, 10^{-10} , 10^{-8} , 10^{-6} M) for 7 days, either in the absence or in the presence of 100 pM E2. Medium containing test compounds was refreshed after 4 days. After 7 days of treatment the CellTiter-Glo Luminescent Cell Viability Assay was performed and proliferation rate was calculated as described above.

2.1.7 Apoptose assay

ELT-3 cells (1,000 per well) were seeded onto 96-well dishes in maintenance medium (2.1.1). After 24 hours medium was changed into phenol red-free DMEM/F12 medium (Invitrogen, Karlsruhe, Germany) containing 1% BSA (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) to starve cells out of hormones overnight. Cells were treated with increasing concentrations of E2 (10^{-10}, 10^{-8} , 10^{-6} M), 1 µM ZK 191703, R5020, PR antagonists (lonaprisan, ZK 137316, onapristone, mifepristone) and SPRMs (J1042, asoprisnil) for 4 days. Treatment with vehicle (0.1% DMSO) served as a negative control. Medium was removed and the Caspase-Glo 3/7 Assay was performed according to the manufacturer's recommendations (Promega, Mannheim, Germany). Apoptosis rate was given as percent of caspase 3/7 activity produced by complete hormonal starvation that was termed as 100%. Statistical significance was assessed by the Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons untreated vs. ER/PR ligand.

2.1.8 Treatments for gene expression analysis

T47D cells (1,000,000 per well) were plated onto 60 cm^2 dishes in 10 % FBS containing phenol red-free RPMI 1640 and were allowed to attach overnight. Cells were then washed twice with PBS, and medium was changed to phenol red-free RPMI 1640 containing 5% charcoal-stripped FBS. After hormonal starvation for 20 hours, 10 nM of the respective PR modulator (R5020, asoprisnil, J1042, J912, lonaprisan, ZK 137316, mifepristone or onapristone, except PRA-910 in a concentration of 100 nM; Table 8) or vehicle (0.1% DMSO) was added for 8 hours. Additionally, PR modulators were administered in combination with 100 pM R5020 for 8 hours to compile antagonistic properties.

ELT-3 cells (600,000 per well) were plated onto 60 cm^2 dishes in maintenance medium (2.1.1) and were allowed to attach overnight. Cells were then washed twice with PBS and medium was changed to basal medium (2.1.1). After hormonal starvation for 18 hours, 10 nM of ER modulator (E2, ZK 190703)

or PR modulator (R5020, lonaprisan), a combination of the modulators or only vehicle (0.1% DMSO) was added for 6, 24 and 48 hours (Table 16).

2.1.9 Cell cycle analysis

T47D cells (200,000 per well) were plated in 6-well plates in their growth medium (2.1.1) and were allowed to attach overnight. Medium was then removed, cells were washed twice with PBS (PAA Laboratories, Cölbe, Germany), and phenol red-free RPMI medium supplemented with 5% charcoal stripped FBS was added. Cells were cultured under steroid hormone-free conditions for two days, and 10 nM of PR modulator (R5020, PRA-910, asoprisnil, J1042, J912, lonaprisan, ZK 137316, mifepristone or onapristone) or vehicle (0.1% DMSO) was added either alone or in combination with 100 pM estradiol. After 24 hours of treatment, cells were harvested by trypsinization, pelleted by centrifugation, and washed once with PBS. Media and washes were retained with the adherent cells. The cells were resuspended in 1 ml of 70% ethanol and pipetted several times to ensure a uniform single-cell suspension. Samples were stored at –20°C until the day of analysis. Samples were then pelleted by centrifugation and washed once with PBS. The cell pellets were resuspended in 0.2 ml PBS containing 1.25 mg/ml ribonuclease A and 50 µg/ml propidium iodide (both from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), pipetted several times to ensure a uniform single-cell suspension, and transferred into a filter cap fitted polystyrene tube (Falcon; BD Biosciences, Heidelberg, Germany). Before analysis cells were incubated at 4°C in the dark for 4 hours. Cells were then analyzed on a fluorescence-activated cell sorting Caliber flow cytometer (FACS Calibur; BD Biosciences), and data were collected using CellQuest Pro software (BD Biosciences). Cells were gated on forward and side scatter to eliminate debris and on the width versus area of the red fluorescent voltage pulse to eliminate cell aggregates. The area of the red fluorescence voltage pulse for the gated cells is proportional to its DNA content, and the cell cycle profile for each sample was estimated using CellQuest Pro software (BD Biosciences). A minimum of 10,000 cells was gated for each sample. Statistical significance was assessed by the Student's T-test (p-value < 0.05 *, p-value < 0.005 **; p-value < 0.001 ***) for the comparisons vehicle vs. PR ligand/E2 (agonism) or E2 vs. PR ligand + E2 (antagonism).

2.1.10 Production of lentivirus in 293FT cells

293FT cells (12,000,000 per flask) were seeded onto 75 cm^2 flasks in their culture medium (2.1.1) and allowed to attach overnight. On the day of transfection, medium was removed from the 293FT cells and was replaced with 6 ml of growth medium containing serum, but no antibiotics. DNA-Lipofectamine 2000 complexes were prepared as recommanded by the manufacturer (Invitrogen, Karlsruhe, Germany). In the evening, medium was removed and 8-12 ml of culture medium were added. Virus containing supernatants were collected every 24 hours and medium was replaced with 8-12 ml of culture medium. Supernatants were harvested by centrifugation (3000 rpm for 5 minutes at 4°C) and then stored at 4°C. To remove cellular debirs, combined viral supernatants were filtered through a sterile 0.45 µm low protein binding filter (Millipore) and then were centrifuged again (18,000 rpm for 2 hours at 4°C). Viral stocks were stored at -80°C or directly titered via HIV-1 P24 ELISA according to

manufacturer's recommendations (Perkin Elmer, Inc., Waltham, MA, USA). A viral concentration that causes maximal transfection rate was used for mammalian cell transduction.

2.1.11 Lentiviral transduction of mammalian cells

ELT-3 cells (150,000 pro well) were seeded onto 6-well plates in their maintenance medium (2.1.1) and allowed to attach overnight. On the day of transduction, culture medium was removed, virus containing medium was prepared as recommended by the manufacturer (Invitrogen) and added to cells. The viral concentration for maximal ELT-3 cell transfection rate correlated with 3.2 µg/ml P24. Total volume of virus containing medium was kept as low as possible (100-150 µl) to further maximize transduction efficiency. Cells were incubated at 37°C for 90 minutes. Virus containing medium was then removed, replaced with 1 ml of fresh, complete culture medium and cells again were incubated at 37°C overnight. Cells were replated into larger-sized tissue culture formats and after 24-48 hours, medium was changed into maintenance medium containing Hygromycin B (Roche, Mannheim, Germany) to select for stably transduced cells.

2.2 Molecular biology

2.2.1 Cloning

Plasmids used for mammalian two-hybrid assays in HeLa cells were generated via TOPO TA Cloning (Invitrogen, Karlsruhe, Germany) and T4 DNA Ligase Kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Initial insert amplification was performed with the AccuPrime Pfx DNA Polymerase (Invitrogen) and fragments were cloned into pCMV-GAL4 and pCMV-NFκB destination vectors (Table 1, Table 2). PCMV-GAL4 and pCMV-NFκB destination vectors as well as the pFR-Luc reporter plasmids were purchased from Stratagene (La Jolla, CA, USA). PCMX-VP16/PR-A and -PR-B were a kind gift from R. Schüle (Gynecology department of the University Hospital of Freiburg, Germany). The LX-H10 sequence (RHWSQSPLLYGLLSDTASGV) fused into the pCMX-GAL4 plasmid was kindly provided by U. Fuhrmann (Bayer Schering Pharma AG, Women's Healthcare, Berlin, Germany).

Table 2. Primer for cloning of SRC-2, SRC-1-NR-D and SRC-3-NR-D into pCMV-GAL4.

Primer	Sequence
SRC-2_HpaI_forward	CGCGGTTAACAGCAGAAGCCACAGGC
SRC-2_HpaI_reverse	CGCGGTTAACTTGGCCAAGTCCACAGG
SRC-1_NR-D_forward	GCGCGGATCCCTGCATAACAATGACAG
SRC-1_NR-D_reverse	GCGCAAGCTTTTATCACACCTTTACATCATCC
SRC-3_NR-D_forward	GCGCGGATCCAGCAGTGTTGAGGGGGC
SRC-3_NR-D_reverse	GCGCAAGCTTTTATCACACTCCTTCCACTTGGGGC

The pGL3-953CdLuc plasmid used for the cyclin D1 luciferase assay was kindly provided by M. Beato (Faculty of Molecular Biology and Cancer Research of the Philipps University of Marburg, Germany) and the pSG5-PR-B plasmid was a kind gift from P. Chambon (Faculty of Medicine of the University of Strasbourg, France) [70].

Plasmids used for gene silencing via short hairpin (sh) RNA in ELT-3 cells were generated using Gateway Cloning (BLOCK-iT U6 RNAi Entry Vector Kit and BLOCK-iT Lentiviral RNAi Expression System; Invitrogen) according to the manufacturer's recommendations. Oligos (Table 3) were purchased from Invitrogen or TIB MOLBIOL (Berlin, Germany).

Table 3. Oligos for cloning of shRNAs into pGT-396_hygro.

Oligo	Sequence
shRNA_ESR1_top	CACCGGACTTGAATCTCCACGATCATTCAAGAGATGATCGTGGAGATTCAAGTCC
shRNA_ESR1_bottom	AAAAGGACTTGAATCTCCACGATCATCTCTTGAATGATCGTGGAGATTCAAGTCC
shRNA_TGFα_top	CACCGCAGTGGTGTCTCACTTCAACTTCAAGAGAGTTGAAGTGAGACACCACTGC
shRNA_TGFα_bottom	AAAAGCAGTGGTGTCTCACTTCAACTCTCTTGAAGTTGAAGTGAGACACCACTGC
shRNA_Inhbb_top	CACCGAAGGCAACCAGAACCTATTTTTCAAGAGAAAATAGGTTCTGGTTGCCTTC
shRNA_Inhbb_bottom	AAAAGAAGGCAACCAGAACCTATTTTCTCTTGAAAAATAGGTTCTGGTTGCCTTC

shRNA = short hairpin RNA; ESR1 = estrogen receptor alpha; TGFα = transforming growth factor α; Inhbb = inhibin β subunit B

2.2.2 RNA preparation and cDNA synthesis

Total RNA was prepared using QIAshredder and RNeasy Mini Kit (both from Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A DNase I (Qiagen) digestion step was included to eliminate genomic DNA. The quality of the total RNA was checked for integrity with RNA LabChips on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., USA) and for concentration on the Peqlab NanoDrop (Peqlab Biotechnology, Erlangen, Germany). Double-stranded cDNA was synthesized from 5 µg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Karlsruhe, Germany).

In case of mammalian tissue, tumors were homogenized using stainless steel beads and the TissueLyser Adapter Set according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Total RNA was prepared as described above.

2.2.3 TaqMan® quantitative real-time PCR assays

The expression levels of selected genes were analyzed with TaqMan® Gene Expression Assays from Applied Biosystems (Foster City, CA, USA) listed in Table 4. Gene-specific primers and probes were used with the Platinum qPCR SuperMix-UDG (Invitrogen, Karlsruhe, Germany) and were incubated at 50°C for 2 minutes followed by 10 minutes at 95°C, and then 40 cycles of PCR as follows: 95°C for 15 seconds, then 60°C for 1 minute in an ABI PRISM 7000 Sequence Detection System (Applied

Biosystems). Data was analyzed using the Sequence Detector Version 2.0 software (Applied Biosystems) and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold change was determined by pairwise comparisons of treatment versus vehicle.

Table 4. TaqMan® gene expression assays used for quantitative real-time PCR.

Gene Name	Gene Symbol	Assay ID	Reference Sequence
homo sapiens			
Cyclophilin A	**PPIA**	Hs99999904_m1	**NM_021130.3**
Kruppel-like factor 4	**EZF**	Hs00358836_m1	**NM_004235.3**
Epidermal growth factor receptor	**EGFR**	Hs00193306_m1	**NM_005228.3**
Cyclin D1	**CCND1**	Hs00277039_m1	**NM_053056.2**
ATPase, Na+/K+ transporting, alpha 1 polypeptide	**ATP1A1**	Hs00167556_m1	**NM_000701.6**
			NM_001001586.1
FK506 binding protein 5	**FKBP51**	Hs00296750_s1	**NM_004117.2**
Progesterone receptor	**PR**	Hs00172183_m1	**NM_000926.4**
Adhesion molecule with Ig-like domain 2	**AMIGO2**	Hs00827141_g1	**NM_181847.3**
Defensin, beta 32	**DEFB32**	Hs01651960_m1	**NM_207469.1**
G protein-coupled receptor, family C, group 5, member A	**GPRC5A**	Hs00173681_m1	**NM_003979.3**
K+ inwardly-rectifying channel, subfamily J, member 3	**KCNJ3**	Hs00158421_m1	**NM_002239.2**
Parathyroid hormone-like hormone	**PTHLH**	Hs00174969_m1	**NM_198964.1**
Zinc finger and BTB domain containing 16	**ZBTB16**	Hs00232313_m1	**NM_001018011.1**
Inhibin, beta B	**INHBB**	Hs00173582_m1	**NM_002193.2**
Jumonji domain containing 2B	**JMJD2B**	Hs00392119_m1	**NM_015015.1**
E2F transcription factor 1	**E2F1**	Hs00153451_m1	**NM_005225.2**
Cyclin-dependent kinase 6	**CDK6**	Hs00608037_m1	**NM_001259.5**
Cyclin-dependent kinase inhibitor 1A	**p21**	Hs00355782_m1	**NM_000389.3**
			NM_078467.1
rattus norvegicus			
Cyclophilin A	**Ppia**	Rn00690933_m1	**NM_017101.1**
Estrogen receptor alpha	**ER1**	Rn00562166_m1	**NM_012689.1**
Progesterone receptor	**PR**	Rn00575662_m1	**NM_022847.1**
Transforming growth factor alpha	**TGFα**	Rn00446234_m1	**NM_012671.1**
Periplakin	**Ppl**	Rn01424894_m1	**XM_220174.4**
			NM_001106976.1
Desmuslin	**Dmn**	Rn00711100_m1	**XM_001055724.1**
			XM_001055657.1
Fibrinogen-like 2	**Fgl2**	Rn00584935_m1	**NM_053455.2**
Inhibin, beta B	**Inhbb**	Rn01753772_m1	**XM_344130.3**
Dehydrogenase/reductase (SDR family) member 7	**Dhrs7**	Rn02395365_m1	**NM_001013098.1**

2.3 Gene expression profiling

2.3.1 Affymetrix GeneChip® expression profiling experiments

Total RNA of T47D cells or ELT-3 cells was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A DNase I (Qiagen) digestion step was

included to eliminate genomic DNA. The quality of the total RNA was checked for integrity with RNA LabChips on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., USA) and for concentration on the Peqlab NanoDrop (Peqlab Biotechnology, Erlangen, Germany). Two µg of total RNA was used to prepare biotinylated and fragmented cRNA following the instruction of the Affymetrix One-Cycle Target Labeling protocol and individual samples were hybridized on the Affymetrix GeneChip arrays (Affymetrix, NASDAQ:AFFX, Santa Clara, CA; USA). Chips were scanned using a GeneChip Scanner 3000 7G (Affymetrix), and scanned images were extracted using the Affymetrix GCOS Software. For T47D cells Affymetrix GeneChip HG-U133Plus2.0 arrays (n = 55) were used. Biological replicates for all treatments (n = 5) and biological replicates for vehicle controls (n = 10) were applied to Affymetrix GeneChip hybridization and analyses. For ELT-3 cells Affymetrix GeneChip Rat230_2.0 arrays (n = 45) were employed. Biological replicates for all treatments and vehicle controls (n = 5) were applied to Affymetrix GeneChip hybridization and analyses.

The primary outcome of Affymetrix analyses is the intensity of cRNA hybridization to respective complementary probe sets indicating the expression level. Probe sets are a combination of 25mer probes representing the most optimal balance between sensitivity and specificity to determine whether or not the complementary sequence of cRNA or cDNA is present in the sample. In section 3 and 4 variations in cRNA hybridization intensity due to PR modulator treatment were referred to as regulation of gene expression. The proteins encoded by the respective genes were referred to as protein products.

Expression analyses were performed using the Expressionist Pro 4.0 software (Genedata AG, Basel, CH). The quality of the data files (CEL format) containing probe level expression data was checked and refined using the Expressionist Refiner software (Genedata AG). Subsequently, refined CEL files were condensed with MAS5.0 and LOWESS normalized using all experiments as a reference.

2.3.2 Unsupervised analysis

Principle Component Analysis (PCA) showing the relationships between individual samples was performed using the Expressionist Analyst Pro 4.0 software (Genedata AG, Basel, CH).

2.3.3 Supervised analysis

After removal of outliers, data was subjected to a number of pairwise comparisons using the Expressionist Analyst Pro 4.0 software (Genedata AG, Basel, CH). Statistical analyses included pairwise comparisons between control samples treated with vehicle (0.1% DMSO) and compound treated samples. Probe sets were regarded to be regulated if they were outside of the triangular region in the Volcano plot (a plot of fold change (FC) versus T-test p-value) with the corner values of an FC of 2.5 or higher and a T-test p-value analogous to ST Q-value < 0.01.

Hierarchical clustering analysis of probe sets significantly regulated by PR antagonists and SPRMs was performed using the Expressionist Analyst Pro 4.0 software (FC > 2, Volcano: FC >2.5-5 and T-test p-value analogous to ST Q-value < 0.01).

Venn intersection analyses for significantly regulated or counter-regulated genes were conducted to identify regulatory overlaps using the Expressionist Analyst Pro 4.0 software.

R5020-regulated genes and PR modulator-counter-regulated genes in combined treatments were assessed by ANOVA analyses (CR < -0.3, threshold selection by manual inspection and ST Q < 0.0001). SOM clustering was carried out using the Expressionist Analyst Pro 4.0 software (quality p-value = 0.04).

Profile distance search for expression profiles was performed using the Expressionist Analyst Pro 4.0 software (correlation coefficient +/- 0.6, p-value < $2x10^{-16}$).

MetaCore pathway analyses were conducted using GeneGo (be-genego.eu.schering.net:8100).

2.4 Protein biochemistry

2.4.1 Western blot

ELT-3 cells were lysed and homogenized in lysis buffer (50 mM Tris-HCl (pH 7.5), 25 mM KCl, 1% Triton X-100, 4 mM EDTA, 10mM sodium fluoride; all from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) containing protease inhibitors (Complete Mini; Roche, Mannheim, Germany), 1 mM dithiothreitol, 1 mM sodium vanadate, and 10 mM β-glycerolphosphat (all from Boehringer Mannheim, Indianapolis, IN). Lysates were incubated on ice for 30 minutes, centrifuged at 4°C for 10 minutes and supernatants were frozen and stored at −20 C. Protein lysates were quantified colorimetrically using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) and 20–30 µg of lysates were separated by 7.5% SDS–PAGE and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked for 1 hour at room temperature in a solution of TBS (150 mM NaCl and 10 mM Tris–HCl (pH 8.0)) containing 0.05% Tween 20 (all from Sigma) and 0.75% Blocking Reagent (Roche) and then were probed with primary antibody (anti-progestrone receptor, 1:500, #MA1-410; Dianova, Hamburg, Germany) at 4°C overnight. β-tubulin was used as a loading control (1:100, #ab7287; Abcam, Cambridge, UK). Horseradish peroxidase conjugated secondary antibody (anti-mouse, 1:3000, #NA931V; Amersham Biosciences) was applied at room temperature for 3 hours. All antibodies were diluted in TBS containing 0.05% Tween 20 and 0.75% Blocking Reagent. Protein expression levels were examined using an ECL Plus kit and ECL Hyperfilm (both form Amersham Biosciences).

2.4.2 Immunofluorescence

ELT-3 cells (5,000 pro well) were seeded onto 12-well plates on sterile coverslips (Fisher Scientific, Pittsburgh, PA, USA) in their maintenance medium. Two days after seeding cells were washed with PBS (PAA Laboratories, Cölbe, Germany) twice for five minutes per wash, were fixed on coverslips in absolute methanol (Carl Roth GmbH, Karlsruhe, Germany) at -20°C for 10 minutes, air-dried and subsequently rinsed with PBS twice. FBS was diluted 1:10 with PBS, added to each coverslip, and incubated for 15 minutes at room temperature. Cells were rinsed three times with PBS for five minutes per wash, and coverslips were overlaid with 50 µl primary antibody (Table 5) diluted in PBS containing 10% FBS. Incubation with antibodies was performed on Parafilm (American National Can Group, Inc., San Diego, CA, USA). As a control for non-specific binding 50 µl PBS containing 10% FBS were utilized in place of antibody. Antibody-coated coverslips were incubated at room temperature for 1 hour and then washed three times with PBS for 5 minutes per wash. Coverslips were overlaid with 200 µl fluorescein-labeled immunoglobulin G (Table 5) diluted in PBS containing 10% FBS and then incubated in the dark at room temperature for 30 minutes. After washing three times with PBS, 500 µl DAPI (100 ng/ml) diluted in PBS was applied for two minutes at room temperature. Cells were rinsed with PBS three times for five minutes per wash. Coverslips were then mounted with Fluoromount G (Dunn Laboratories, Inc., Roswell, GA, USA), air-dried in the dark overnight and examined under a AxioVision microscope (Carl Zeiss AG, Göttingen, Germany) equipped with epifluorescent optics (358 nm, 488 nm or 568 nm).

Table 5. Antibodies utilized for immunofluorescence staining.

Antibody	Dilution	#
primary		
anti-smooth muscle actin	1:100	ab18147[1)]
anti-desmin	1:20	ab6322[1)]
anti-estrogen receptor alpha	1:100	sc-542[2)]
anti-progesterone receptor	1:200	ab2764[1)]
secondary		
anti-mouse Alexa Fluor 568	1:1000	A-11031[3)]
anti-mouse Alexa Fluor 488	1:1000	A-11029[3)]
anti-rabbit Alexa Fluor 568	1:1000	A-11036[3)]

1) Abcam, Cambridge, UK; 2) Santa Cruz Biotechnology, Inc, Santa Cruz, CA, US; 3) Molecular Probes, Biocompare, Inc., San Francisco, CA, US

2.5 In vivo experiments

2.5.1 Animals

Fox Chase female SCID (severe combined immunodeficiency) mice (5-6 weeks old,~ 20 g;) were obtained from Charles River Laboratories (Sülzfeld, Germany). Mice are immunodeficient regarding to

T-and B-lymphocytes and were checked by the breeder for < 100 ng immunoglobulin/ml blood (proven non-leaky). They were kept under specific pathogen free (SPF) housing and feeding conditions in Scantainers (Scanbur Ltd., Karslunde, Denmark) in filter covered polycarbonate cages (10 animals/group; Makrolon type III, Fa. Becker, Castrop-Rauxel, Germany) with dust-free softwood granulate (Fa. Rettenmeier & Söhne, Rosenberg, Germany). Feeding conditions were ad libitum and pellets were autoclaved and special for immunodeficient mice breeding (fortfid; Fa. Sniff, Soest, Germany). All manipulations, including cage transfers twice per week, were carried out under clean benches (Lamin Flow Box HERAsafe; Fa. Heraeus, Hanau, Germany).

All animal experiments were conducted according to German animal protection laws.

2.5.2 Establishment of an ELT-3 cell induced xenograft system in SCID mice

ELT-3 cells have previously been shown to be tumorigenic when injected into athymic nude mice [48]. The tumorigenity in Fox Chase SCID mice, and furthermore, the ability of 17β-estradiol to modulate the growth of tumors in a SCID mouse xenograft system was determined by inoculating 5-6 week-old intact SCID mice (Charles River) subcutaneously (sc) with $5x10^6$ cells over the right hip. Three days before inoculation mice were randomized, separated into four treatment groups (n = 5) and implanted with pellets of 17β-estradiol (one 1.7-mg or 0.36-mg 60-day release pellet in each mouse; Innovative Research of America, Toledo, OH, USA) sc in the interscapular area, ovariectomized or left untreated. Immediately before inoculation cells were harvested during log phase growth and resuspended in 0.1 ml of serum-free RPMI 1640 medium (PAA Laboratories, Cölbe, Germany) or serum-free RPMI 1640 medium/MatriGel (1:1, v/v; BD biosciences, Erembodegem, Belgium). Animals were observed twice a week post inoculation and tumor development was measured with a sliding caliper (Absolute Digimatic Caliper No. 500; Fa.Mitutoyo, Andover, UK). The mean area of each tumor was calculated as the product of the longest diameter and its perpendicular. Mice were killed by cervical dislocation when tumors grew greater than 150 mm^2 or approximately 67 days after inoculation.

2.5.3 Genetic modulation of ELT-3 tumor growth

Inoculation of wildtype ELT-3 cells or ELT-3 cells with silenced estrogen receptor alpha expression was performed in 0.1 ml of serum-free RPMI 1640 medium as described above. All SCID mice were supplemented with 17β-estradiol beginning three days before inoculation. 17β-estradiol was given as a depot (0.1 mg/kg estradiol-valerate in ethanol/arachidis oleum (1:9, v/v)) sc every seven days. Three days before inoculation mice were randomized, separated into four treatment groups (n = 10) and ovariectomized or left untreated. Animals were observed twice a week post inoculation of ELT-3 cells or ELT-3 cells carrying a knock down. Analysis as well as finalization of the experiment was performed as described above.

2.5.4 Pharmacologic modulation of ELT-3 tumor growth

Inoculation of ELT-3 cells was performed in 0.1 ml of serum-free RPMI 1640 medium as described above. All SCID mice were supplemented with 17β-estradiol as a depot as described above. Animals were observed twice a week post inoculation. If established tumors reached 15 mm^2 (~ 39 days post inoculation) in the most of the mice, mice were randomized, separated into treatment groups (n = 10), ovariectomized (simultaneously to treatment start) or treated with solvent control (ethanol/arachidis oleum (1:9, v/v)) or test compound (ZK 191703, lonaprisan, mifepristone: all 30 mg/kg per os, daily) for 2 weeks. Tumor growth was observed twice a week within the treatment period. Analysis and finalization of the experiment was performed as described above. Tumors were removed, weighed and a portion of each tumor was frozen at -70°C for subsequent analyses. The remainder of the sample was fixed in 3.7% neutral buffered formalin for histological examination. Fixed tumor samples were embedded in paraffin by routine methods, sectioned, stained with hematoxylin and eosin, and examined under a microscope.

2.5.5 Statistical analysis

Baseline-adjusted area under curves (AUCs) were calculated using the trapezoidal rule.

The inhibition was calculated as $\frac{C^+ - \text{substance}}{C^+ - C^-}$ for comparisons of compounds, with C^+ = estradiol supplemented and vehicle treated mice and C^- = ovarectomized mice, or $\frac{C^+ - \text{substance}}{C^+}$ for comparisons of different injected cells, with C^+ = wildtype cells.

Usually, Fieller confidence intervals [1] are used in this situation. However, there was relevant heterogeneity in the data, and also the normality of the data could be questioned, which may lead to bias in the estimation of the confidence intervals [2]. Therefore, in addition the one-sided 95% confidence intervals were obtained by bootstrapping (1000 samples from the original data) [3].

For the comparison of the substances lonaprisan versus mifepristone, a two-sided 95% confidence interval for the quotient $\frac{lonaprisan}{mifepristone}$ was calculated.

For the comparison of the ELT-3 cells stably transformed with Esr1-shRNA versus control-shRNA, a two-sided 95% confidence interval for the quotient $\frac{ER1 - shRNA}{control - shRNA}$ was calculated in a similar way as described before. No correction for multiple testing was applied.

3. Results

3.1 Analyses of PR modulators' mechanism of action

PR modulators are known to display different profiles in vivo, in particular SPRMs and PR antagonists [115, 116]. To identify a suitable classification system, the following analyses of PR modulators' mode of action included the non-steroidal ligand PRA-910 [113, 114, 143], selective progesterone receptor modulators (J1042, asoprisnil, J912) which exhibit tissue selective agonistic activity in some experimental settings in vivo [116], the mixed antagonist mifepristone which exerts agonistic potential with respect to specific cellular cues [122], classical antagonists (ZK 137316, onapristone) and the pure antagonist lonaprisan. Chemical structures of all PR modulators are shown in Figure 3. Previously described characteristics of the PR modulators are listed Table 6.

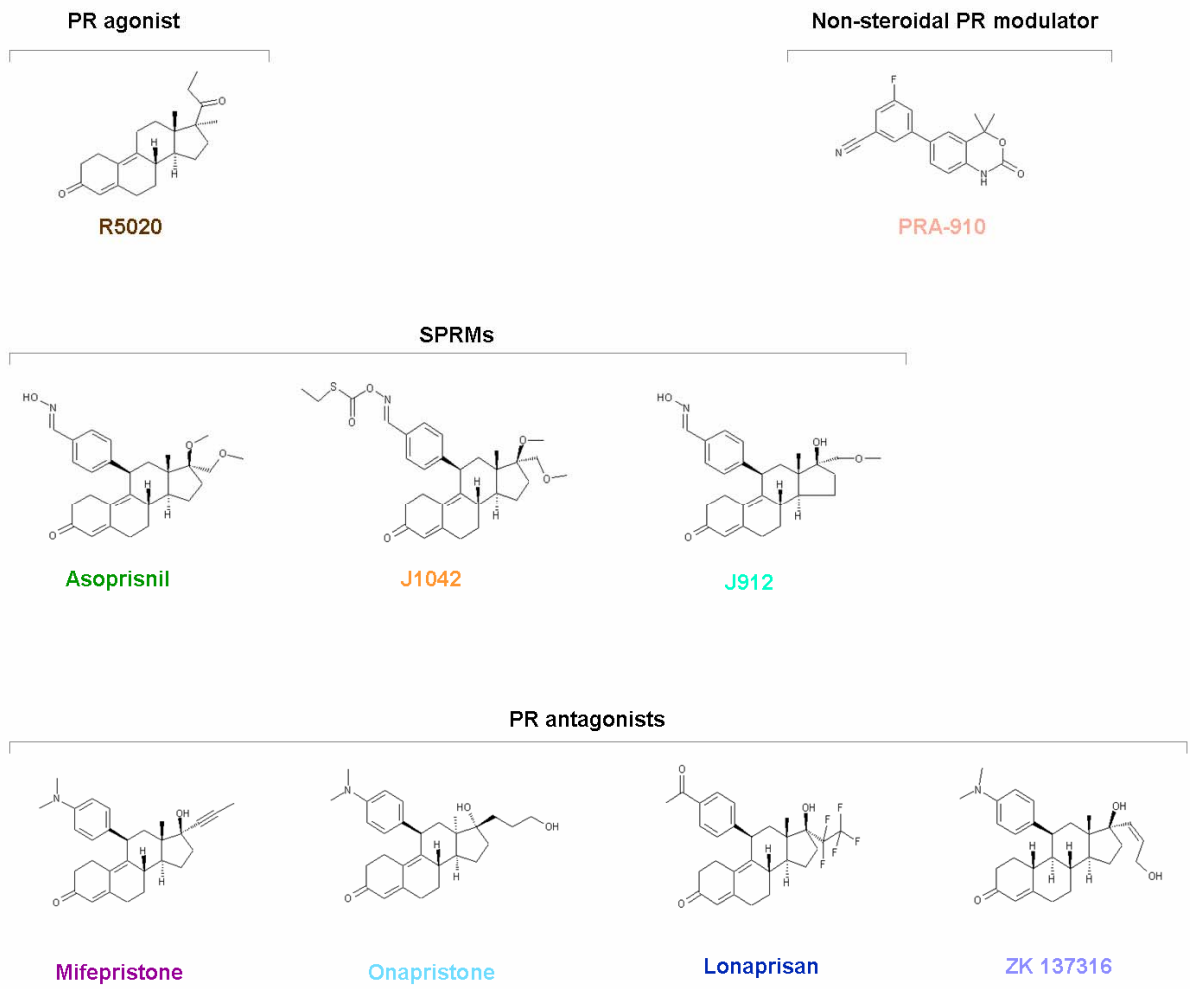

Figure 3. Chemical structures of different types of PR modulators described so far. Steroidal PR agonist: R5020. Steroidal SPRMs: Asoprisnil, J1042, J912. Steroidal PR antagonists: Mifepristone, Onapristone, Lonaprisan, ZK 137316. Non-steroidal PR modulator: PRA-910.

Table 6. Properties and previous classification of used PR ligands based on published data [115, 116].

PR modulator	Other designations	In vivo	Previous classification		
			Category	Type*	Structure
R5020	promegestone	agonist	agonist; standard	-	steroidal
PRA-910	tanaproget derivative	agonist / antagonist	partial agonist	-	non-steroidal
J1042	mesoprogestine	SPRM	SPRM	II	steroidal
Asoprisnil	J867	SPRM	SPRM	II	steroidal
J912	-	SPRM	SPRM	II	steroidal
Mifepristone	RU486	antagonist	antagonist; standard	II	steroidal
ZK 137316	-	antagonist	antagonist	II	steroidal
Onapristone	ZK 98299	antagonist	antagonist	I	steroidal
Lonaprisan	ZK 230211	antagonist	antagonist	III	steroidal

3.1.1 Antagonistic activity in a cellular transactivation assay

In general, the PR agonistic and antagonistic activities of synthetic PR modulators can be determined by cellular transactivation assays in vitro. SK-NM-C cells stably expressing the full-length PR isoform A or B were used to characterize the ligand-dependent PR transcriptional activity for the selected PR modulators from a reporter gene which contains the complex mammalian mammary tumor virus (MMTV) long-terminal repeat (LTR) progesterone responsive element (PRE) [144]. Cells were treated with increasing concentrations of PR ligand only for agonistic activity or in the presence of 100 pM R5020 for antagonistic profiles. All transcriptional activities at the MMTV promotor were compared to the standard agonist R5020 or the standard antagonist mifepristone, respectively. In most cases, the results for modulation of PR transcriptional activity were similar for the PR isoforms A and B.

No difference in efficacy was observed between pure antagonists, mixed antagonists and designated SPRMs. Neither mifepristone, ZK 137316, onapristone and lonaprisan nor J1042, asoprisnil and J912 exhibited any agonistic activity at the MMTV promoter (Figure 4A, B, Table 9), but full antagonistic effects when tested in combination with R5020 (Figure 4C, D, Table 9). The SPRMs displayed antagonistic potencies comparable to mifepristone (IC_{50} = 0.05 nM). However, PR antagonists differed in their potencies. In particular, lonaprisan represented the most potent antagonist of the modulators, with a 10-fold higher potency than mifepristone. In contrast, the classical antagonist onapristone and the non-steroidal PR modulator PRA-910 displayed reduced antagonistic potency (IC_{50} = 3.3 nM; Figure 4C, D). Furthermore, PRA-910 showed a maximal antagonistic efficacy of 62% and slight agonistic effects (max. efficacy = 52%). The agonistic potency of PRA-910 was markedly lower compared to the standard R5020 (EC_{50} = 130.6 nM vs. EC_{50} = 0.05nM; Figure 4B).

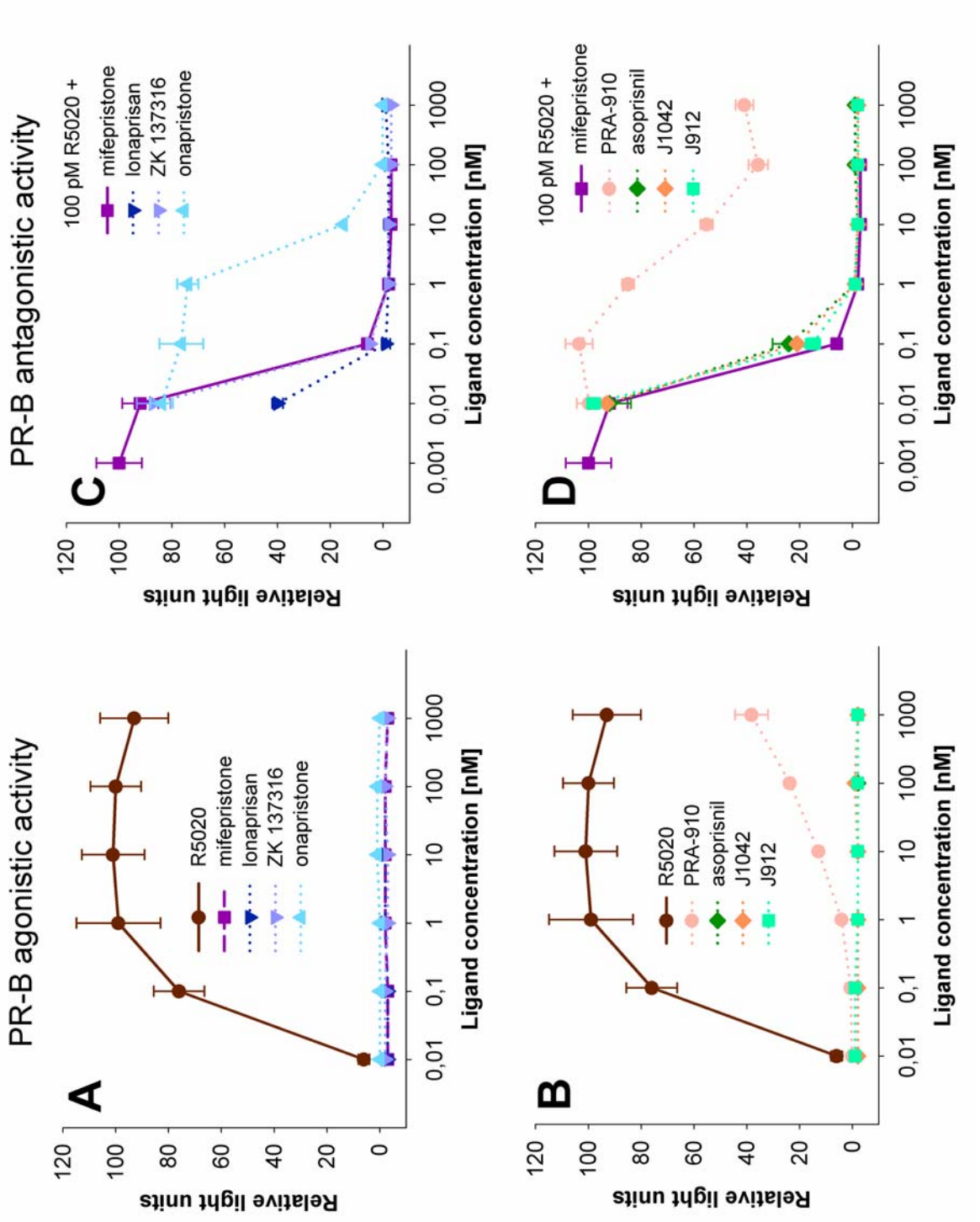

Figure 4. Transcriptional activity at the MMTV promoter. PR antagonists and SPRMs in transactivation assays in SK-NM-C VIII-1.1 cells. A-B) Agonistic activity of ligand-bound PR-B. Cells were treated for 24 hours with vehicle or increasing concentrations (10^{-11} to 10^{-6} M) of PR modulator. C-D) Antagonistic activity of ligand-bound PR-B. Cells were treated for 24 hours with 100 pM R5020 plus increasing concentrations (10^{-11} to 10^{-6} M) of PR modulator. Each graph represents at least two independent experiments and error bars denote CI (95%) of triplicate wells. Transcriptional activities at the MMTV promotor were similar for PR-A (data not shown).

Taken together, the PR modulators analyzed displayed different potencies in SK-NM-C cell-based transactivation assays. However, SPRM activity could not be differentiated from mixed PR antagonist activity, as observed for mifepristone, by transactivation assays in vitro.

3.1.2 Influence on protein interaction properties of the human progesterone receptor

Activation or inhibition of progesterone receptor action is a consequence of PR modulator-induced alterations in the receptor conformation. Conformational changes are responsible for the formation of a receptor surface with binding pockets and thus affects the ability of the PR to interact with the DNA, but also with other interacting proteins such as coactivators and corepressors or kinases.

Using a mammalian two-hybrid assay system, the interaction properties of full-length PR isoforms and an assortment of cofactors were analyzed for selected PR modulators. In this system, plasmids expressing the full-length human PR fused to the strong activation domain of NFκB in pCMV-AD or the strong activation domain of VP16 in pCMX, respectively, were used in combination with plasmids expressing the respective cofactor fused to the GAL4-DNA binding domain in pCMV-BD. The ability of the PR/NFκB or PR/VP16 fusion to activate transcription from a GAL4-responsive reporter plasmid (pFR-Luc) was utilized as a readout for the interaction between PR and the cofactor.

Analysis of human cell lines with low or no endogenous expression of progesterone receptor isoforms A and B (HeLa, SK-NM-C, PC-3, MCF-7, HEK-293 cells) identified HeLa cells as the cell line with the lowest expression level of selected coactivators and corepressors on both mRNA and protein level. Furthermore, HeLa cells were identified to have low basal GAL4-mediated transcriptional activity (data not shown). The specificity of the HeLa cell-based assay system was confirmed by using control experiments which revealed no induction of luciferase expression in cells transfected with a transactivation domain-containing plasmid that lacks PR-fusion (data not shown). Absence of luciferase expression was also observed in cells which were transfected with a GAL4-containing plasmid that lacks cofactor-fusion (data not shown).

Tested cofactors included members of the p160 family of steroid receptor coactivators (SRC-1, SRC-2 and SRC-3) [145-147] in full-length and truncated forms covering the NR interaction domain. Coactivators of the SRC-family were chosen, because they have been shown to be essential for hormone-induced transcription [148]. In a phage peptide library of $(X)_7LxxLL(X)_7$ peptides differing in sequences flanking the LxxLL core motif of coactivators which is critical for nuclear receptor interaction, the LX-H10 peptide was identified to be useful in determining receptor selectivity [149, 150]. Therefore, PR interaction with the LX-H10 peptide was analyzed to investigate PR isoform-specific recruitments. The nuclear receptor corepressor (NCoR) and the silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) were selected as corepressors because they have been shown to be essential for the activity of ER and PR antagonists [151]. In the presented experiments, NCoR and SMRT were used in truncated forms covering the NR interaction domains [152].

In most cases, the results for cofactor interactions induced by the respective PR modulator were similar for PR isoform A and B. Hence, Figure 5 and Figure 6 display the representative interaction profiles of isoform B.

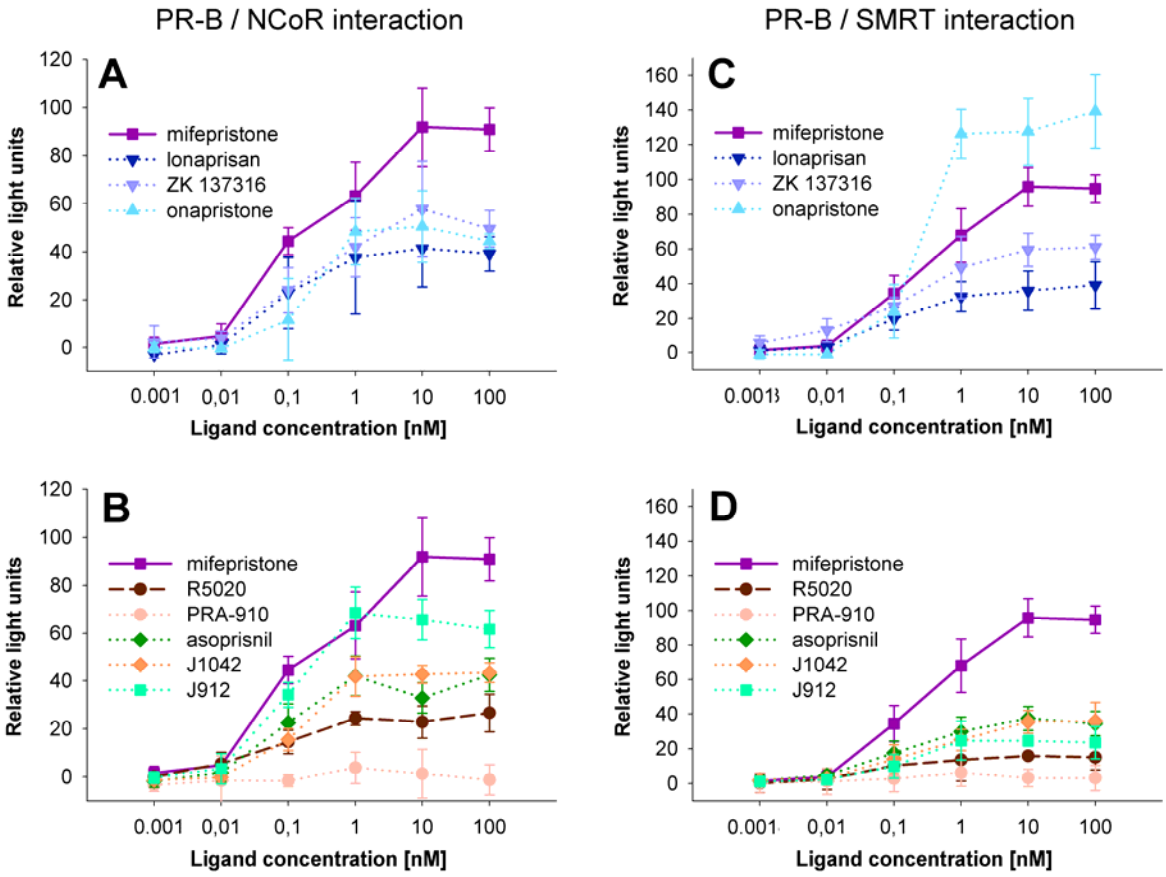

Figure 5. PR modulator-induced interactions of PR-B with the corepressors NCoR and SMRT in a mammalian two-hybrid assay system in HeLa cells. Transiently transfected cells were treated for 24 hours with vehicle or increasing concentrations (10^{-12} to 10^{-7} M) of PR modulator. A-B) PR-B interaction with NCoR induced by PR antagonists (A) or SPRMs (B). C-D) PR-B interaction with SMRT induced by PR antagonists (C) or SPRMs (D). Each graph represents at least two independent experiments and error bars denote CI (95%) of triplicate wells. Interaction profiles for PR-A were similar (data not shown).

For all types of steroidal agonists and antagonists, interactions of the PR isoforms were observed with coactivators and corepressors, although to different degrees. As expected, the interactions with corepressors induced by agonists were not as prominent as for the antagonists or SPRMs (Figure 5). Moreover, PR antagonists differed in potency and efficacy.

The highest efficacy for ligand-induced association with NCoR was observed for the mifepristone-bound PR-B (Figure 5A, B). Onapristone exhibited the highest efficacy to recruit SMRT when bound to PR isoform B (Figure 5C), however, not when bound to PR isoform A (data not shown). The potency of onapristone-bound PR-B to induce SMRT interaction was similar to mifepristone-bound, although onapristone displayed lower potency for SMRT recruitment to PR isoform A (data not shown). Beyond this, no isoform-selective recruitments of corepressors were observed for the various types of steroidal modulators in this experimental setting. The recruitment of the corepressors NCoR and SMRT were not significantly different for the SPRMs asoprisnil, J1042, and J912. Interestingly, the non-steroidal PR modulator PRA-910 did not induce PR association with NCoR and SMRT.

In assays analyzing the interactions with the full-length coactivators SRC-1, SRC-2 and SRC-3, no significant differences were identified for the different types of ligands (Table 7). However, the interaction profiles with the nuclear receptor interacting domain (NR-D) of SRC-3 showed that this recruitment is exclusive to agonistic ligands. Antagonists and SPRMs did not induce interactions with SRC-3-NR-D (Table 7). In particular, the interaction profile of the non-steroidal PR modulator PRA-910 differed from all types of steroidal ligands. PRA-910-bound PR did not interact with tested cofactors, neither with the selected corepressors nor with the coactivators in full-length nor in truncated form (Figure 5B, D). The only exception was the LX-H10 peptide (Figure 6B).

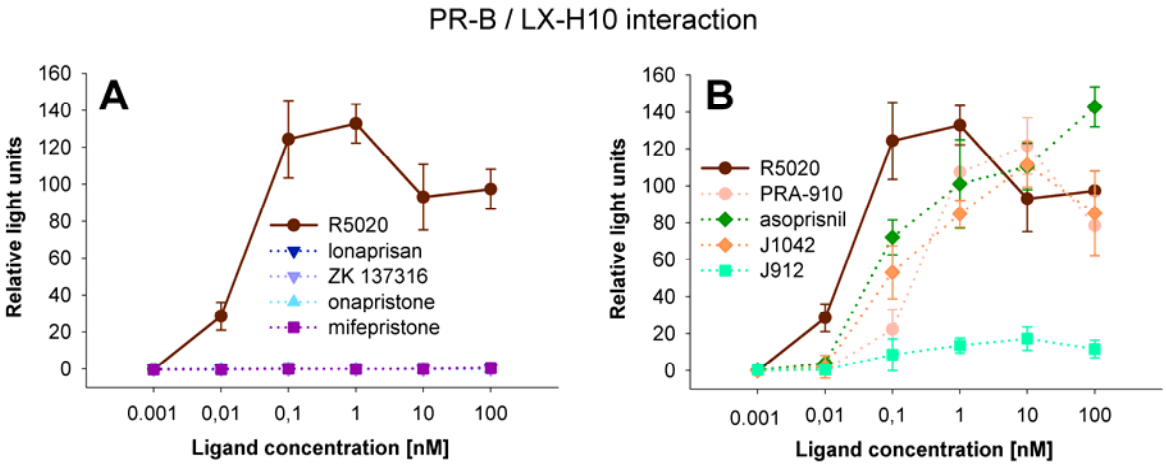

Figure 6. SPRM- and PR antagonist-induced interaction of PR-B with the LX-H10 peptide in a mammalian two-hybrid assay system in HeLa cells. Transiently transfected cells were treated for 24 hours with vehicle or increasing concentrations (10^{-12} to 10^{-7} M) of PR antagonists (A) or SPRMs (B). Each graph represents at least three independent experiments and error bars denote CI (95%) of triplicate wells. Interaction profiles for the PR-A were similar and are not shown.

The efficacy of PRA-910-induced interaction with LX-H10 peptide was similar to the full agonist R5020, albeit the potency was ten times lower. In addition, LX-H10 peptide interaction profiles revealed a significant difference between steroidal antagonists and SPRMs. Antagonist-bound PR-B did not show any recruitment of LX-H10 peptide (Figure 6A), in contrast to SPRM-bound PR-B (Figure 6B). The maximum efficacy of LX-H10 peptide interaction observed for asoprisnil- and J1042-bound PR-B was similar to agonist R5020 (Figure 6B) whereas the efficacy of J912-bound PR-B was significantly lower (max. efficacy = 14%). In addition, the potency of recruiting activity was about five times stronger for R5020- than for SPRM-bound PR-B. An isoform-specific recruitment of LX-H10 as reported by Giangrande et al. [150] was not observed.

Beyond its classical function as a ligand-activated transcription factor, the human progesterone receptor isoform B rapidly activates cytosolic signaling pathways [105, 153], including downstream pathways of SH3 domain-containing factors like c-Src kinase. The rapid activation of c-Src kinase signaling by PR ligands was shown to be transcription-independent and to contribute to the effects on cell proliferation [154]. To analyze the ligand-induced association with c-Src kinase for different PR modulators, mammalian two-hybrid assays were performed in HeLa cells. Cells were transfected with plasmids expressing the full-length human PR-B fused to the strong activation domain of NFκB in pCMV-AD and plasmids expressing the c-Src kinase fragment fused to the GAL4-DNA binding domain in pCMV-BD. The interaction between PR-B and c-Src kinase induced by the respective ligand was assessed by measuring the ability of the NFκB/PR-B fusion to activate transcription from a GAL4-responsive reporter plasmid (pFR-Luc).

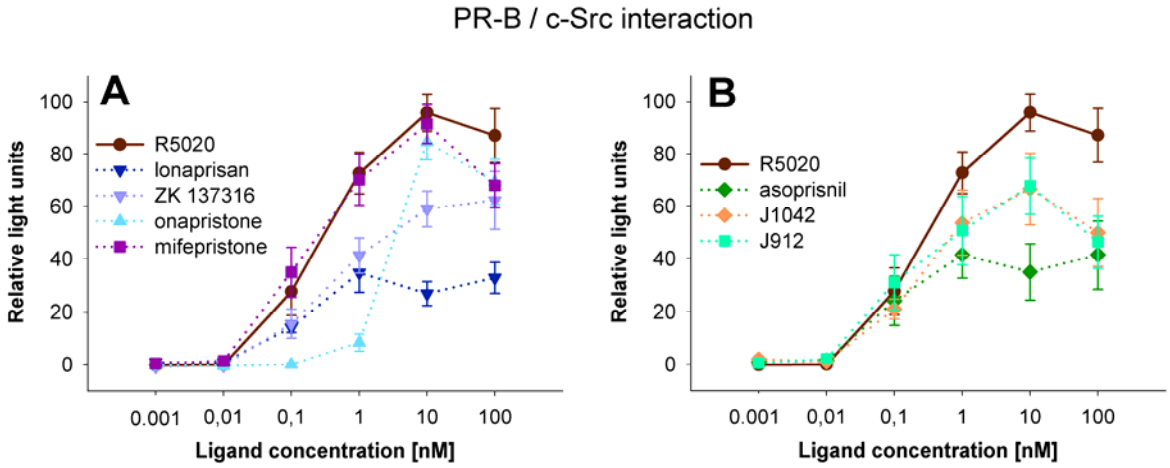

Figure 7. SPRM- and PR antagonist-induced interaction of PR-B with c-Src kinase in a mammalian two-hybrid assay system in HeLa cells. Transiently transfected cells were treated for 24 hours with vehicle or increasing concentrations (10^{-12} to 10^{-7} M) of PR antagonists (A) or SPRMs (B). Each graph represents at least two independent experiments and error bars denote CI (95%) of triplicate wells.

PR agonists, PR antagonists and SPRMs all induced PR-B interaction with c-Src kinase in HeLa cells, although to different degrees (Figure 7A, B). They showed significant differences in their efficacies and potencies to recruit c-Src kinase, in particular the PR antagonists (Figure 7A). Compared to the antagonists mifepristone and onapristone which clearly displayed strong induction of interaction, lonaprisan-bound PR recruited c-Src kinase with significatnly lower efficacy (max. efficacy = 33% vs. max. efficacy = 90-100%). The potencies of induced interactions were comparable for mixed PR antagonist mifepristone and PR agonist R5020 (EC_{50}=1.2 x 10^{-10} M; Figure 7A). Onapristone displayed a 30-fold lower potency. Maximal efficacies of about 40-60% and potencies comparable to R5020-bound PR demonstrated an intermediate interaction state with c-Src kinase for SPRM-bound PR.

As a control, plasmids expressing c-Src kinase without the SH3 domain were used. In these assays, ligand-induced recruitment to the PR was completely abolished (data not shown).

To determine whether the differences in PR-B/c-Src-interaction influence the expression of respective downstream genes, cyclin D1 reporter gene assays in U2OS cells were performed. Cyclin D1 expression is not controlled by the direct transcriptional activity of PR as the cyclin D1 promoter lacks consensus PREs [106].

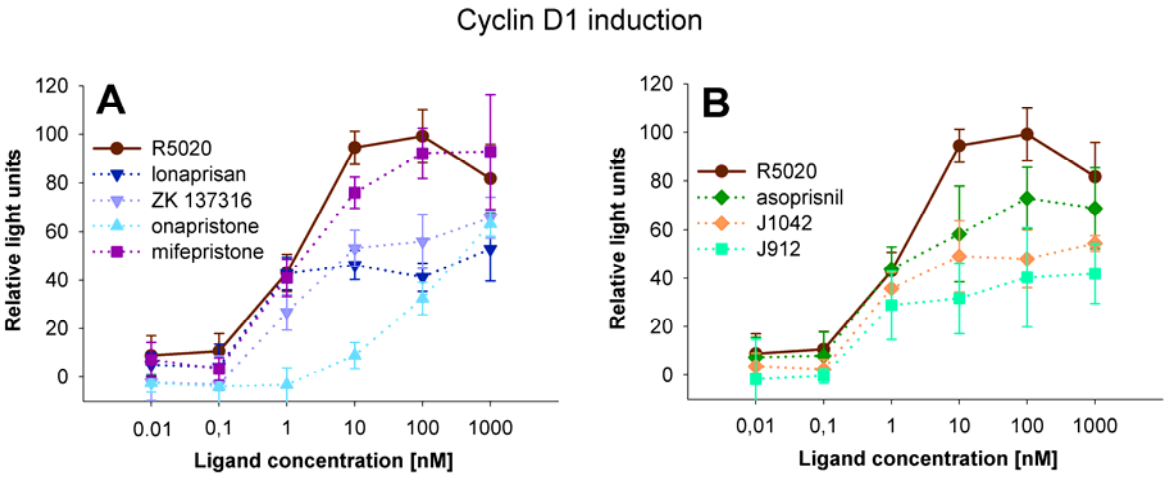

Figure 8. SPRM- and PR antagonist-induced cyclin D1 promotor activity in a reporter gene assay in U2OS cells. Transiently transfected cells were treated for 18 hours with vehicle or increasing concentrations (10^{-11} to 10^{-6} M) of PR antagonists (A) or SPRMs (B). Each graph represents at least two independent experiments and error bars denote CI (95%) of triplicate wells.

Consistent with c-Src kinase interaction, the cyclin D1 promoter was highly activated in response to R5020 treatment (Figure 8A). Among the antagonists analyzed, lonaprisan-bound PR displayed the lowest induction of cyclin D1 gene promotor activity (max. efficacy = 47%). The effects of mifepristone were similar to R5020 (Figure 8A) whereas the onapristone-liganded PR was maximal effective, but showed a markedly weaker potency (EC_{50}=1.3 x 10^{-7} M). SPRM-bound PR displayed cyclin D1 promoter activity of an intermediate state (max. efficacies = 40-70%). No significant differences where observed within the group of SPRMs (Figure 8B).

In summary, profound differences between SPRMs and PR antagonists were observed in their induced interactions of PR with the copressors NCoR and SMRT. In particular, the PR interaction profile with the LX-H10 peptide clearly distinguished SPRM-like from PR antagonist-like activities. Additionally, they differently recruited c-Src kinase to PR isoform B. A unique interaction profile was identified for the non-steroidal PR modulator PRA-910, distinguishing it from steroidal PR modulators.

Table 7. Summary of PR modulator-induced PR interaction profiles.

PR interaction with	PR agonists		SPRMs			PR antagonists			
	R5020	PRA-910	Asoprisnil	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
genomic cofactors									
NCoR, SMRT	+	-	+++	+++	+++	+++	+++	+++	+++
SRC-1, SRC-2, SRC-3	+	-	+	+	+	+	+	+	+
SRC-3-NR-D	+++	-	-	-	-	-	-	-	-
LX-H10 (AF-2 interacting motif)	+++	+++	+++	+++	+	-	-	-	-
non-genomic cofactors									
c-Src kinase	+++	n.d.	++	++	++	+++	+++	++	+

+++ = strongly induced interaction, + = induced interaction, - = no induced interaction, n.d. = not determined; red = profound differences in efficacies/potencies of cofactor interaction within the group, black = moderate differences in efficacies/potencies of cofactor interaction within the group.

3.1.3 Gene expression profiles in T47D cells

In order to assess whether the observed differences in PR interaction properties directly reflect different gene expression profiles induced by PR modulators, a global gene expression profiling study was performed. The study was conducted in the progesterone-responsive T47D cell line derived from human mammary tumors. T47D cells allow for the comparison to other global PR ligand expression profiling studies already published [155-158] and provide a suitable in vitro tool to analyze PR action.

Initial experiments to optimize incubation conditions provided consistently high gene expression of known PR target genes, such as kruppel-like factor 4 (EZF), epidermal growth factor receptor (EGFR), cyclin D1 (CCND1), FK506 binding protein 5 (FKBP51), Na^+/K^+ transporting ATPase alpha 1 polypeptide (ATP1A1), and the progesterone receptor itself (Figure 9) [157, 158], after 8 hours of treatment. Cells were incubated with PR ligand concentrations identified to exhibit full efficacies in transactivation assays (Table 8; Figure 4). In combined treatments, R5020 was used in a concentration which was shown to evoke half maximal transactivation efficacy (EC_{50} = 100 pM) to enable sustained counter-regulation by the respective PR modulator.

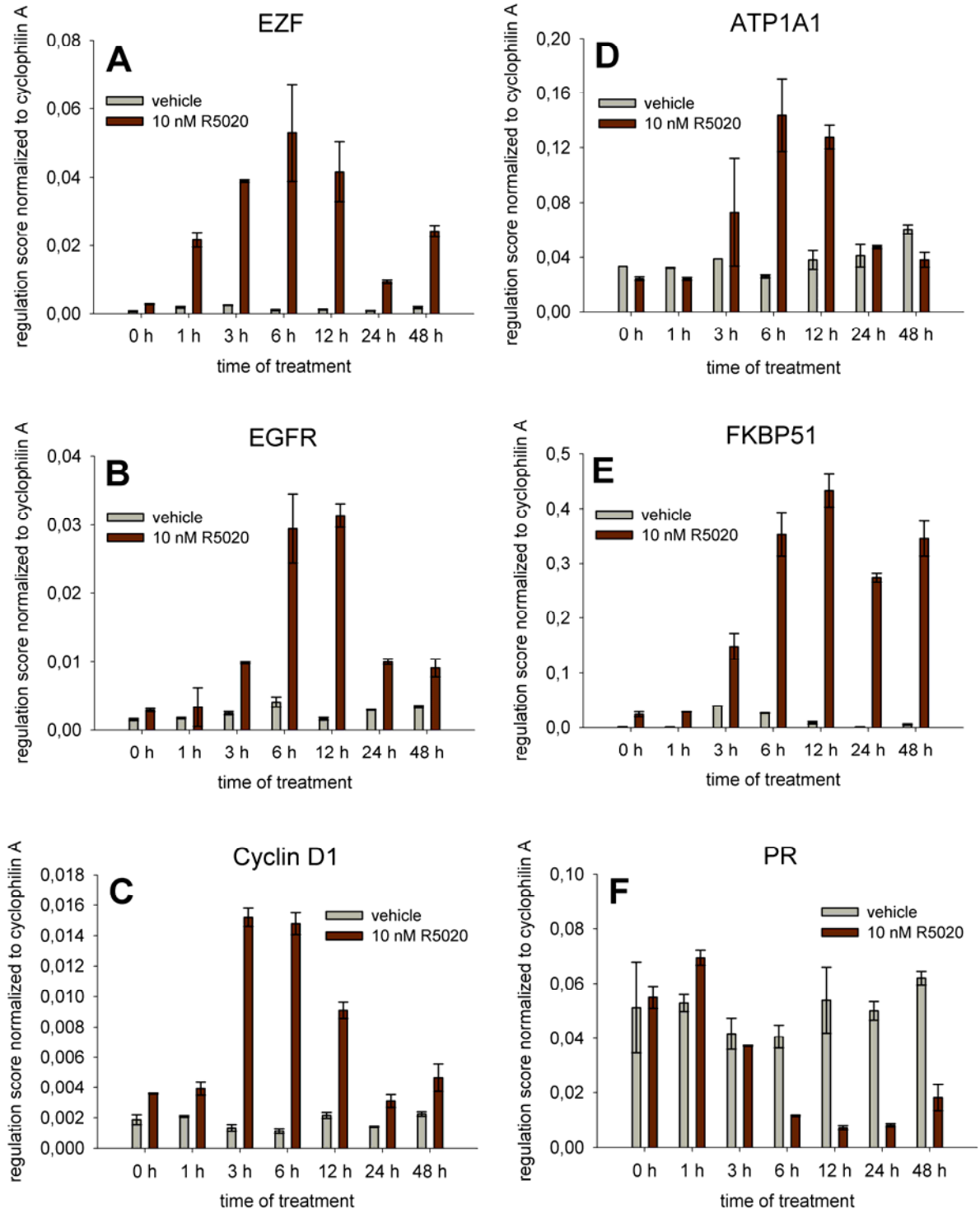

Figure 9. Time course of reference gene expression levels analyzed via quantitative real-time PCR. T47D cells were treated with vehicle or 10 nM of PR agonist R5020 for 1, 3, 6, 12, 24 and 48 hours or were left untreated. RNA expression levels of EZF (A), EGFR (B), cyclin D1 (C), ATP1A1 (D), FKBP51 (E), and PR (F) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. Each graph represents at least two independent, time-separated experiments and error bars denote CI (95%) from duplicate wells.

Table 8. PR modulator treatments for Affymetrix GeneChip analysis in T47D cells.

Probe*	Treatment	Vehicle	100 pM R5020	PR ligand	
				Compound	c [nM]
1	-	-	-	-	
2**	+	+	-	-	
3	+	+	-	R5020	10
4	+	+	-	PRA-910	100
5	+	+	-	Asoprisnil	10
6	+	+	-	J1042	10
7	+	+	-	J912	10
8	+	+	-	Mifepristone	10
9	+	+	-	Onapristone	10
10	+	+	-	ZK 137316	10
11	+	+	-	Lonaprisan	10
12	+	+	+	-	
13	+	+	+	Asoprisnil	10
14	+	+	+	J1042	10
15	+	+	+	Mifepristone	10
16	+	+	+	Lonaprisan	10

* = 5 replicates; ** = 10 replicates

Gene expression profiles were analyzed via Affymetrix GeneChip HG-U133Plus2.0 arrays. In the following sections, variations in cRNA hybridization intensity due to PR modulator treatment are referred to as ligand-induced regulation of gene expression. The encoded proteins are referred to as protein products. The initial simultaneous assessment of gene expression values in treated cells (see 2.3.3) revealed uniform overall expression and distribution of transcripts, indicating that expression profiles were consistent with established standards for gene expression analyses.

The principle component analysis (PCA) of PR modulator-induced gene expression profiles depicts the variance among samples. In the three-dimensional representation, the distance between two plotted spheres is inversely proportional to the degree of similarity between the two groups' gene expression profiles using all probe sets on the Affymetrix GeneChips.

The PCA revealed four main cluster areas, alongside component 1. Samples from the same treatment group clustered together. Differentially treated groups were clearly separated from each other (Figure 10A, B). The largest variance to vehicle samples was observed for R5020 samples, the smallest variance for antagonist samples (Figure 10A). PRA-910 samples showed the highest similarity to R5020 samples, however, they were in an intermediate position between antagonists and R5020. Samples which were treated with J1042 and asoprisnil clustered collectively in a separate group, slightly apart from the PR antagonist samples. Interestingly, J912 samples plotted much closer to antagonist than to J1042 and asoprisnil samples. In addition, alongside component 2 and 3, a separation with low but significant variance was observed between J912, mixed, classical and pure antagonist samples (Figure 10B), demonstrating unique properties on T47D cell gene expression in the absence of PR agonist.

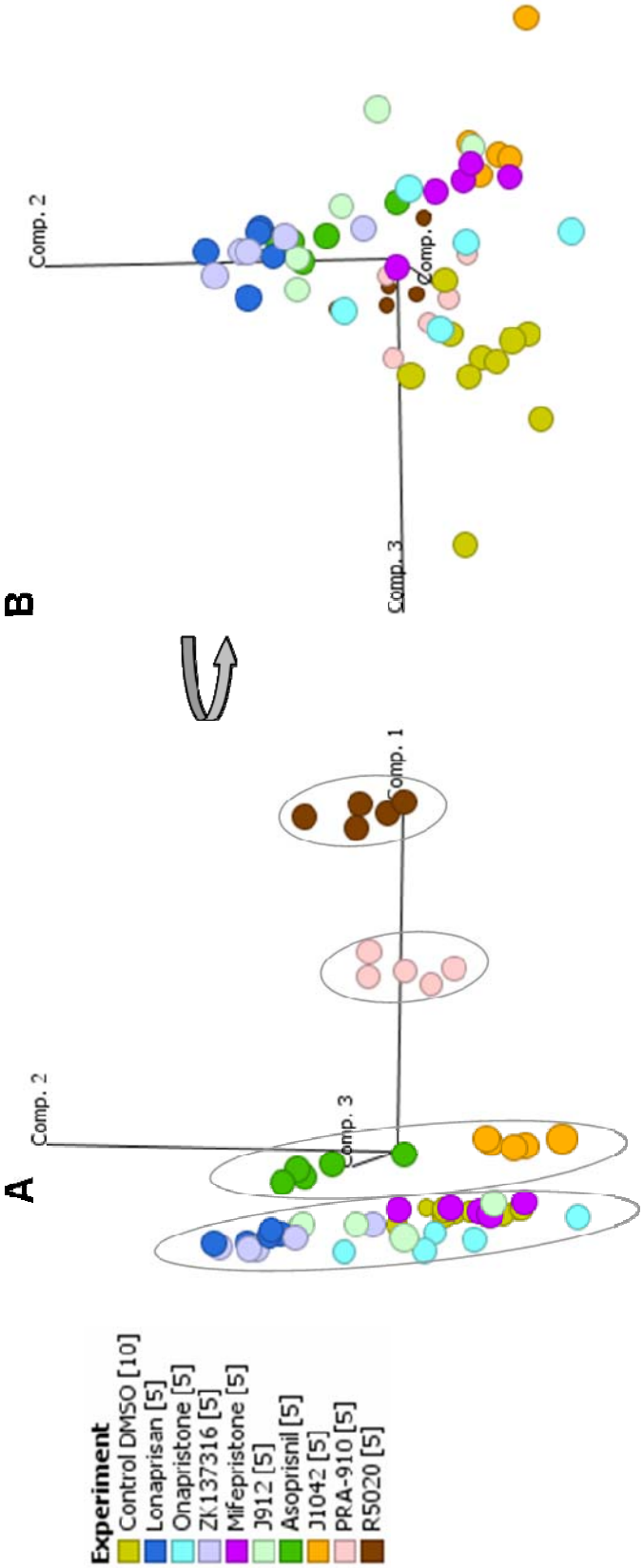

Figure 10. Principle Component Analysis (PCA) of T47D cell expression profiles. Samples are colored according to PR ligand treatment and the number of biological replicates is given in brackets. Each plotted sphere represents the expression profile of an individual sample based on the projection of the data on the first three principal components, accounting for most of the variability in the data (labeled axes). The PCA is shown for two different angular fields (A, B; 90°-rotation on component 2).

To specify the progesterone-independent effects of PR modulators as seen in the PCA, pairwise comparisons (treatment vs. vehicle) were conducted. A total of 1981 genes was identified to be significantly regulated by R5020. In Table 14, the PR modulator-induced expression profiles were organized by similarity to R5020, based on the variance in the PCA (Figure 10A). The entire number of significantly regulated genes was presented in the diagonal. Genes identified in two corresponding sets (column / row) were shown in the upper right. For example, eleven of mifepristone-regulated genes were also observed in J912-treated cells. Genes exclusively identified in one of the two sets (column / row) were shown in the lower left. The highest similarity was identified for genes regulated by J1042 and asoprisnil (54% overlap) as well as for genes regulated by the pure antagonist lonaprisan and the classical antagonist ZK 137316 (77% overlap). In general, all SPRMs and all antagonists displayed an overlap in regulated genes, except onapristone. The transcriptional effect of onapristone was minimal whereas other PR antagonists, J1042, asoprisnil and J912 clearly exhibited progesterone-independent effects on gene expression in T47D cells (Table 14).

The PR antagonists lonaprisan and ZK 137316 as well as J912 predominantly down-regulated gene expression (> 80% of regulated genes; Figure 11B). Mifepristone and asoprisnil tended to result in a down-regulation of genes (~ 60% of regulated genes). In contrast, more than 58% of regulated genes were up-regulated after treatment with J1042, PRA-910 and R5020 (Figure 11A, B).

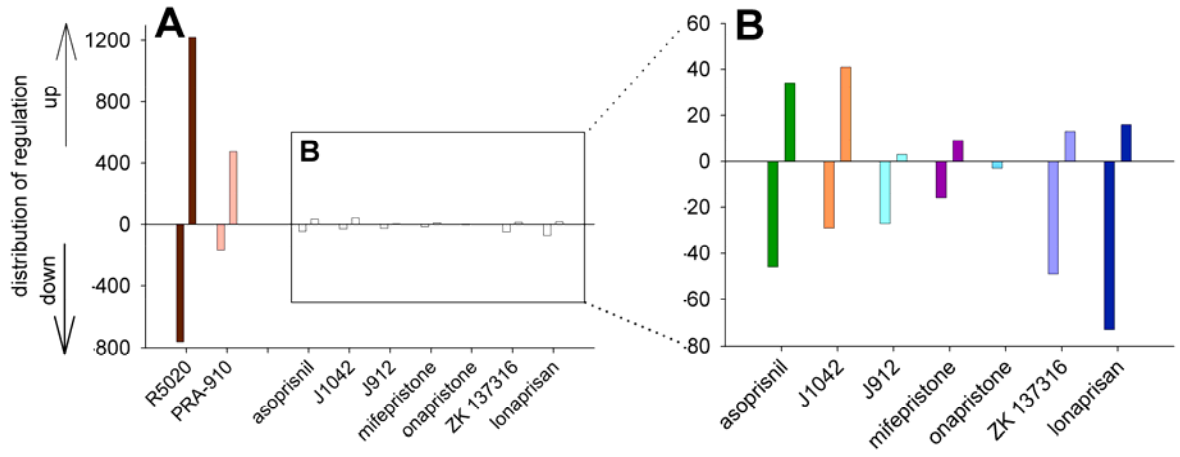

Figure 11. Distribution of significantly regulated genes from pairwise comparisons (treatment vs. vehicle; FC > 2, Volcano: FC > 2.5 - 5 and p-Value analogous to ST Q < 0.01 from T-test) shown for all PR ligands (A) and for PR antagonists / SPRMs in more detail (B).

To identify clusters of genes similarly regulated by PR modulators, hierarchical clustering analysis was conducted using gene expression data of replicate treatment samples based on pairwise comparisons (treatment vs. vehicle) and the combined list of 199 genes which were significantly regulated by PR antagonists, J912, asoprisnil and J1042.

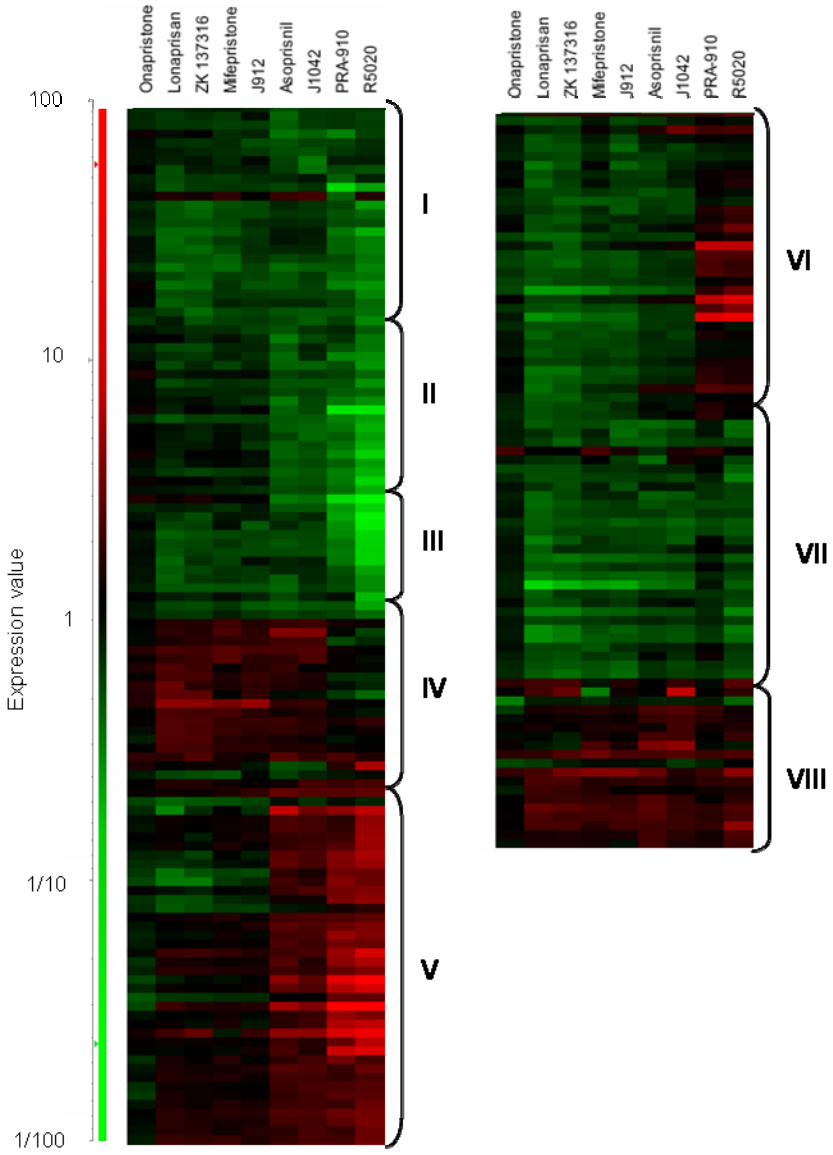

Figure 12. Hierarchical clustering analysis of genes significantly regulated by PR antagonists and SPRMs (treatment vs. vehicle; FC > 2, Volcano: FC > 2.5 - 5 and p-Value analogous to ST Q < 0.01 from T-test). Red: up-regulated; green: down-regulated.

In cluster area III, IV and VI, R5020 and PRA-910 samples segregated from antagonists and SPRMs according to trend and intensity of gene regulation (Figure 12). J1042 and asoprisnil samples partially clustered with R5020 and PRA-910 samples in area II and V, confirming the previously reported observation of separation from antagonists in the PCA (Figure 10A). J912 samples were observed to predominantly cluster with antagonists and therefore were shown to segregate from J1042 and asoprisnil. Nearly similar effects for all types of ligands were found in parts I, VII and VIII, except onapristone which was shown to act as a very particular PR ligand.

To investigate the inhibitory properties of PR modulators on agonist-induced genomic signaling as demonstrated in cellular transactivation assays (Figure 4), gene expression profiles in combined treatments of R5020 with mifepristone, lonaprisan, asoprisnil and J1042 were analyzed (Table 8).

ANOVA analyses and self-organizing maps (SOM clustering) were utilized to identify genes which were regulated by R5020 and significantly counter-regulated by the respective PR modulator. Clusters of genes in white boxes were regulated by R5020 and significantly counter-regulated by the PR modulator (Figure 13). Clusters of genes in blue boxes were not significantly counter-regulated by the PR modulator. The number of genes forming the respective cluster was visualized by the size of the dots.

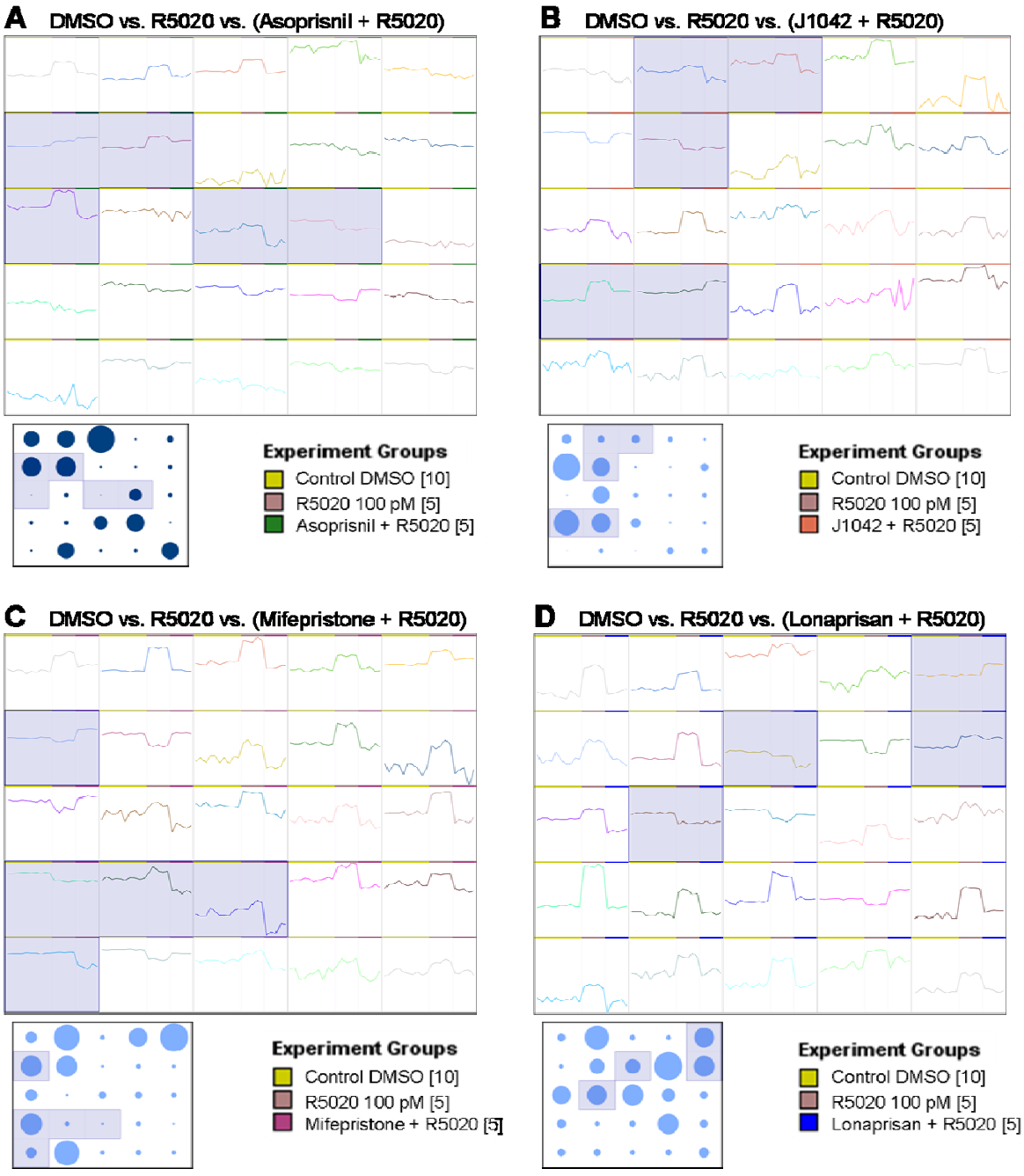

Figure 13. SOM clustering for genes which were regulated by R5020 and counter-regulated by PR modulators (p-value = 0.04, CR-score < -0.3). Cluster of genes significantly counter-regulated by asoprisnil (A), J1042 (B), mifepristone (C) and lonaprisan (D) are shown in white boxes. Cluster of genes not significantly counter-regulated are shown in blue boxes. The size of the dots corresponds to the number of genes in the respective section.

For the majority of R5020-regulated genes, a significant counter-regulation by the respective PR modulator was identified. Therefore, PR modulators dominate R5020 effects in T47D cells in combined treatments. However, the counter-regulating effects differed and demonstrated distinct antagonistic

properties on agonist-induced expression profiles. The dominance of PR modulators' effects in combined treatments as well as the differences for various modulators were confirmed for selected genes using quantitative real-time PCR (see below, Figure 14).

Taken together, gene expression profiles in T47D cells were significantly distinct for PR agonists, SPRMs, PR antagonists, and non-steroidal PR modulators. Each PR modulator exhibited unique progesterone-independent properties as well as specific antagonistic activities on R5020-induced gene expression. The SPRMs asoprisnil and J1042 clearly separated from PR antagonists in their expression profiles. Interestingly, J912 displayed higher similarities to the pure antagonist lonaprisan and the classical antagonist ZK137316 than to the SPRMs asoprisnil and J1042.

3.1.4 SPRM-regulated gene transcripts

J1042 and asoprisnil showed very similar profiles in the gene expression profiling study.Venn intersection analyses for regulated genes identified 29 unique genes which were significantly changed in their expression levels after asoprisnil and J1042 treatment, but not after J912, mixed, classical or pure antagonist treatment. Gene ontology assessment and arrangement of differentially expressed genes into similar functional categories indicated that the protein products are involved in a variety of biological processes like signal transduction, membrane effects, transcriptional and nucleic acid processing activities, hormone activities, cell cycle control, metabolic processes and defense response (Table 15). Four genes have not been annotated so far.

Four up-regulated and two down-regulated genes were selected to confirm the identified differences in expression levels using quantitative real-time PCR as an independent method. The genes chosen for validation were member 3 of subfamily J of potassium inwardly-rectifying channels (KCNJ3), member A of group 5 of family C of G protein-coupled receptors (GPRC5A), the adhesion molecule with immunoglobulin (Ig)-like domain 2 (AMIGO2), the parathyroid hormone-like hormone (PTHLH), the zinc finger and BTB domain containing protein 16 (ZBTB16) and defensin beta 32 (DEFB32). The selected genes are factors involved in different biological processes like ion flux, membrane-bound receptor signaling, cell adhesion, hormone activity and transcription.

Induction of KCNJ3 and GPRC5A transcription as well as repression of AMIGO2 transcription was specific for J1042 and asoprisnil, and also J912 (Figure 14A-C); the latter was not apparent from global gene expression profiling (Table 15). Neither mixed nor pure antagonists affected transcription of these genes with statistical significance, as expected from microarray analysis. In particular, the increase in KCNJ3 mRNA expression was exclusive for the SPRMs J1042, asoprisnil and J912.

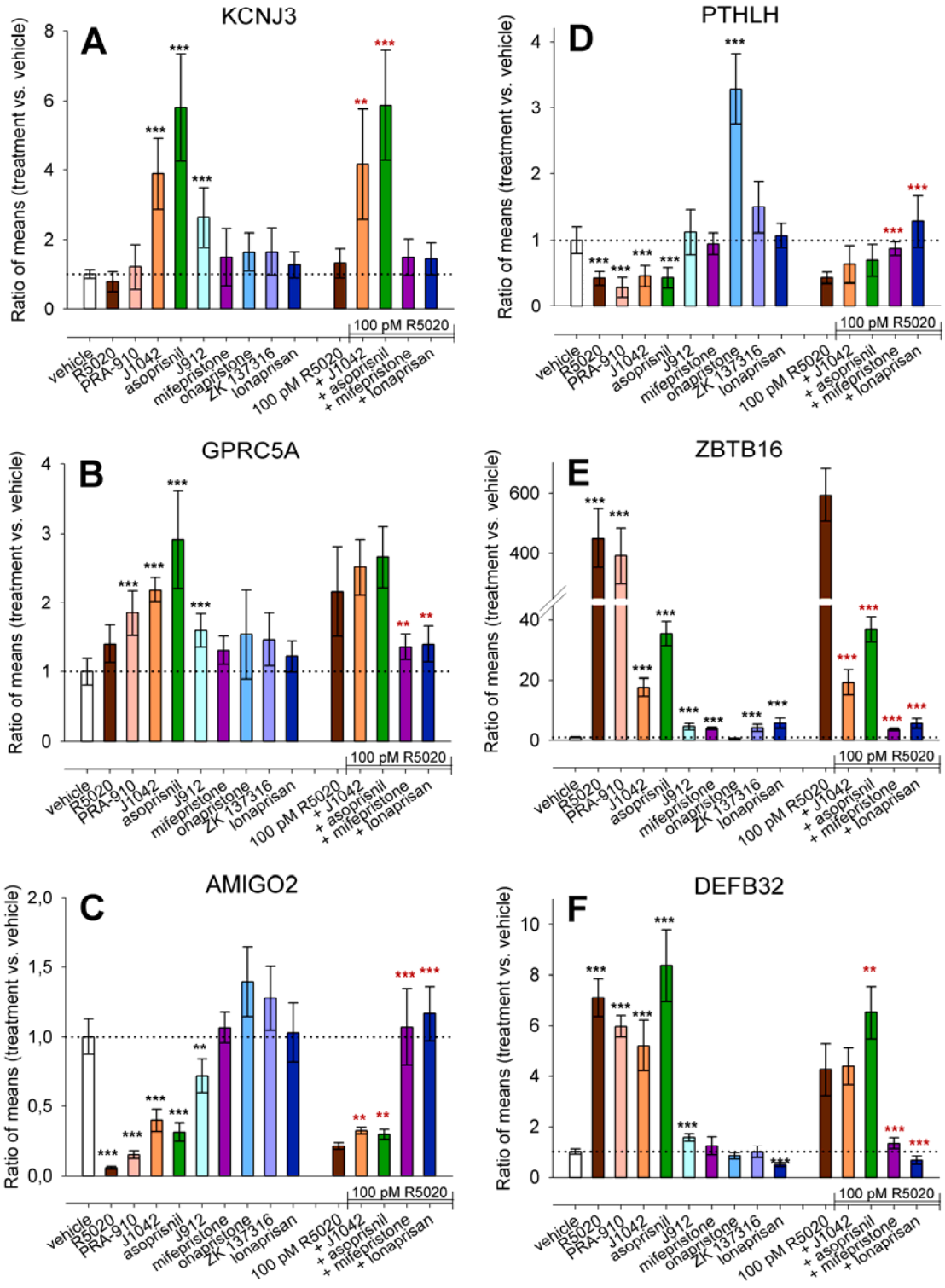

Figure 14. Confirmation of SPRM-specific downstream genes using quantitative real-time PCR. Left part of diagrams: Agonistic profile. T47D cells were treated with vehicle or 10 nM of PR ligand (100 nM PRA-910) for 8 hours. Right part of diagrams: Antagonistic profile. T47D cells were treated with 100 pM R5020 plus vehicle or 10 nM of PR modulator for 8 hours. RNA expression levels of KCNJ3 (A), GPRC5A (B), AMIGO2 (C), PTHLH (D), ZBTB16 (E), and DEFB32 (F) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of three independent, time-separated experiments. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons PR ligand vs. vehicle [agonism, black stars] or (PR ligand + R5020) vs. R5020 [antagonism, red stars].

Besides the regulation of GPRC5A-and AMIGO2-transcript levels, the transcription of PTHLH, ZBTB16 and DEFB32 was influenced by R5020 and PRA-910 (Figure 14D-F). Additionally, AMIGO2 and ZBTB16 gene expression was shown to be regulated by R5020 and PRA-910 even stronger than by SPRMs (Figure 14C, E, Table 15).

The effects of J912 on ZBTB16- and also DEFB32- and PTHLH-transcript levels were minimal or absent. Therefore, J912 corresponded to PR antagonists with regard to the regulation of PTHLH, DEFB32 and ZBTB16, but to asoprisnil and J1042 with regard to the regulation of KCNJ3, GPRC5A and AMIGO2.

The antagonistic effects of asoprisnil and J1042 on R5020-induced gene expression detected in SOM clustering (Figure 13) were confirmed for the selected genes KCNJ3, GPRC5A, AMIGO2, PTHLH, ZBTB16 and DEFB32 using real-time PCR (Figure 14). The SPRMs generally dominated the effects on gene expression in combined treatments with R5020. However, counter-regulation of R5020 effects was stronger for PR antagonists than for SPRMs.

Taken together, the SPRMs asoprisnil and J1042 displayed regulation of gene expression levels which were not influenced by mixed, classical and pure antagonists. In particular, the induction of KCNJ3 transcription in T47D cells was absolutely exclusive to asoprisnil, J1042 and J912, after 8 hours of treatment. Moreover, J912 exhibited ambivalent properties for the regulation of selected genes and represented an intermediate state between SPRM and antagonist. In combined treatments with R5020, asoprisnil and J1042 dominated the agonistic effect, although to a minor degree than observed for the mixed antagonist mifepristone and the pure antagonist lonaprisan.

3.1.5 PR antagonist-regulated gene transcripts

In T47D cells, PR modulators revealed progesterone-independent effects which differed depending on the type of ligand. To some extent, the ligand-specific effects overlapped, but the degrees of concurrent regulations varied (Table 14). To determine whether there are exclusive effects not only for SPRMs, but also within the three different classes of antagonists, onapristone-, mifepristone- and lonaprisan-regulated genes were filtered for genes exclusively regulated by one of these three antagonists only. The effects of R5020 on selected genes were either not statistically significant or otherwise contradictory to the effect of the PR antagonist. Categorical lists of genes exclusively regulated by type I-, II- or III PR antagonists are shown in Table 16-18.

The specific effects of lonaprisan were diverse and included the regulation of factors which are important for hormone activity, membrane effects, control of cell proliferation and apoptosis, nucleic acid and protein processing. The biological functions of four protein products (C6orf141, FAM107B, C8orf46, C9orf91) have not been described so far. Lonaprisan predominantly down-regulated genes, in particular genes whose protein products are involved in FSH hormone activity, cell adhesion and cytoskeletal interactions (except FYVE, RhoGEF and PH domain containing 4 (FGD4) which were up-regulated).

Interestingly, the onapristone-bound PR, which regulated only three genes with statistical significance (Table 14), exclusively down-regulated a gene whose protein product is involved in nucleic acid processing (JMJD2B). No annotation is given for the other two genes specifically regulated by onapristone.

The protein products of mifepristone-modulated genes are mainly involved in angiogenesis and protein processing. The biological functions of two genes (C1orf116, FAM129B) have not been described so far. Angiopoietin 1 was the only one specifically and significantly down-regulated by mifepristone, the other specific genes were up-regulated.

Among the PR antagonist-regulated genes, the inhibin β subunit B (Inhbb) and the jumonji domain containing 2B (JMJD2B) might be important for PR antagonists' action. Inhbb was regulated by lonaprisan, but was not significantly influenced by mifepristone or onapristone. The protein produced of this gene is of interest as it regulates pituitary FSH secretion and thereby indirectly regulates estrogen and progesterone hormone activity [159]. The onapristone-bound PR displayed only marginal transcriptional activity, anyhow, it specifically down-regulated jumonji domain containing 2B (JMJD2B) which is important for transcriptional control [160, 161].

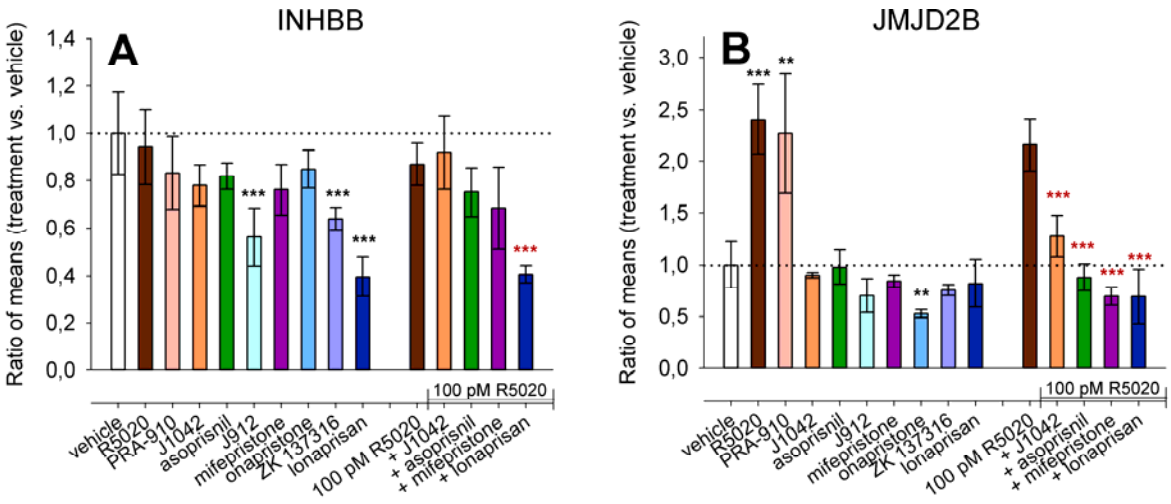

Figure 15. Confirmation of lonaprisan- and onapristone-regulated genes using quantitative real-time PCR. Left part of diagrams: Agonistic profile. T47D cells were treated with vehicle or 10 nM of PR ligand (100 nM PRA-910) for 8 hours. Right part of diagrams: Antagonistic profile. T47D cells were treated with 100 pM R5020 plus vehicle or 10 nM of PR modulator for 8 hours. RNA expression levels of Inhbb (A) and JMJD2B (B) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of three independent, time-separated experiments. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons PR ligand vs. vehicle [agonism, black stars] or (PR ligand + R5020) vs. R5020 [antagonism, red stars].

Real-time PCR experiments confirmed a significant down-regulation of Inhbb gene expression after lonaprisan-, but also after ZK 137316- and J912-treatment (Figure 15A). Therefore, the effect was not exclusive to lonaprisan. However, it was exclusive to modulators that share most of the characteristics of lonaprisan-induced gene expression profile (Table 14). Onapristone and mifepristone did not regulate Inhbb. Moreover, the repression of Inhbb expression in the presence of R5020 was strongest after lonaprisan treatment. The down-regulation of JMJD2B gene expression was confirmed to be unique for onapristone. R5020 and PRA-910 treatment displayed contradictory regulation of JMJD2B transcripts (Figure 15B). Mifepristone and lonaprisan exhibited no effect on JMJD2B expression themselves, but antagonized the increased expression induced by R5020 in combined treatments.

Pathway analyses were conducted for all genes which were significantly regulated by one type of PR antagonist only (I, II or III), with a fold change larger than 1.5 (Table 9). The limit fold change was reduced from 2.0 to 1.5 to enhance the repertoire of specific PR antagonist-regulated genes. This procedure further increased the significance of affected pathways.

Table 9. Pathways significantly regulated by lonaprisan, onapristone and/or mifepristone.

	Lonaprisan	Onapristone	Mifepristone
PPAR pathway	+	-	-
RXR-dependent regulation of lipid metabolism via PPAR, RAR, VDR	+	-	-
PGE2 in immune and neuroendocrine system interactions	+	-	-
Regulation of G1/S transition	+	-	-
Regulation of fatty acid synthase activity in hepatocytes	+	-	-
PGE2 common pathways	+	-	+
Beta-adrenergic receptors regulation of ERK	+	-	+
WNT signaling pathway	+	-	+
PGE2-induced pain processing	+	-	-
GPCRs in the regulation of smooth muscle tone	+	-	-
PKA signaling	+	-	-
Non-genomic (rapid) action of Androgen Receptor	-	+	-
Endothelin-1/EDNRA transactivation of EGFR	-	+	-
PTEN pathway	-	+	-
Endothelin-1/EDNRA signaling	-	+	-
Androgen Receptor nuclear signaling	-	+	-
Human NKG2D signaling	-	-	+
Beta-adrenergic receptors transactivation of EGFR	-	-	+
NKG2D signaling (murine)	-	-	+

Number of regulated genes in this pathway signifcant ($p < 0.01$; +) or not significant ($p > 0.01$; -).

PR bound to the type III antagonist lonaprisan shared highest activity in the regulation of genes whose protein products are involved in nuclear signaling (PPAR, RAR, VDR) and, notably, the regulation of cell cycle progression (G1/S transition; Figure 16). The type I antagonist onapristone predominantly modulated genes which encode proteins involved in genomic and non-genomic androgen receptor signaling and endothelin 1 signaling. The type II antagonist mifepristone regulated genes whose protein products are important for beta-adrenergic receptor pathways and WNT signaling.

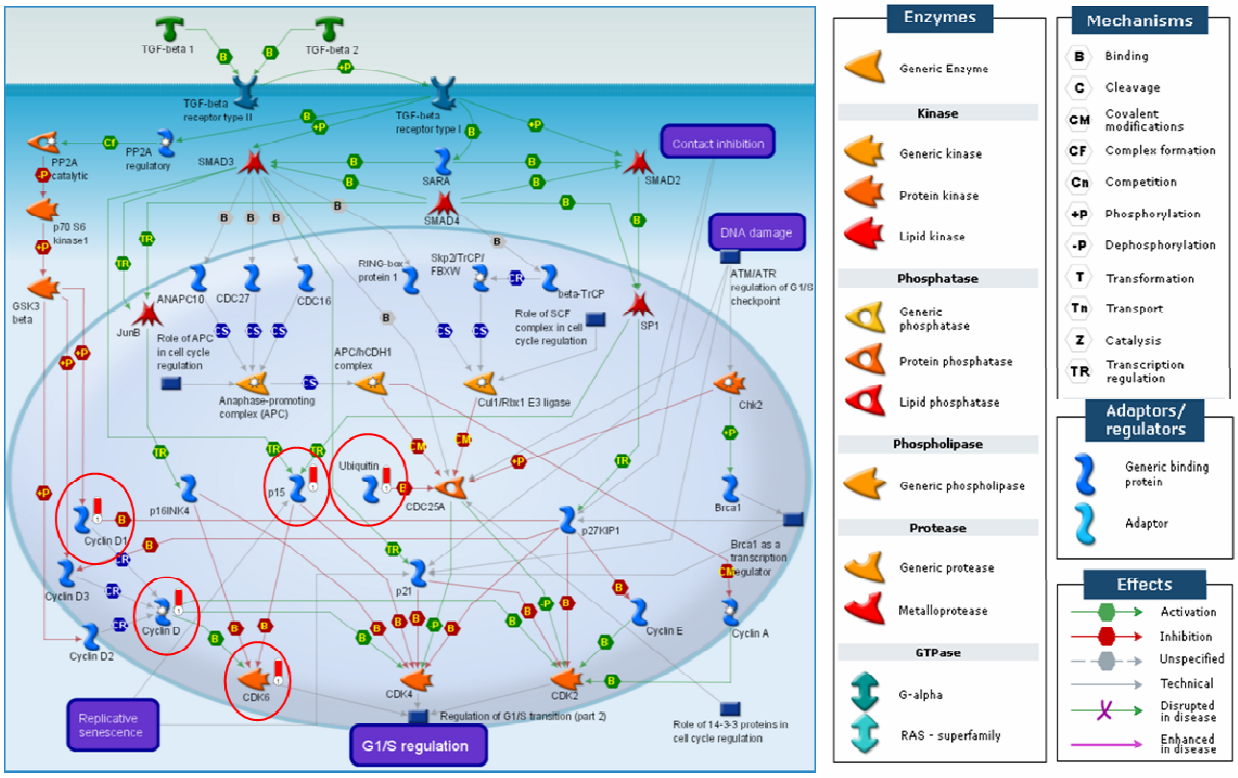

Figure 16. Regulation of G1/S transition. Influence of lonaprisan on selected pathway members analyzed via MetaCore pathway analyses. Red circles indicate genes significantly regulated by lonaprisan (FC > 1.5, Volcano: FC > 2.5 - 5 and p-Value analogous to ST Q < 0.01 from T-test). Expression levels of genes which were inserted into pathways analysis are not quantitative. Only information of qualitative significant regulation is given.

Additionally, pathway analyses were conducted for all genes which were regulated by R5020 and significantly counter-regulated (CR < -0.3) by lonaprisan and mifepristone (Table 10).

Table 10. R5020-regulated pathways significantly counter-regulated by lonaprisan and/or mifepristone.

	Lonaprisan_CR	Mifepristone_CR
Oncostatin M signaling via JAK-Stat in human/mouse cells	+	+
Non-genomic (rapid) action of Androgen Receptor	+	+
EGFR signaling via PIP3	+	+
Ligand-independent activation of ESR1 and ESR2	+	+
Start of DNA replication in early S phase	+	+
ChREBP regulation pathway	+	+
Leptin signaling via PI3K-dependent pathway	+	+
Regulation of G1/S transition	+	+
Androgen Receptor nuclear signaling	+	-
EGFR signaling via small GTPases	-	+
ESR1 regulation of G1/S transition	-	+
EGF signaling pathway	-	+
Role of heterochromatin protein 1 (HP1) family in transcriptional silencing	-	+
NF-AT signaling in Cardiac Hypertrophy	-	+

Number of regulated genes in this pathway signifcant (p < 0.01; +) or not significant (p > 0.01; -).

The protein products of genes counter-regulated by both antagonists are crucial for non-genomic signaling pathways, in particular ligand-independent activation of estrogen receptors and, notably, cell cycle progression (Figure 17). Moreover, genes which were specifically counter-regulated by mifepristone affect EGF/EGFR signaling pathways and estrogen receptor mediated G1/S transition.

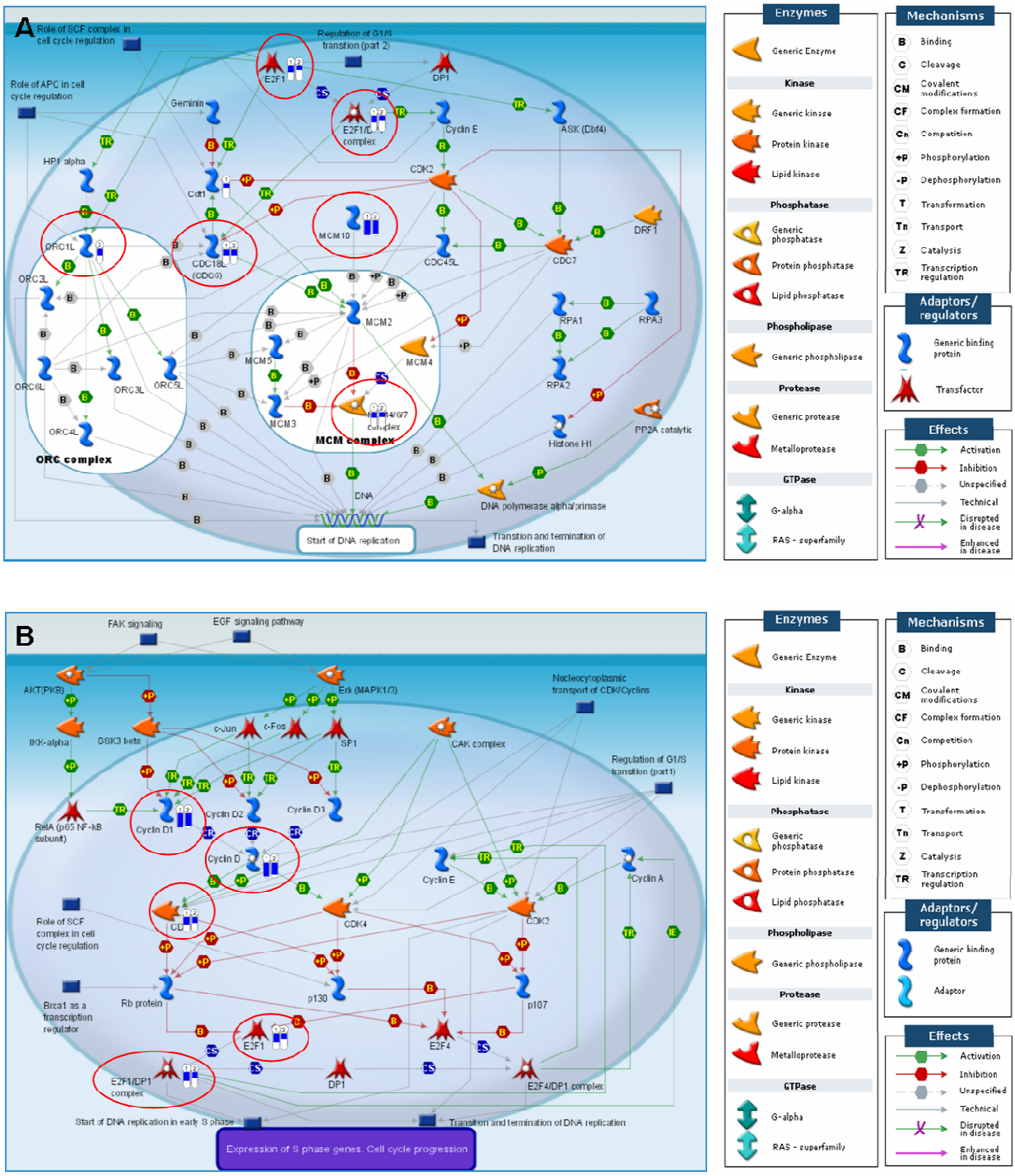

Figure 17. R5020 regulation and lonaprisan / mifepristone counter-regulation of selected genes involved in initiation of DNA replication in early S phase (A) and G1/S transition (B) as revealed by MetaCore pathway analyses. Red circles indicate genes significantly counter-regulated by lonaprisan (1) and mifepristone (2) (ANOVA ST Q < 0.0001 and CR < -0.3). Expression levels of genes which were inserted into pathways analysis are quantitative.

PR antagonists cumulatively displayed regulation of genes which are involved in non-genomic signaling and cell cycle control (Figure 17, Table 9, Table 10). In combination with the results from mammalian two-hybrid assays analyzing the induction of PR-B/c-Src interaction (Figure 7, Figure 8), the differences in gene expression profiles indicate distinct modulation of non-genomic signaling pathways by various PR antagonists. The central role of non-genomic signaling pathways in regulating a variety of cellular processes such as cell cycle control and cell proliferation [154] implicated further studies which focused on these biological function of PR action. To confirm the influence of PR antagonists on the expression levels of genes which are relevant for cell cycle control, cyclin D1 and E2F1 transcript levels were examined using real-time PCR. The selected genes have been described to contribute to hormone-dependent cell cycle progression previously [162-166]. In the microarray analysis, both genes were significantly induced by the PR agonist R5020. In turn, pathway analyses demonstrated that cyclin D1 and E2F1 are significantly counter-regulated by lonaprisan and mifepristone (Figure 16, Figure 17).

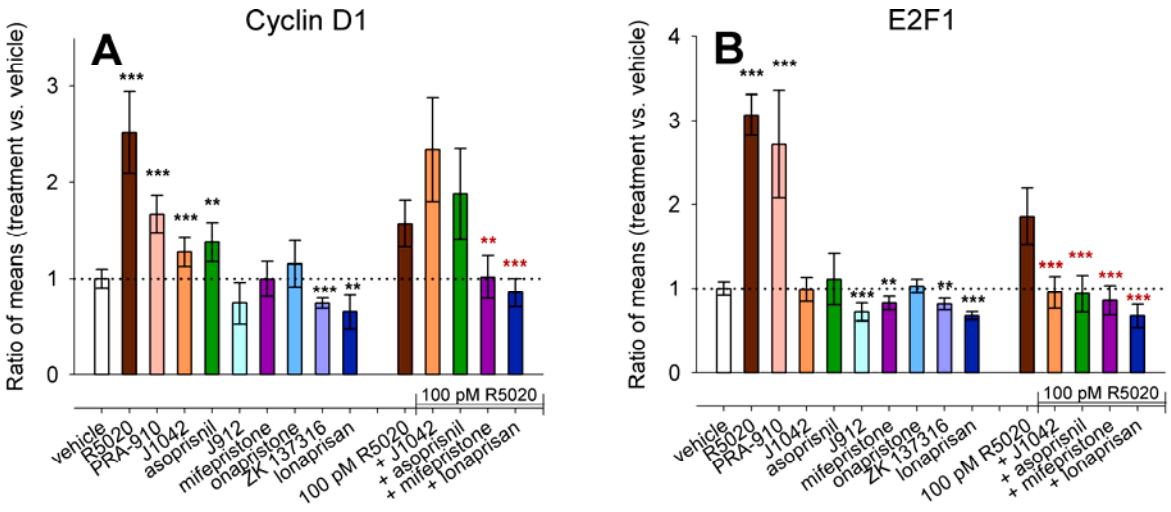

Figure 18. PR antagonist-regulated genes, which are relevant for cell cycle control, confirmed via quantitative real-time PCR. Left part of diagram: Agonistic profile. T47D cells were treated with vehicle or 10 nM of PR ligand, except PRA-910 in a concentration of 100 nM, for 8 hours. Right part of diagram: Antagonistic profile. T47D cells were treated with 100 pM R5020 plus vehicle or 10 nM of PR modulator for 8 hours. RNA expression levels of cyclin D1 (A) and E2F1 (B) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of three independent, time-separated experiments. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons PR ligand vs. vehicle [agonism, black stars] or (PR ligand + R5020) vs. R5020 [antagonism, red stars].

Real-time PCR experiments confirmed a significant induction of cyclin D1 gene expression in T47D cells after 8 hours of treatment with R5020 and also after treatment with PRA-910, asoprisnil and J1042 (Figure 18A). In contrast, lonaprisan and ZK 137316 repressed cyclin D1 gene expression. E2F1 transcript levels were significantly induced by R5020, but not by the SPRMs asoprisnil and J1042

(Figure 18B). The SPRM J912 even showed a significant repression of E2F1 transcription, similar to lonaprisan, ZK 137316, and mifepristone. Onapristone did not significantly regulate any of the genes.

In combined treatments, the agonistic effect of R5020 on cyclin D1 expression was reversed by lonaprisan and mifepristone. However, the R5020-induced increase in E2F1 transcript levels was counter-regulated by PR antagonists as well as by asoprisnil and J1042.

In summary, the PR antagonists onapristone, mifepristone and lonaprisan displayed specific progesterone-independent effects on T47D cell gene expression, in particular on the regulation of genes relevant for cell cycle progression. After 8 hours of treatment, cell cycle associated genes were predominantly influenced by type II and III PR antagonists.

3.1.6 Inhibition of estradiol-induced cell cycle progression

To determine whether the differences in cell cycle regulator gene expression have an impact on T47D cell cycle phases, propidium iodide staining and FACS analyses were performed after treatment with PR modulators and estradiol. Estradiol (E2) is known to induce cell cycle progression and proliferation of breast cancer cells even more prominent than progesterone [162, 167, 168]. The effects of PR agonists, SPRMs and PR antagonists on T47D cell cycle phases were analyzed after 24 hours of treatment both alone and in combination with estradiol to examine progesterone-independent effects as well as inhibitory effects on estradiol action.

The agonist R5020 revealed stimulatory effects. It enhanced S phase entry as well as G2/M phase entry (Figure 19B, C). Similar effects were observed for the non-steroidal PR modulator PRA-910 and the SPRMs asoprisnil and J1042. All of them reduced the proportion of cells in G0/G1 phase (Figure 19A). The PR antagonists mifepristone and onapristone as well as J912 did not affect cell cycle phases per se (Figure 19A-C). The strongest antagonistic effects were identified for the potent PR antagonist lonaprisan which significantly repressed cell cycle progression per se (Figure 19B). Similar effects were observed for ZK 137316.

The E2-induced increase in S and G2/M phases was inhibited neither by PR ligands with agonistic properties such as R5020, PRA-910, asoprisnil and J1042 nor by the classical antagonist onapristone (Figure 19D-F). However, the mixed antagonist mifepristone antagonized the E2-induced increase in G2/M phase, albeit it did not affect the increased proportion of cells in S phase. Lonaprisan inhibited both the E2-induced increase in the proportion of cells passing S phase and G2/M phase. Similar effects were identified for ZK 137316 and J912. Therefore, lonaprisan or compounds with related effects maintained cells in G0/G1 phase and diminished the E2-induced shift in cell cycle phases that underlies enhanced proliferation.

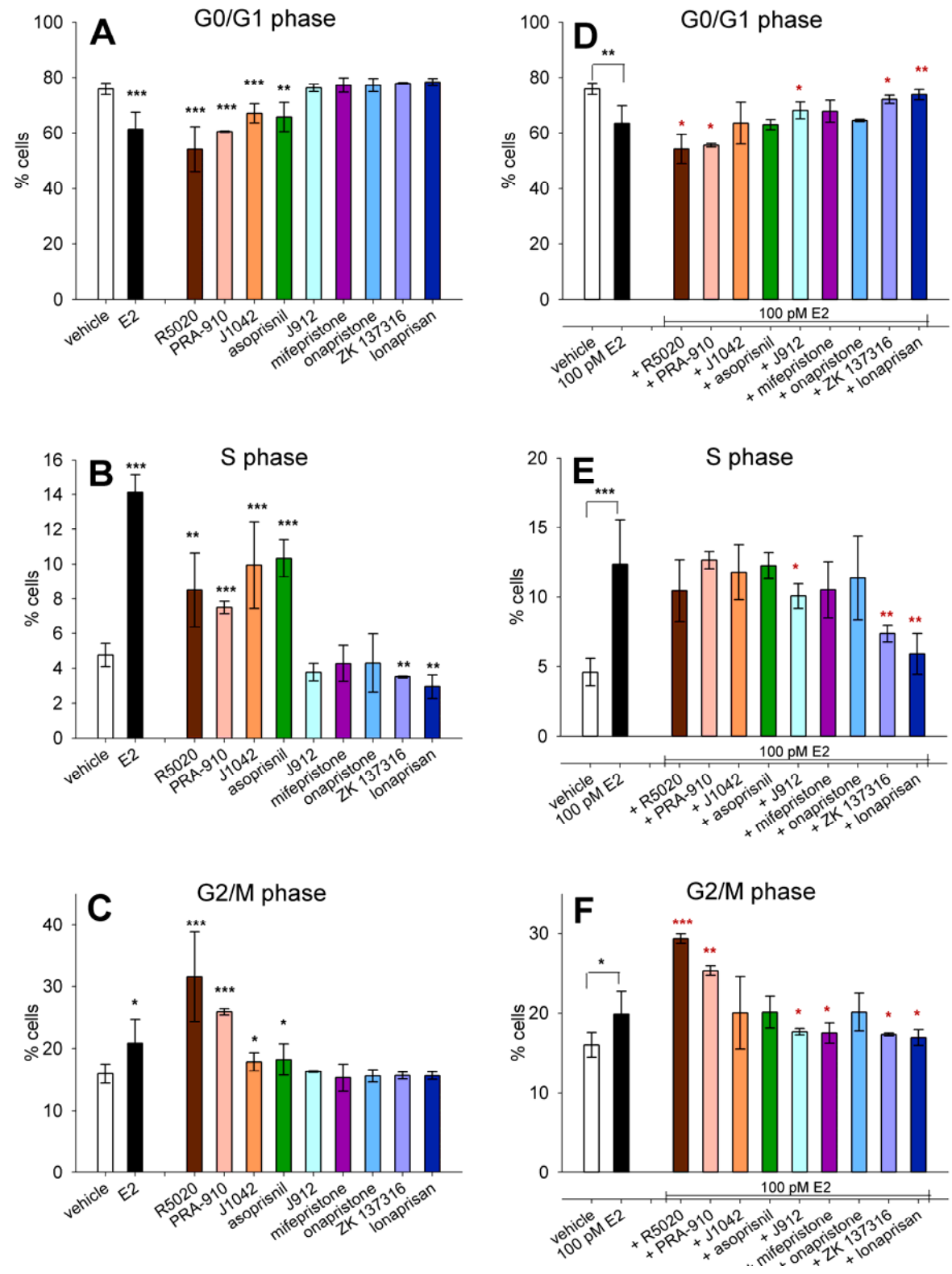

Figure 19. Distribution of T47D cell cycle phases after treatment with PR modulators. Cell cycle phases were determined using propidium iodide staining and FACS analysis. A-C) Agonistic profile. Cell cycle phases after 24 hours of treatment with vehicle, 10 nM estradiol or 10 nM of PR ligand. D-F) Antagonistic profile. Cell cycle phases after 24 hours of treatment with 10 nM PR ligand in the presence of 100 pM estradiol. Each graph represents at least two independent experiments and error bars denote CI (95%) of triplicate wells. Statistical significance was assessed by Student's T-test (p-value < 0.05 *, p-value < 0.005 **; p-value < 0.001 ***) for the comparisons PR ligand/E2 vs. vehicle [agonism, black stars] or (PR ligand + E2) vs. E2 [antagonism, red stars].

Concomitant with the differences in T47D cell cycle phases distribution, real-time PCR experiments revealed distinct expression levels of the cell cycle regulators cyclin-dependent kinase 6 (CDK6) and cyclin-dependent kinase inhibitor 1A (p21) after treatment with different PR modulators. CDK6 and p21 are important for G1 phase transition and have been reported to be responsible for PR antagonist-induced inhibition of cell cycle progression in T47D cells [169].

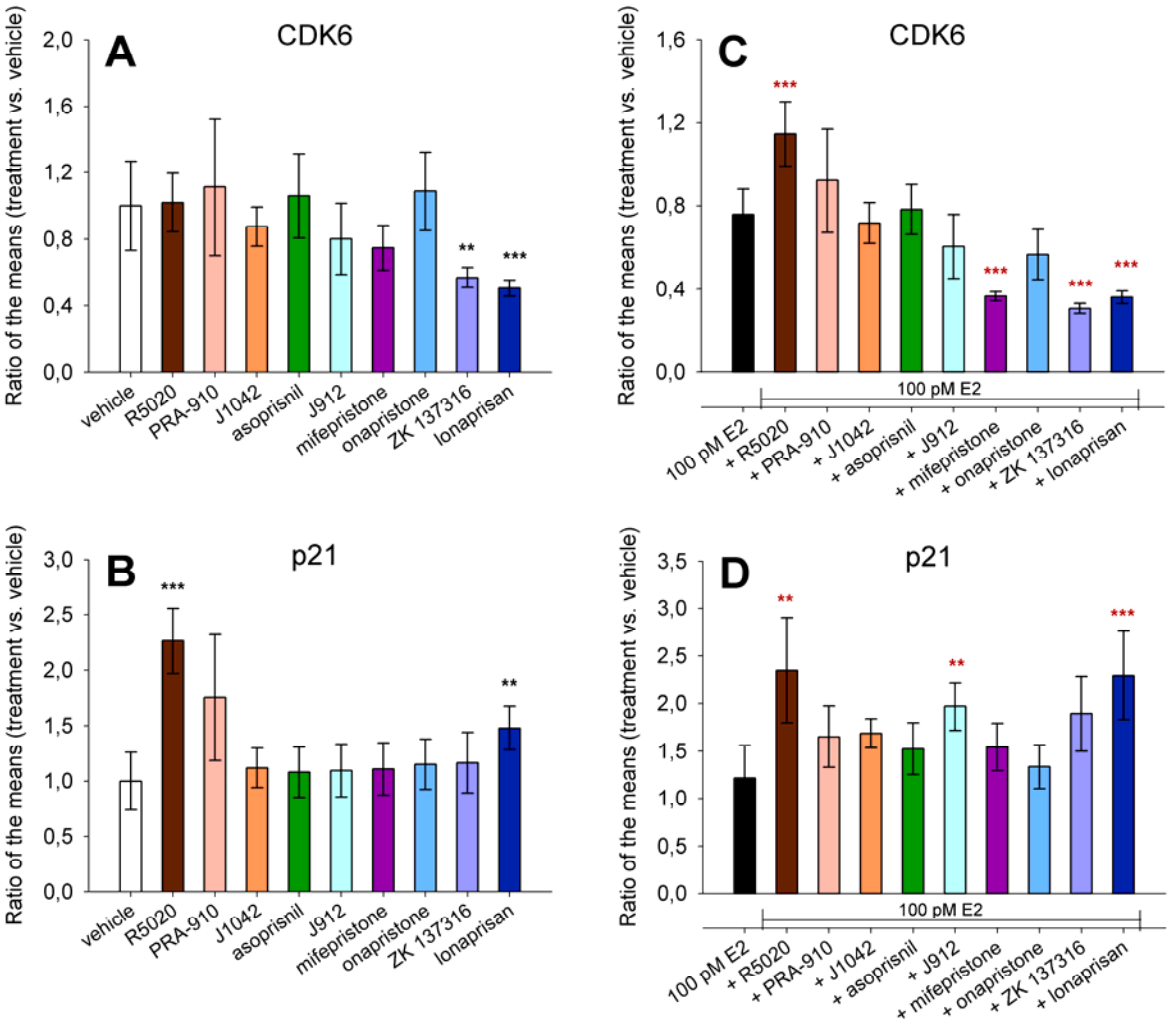

Figure 20. Expression levels of cell cycle regulator genes in T47D cells analyzed via quantitative real-time PCR. T47D cells were treated with vehicle or 10 nM of PR modulator (A-B) or 10 nM of PR modulator in the presence of 100 pM estradiol (C-D) for 24 hours. RNA expression levels of CDK6 (A,C) and p21 (B,D) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of three independent, time-separated experiments. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons PR ligand/E2 vs. vehicle [agonism, black stars] or (PR ligand + E2) vs. E2 [antagonism, red stars].

After 24 hours of treatment, lonaprisan and ZK 137316 significantly inhibited expression of the cell cycle progressor CDK6. Lonaprisan simultaneously increased the transcript level of the CDK inhibitor p21. Interestingly, similar effects on p21 gene expression were observed for R5020.

R5020, lonaprisan and also J912 significantly enhanced p21 transcription in the presence of estradiol. However, R5020 simultaneously induced CDK6 expression in the presence of estradiol compared to estradiol alone, while mifepristone, lonaprisan and ZK 137316 decreased CDK6 transcript levels. The type I antagonist onapristone did not affect CDK6 and p21 transcription per se and did not antagonize the E2-induced expression levels.

Taken together, R5020, the non-steroidal PR modulator PRA-910, asoprisnil and J1042 displayed stimulating effects on T47D cell cycle progression, although to different degrees. PR antagonists, in particular type III PR antagonists, revealed strong functional anti-estrogenic effects on cell cycle progression and expression of cell cycle associated genes, but type I antagonists did not.

3.1.7 Inhibition of estradiol-induced proliferation

Estradiol and progesterone are known to stimulate T47D cell proliferation [170]. However, the effects of PR ligands are discussed controversially and depend on the experimental setup and the type of ligand examined [168]. To investigate the agonistic and antagonistic impact of various PR modulators on cell proliferation as the biological consequence of cell cycle control, T47D cell viability was analyzed after five days of hormonal deprivation and subsequent supplementation with PR modulators in the absence as well as in the presence of estradiol. Single treatments were used to examine progesterone-independent effects and combined treatments with estradiol were used to investigate inhibitory effects on estradiol action.

When cells were incubated with the PR modulator only, R5020, asoprisnil and J1042 showed a tendency for stimulating effects (Figure 21A, E, F). However, in single treatment with the pure PR antagonist lonaprisan, T47D cell proliferation was significantly inhibited (Figure 21D).

The E2-induced T47D cell proliferation was antagonized by all PR modulators. R5020 and lonaprisan displayed the strongest inhibitory effects starting from concentrations of 100 pM (Figure 21).

In summary, the progesterone receptor exhibited ligand-specific effects on T47D cell cycle progression and cell proliferation, depending on the experimental conditions and the timepoint examined. The type III PR antagonist lonaprisan demonstrated the strongest functional, non-competitive ER antagonistic and antiproliferative properties in the T47D cell model system.

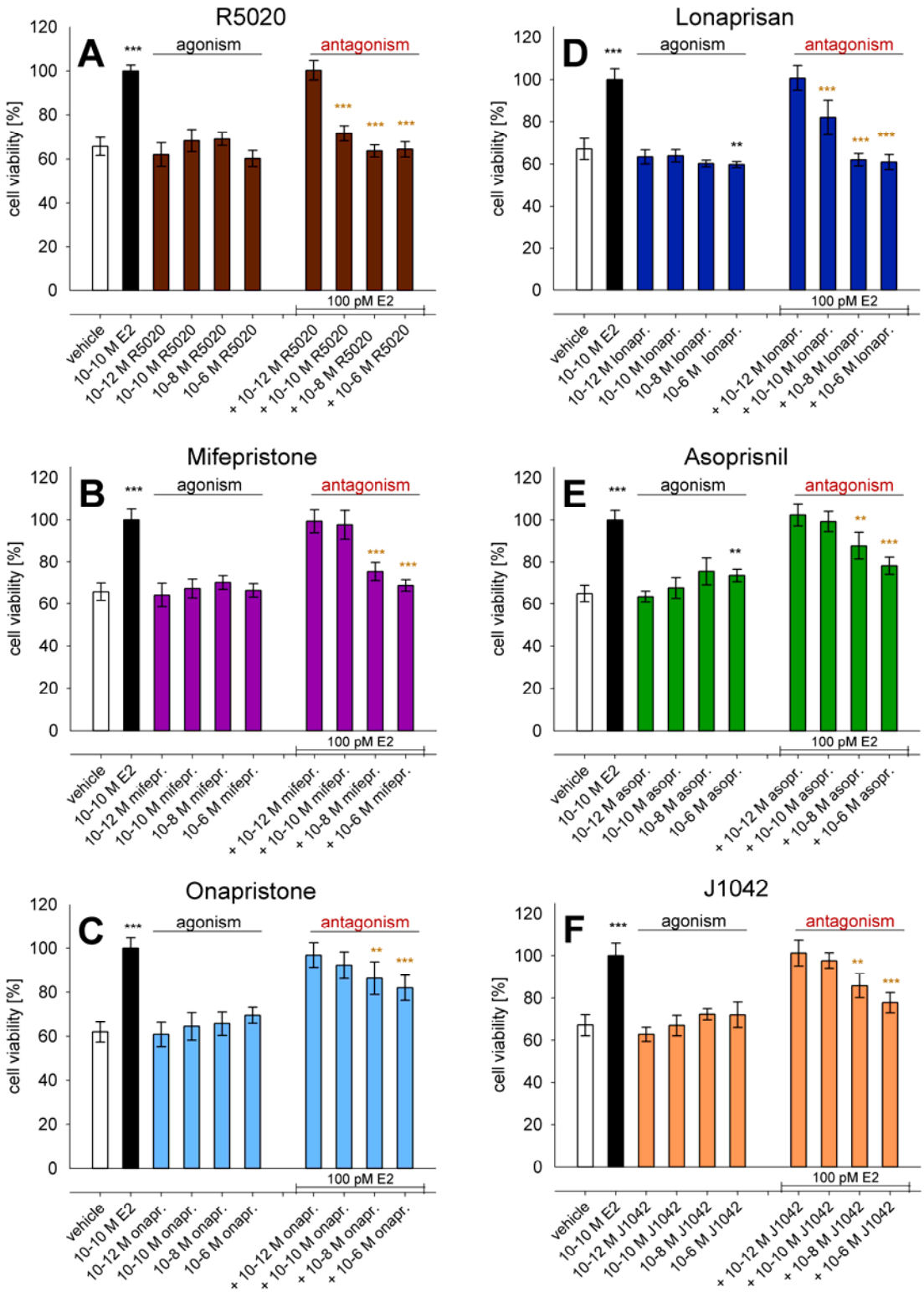

Figure 21. Effects of PR modulators on T47D cell proliferation. Left part of diagrams: Agonistic profile. T47D cells were cultured in serum-deprived medium supplemented with increasing concentrations of R5020 (A), mifepristone (B), onapristone (C), lonaprisan (D), asoprisnil (E), J1042 (F) for 5 days. Right part of diagrams: Antagonistic profile. T47D cells were cultured in serum-deprived medium supplemented with increasing concentrations of PR ligand and 100 pM estradiol for 5 days. Cell viability was obtained by CellTiter-Glo assays. Vehicle served as negative control and 100 pM estradiol as positive control. E2-induced proliferation was arbitrarily set as 100% and as parameter for cell proliferation, the percent change of cell viability was determined for each treatment. Each graph represents at least two independent experiments and error bars denote CI (95%) of sexduplicate wells. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons PR ligand/E2 vs. vehicle [agonism, black stars] or (PR ligand + E2) vs. E2 [antagonism, red stars].

3.2 Functional analyses of PR modulators in a uterine leiomyoma model system

3.2.1 Establishment of an in vitro / in vivo model system for uterine leiomyomas

Model systems for uterine leiomyomas are rare. A well characterized model system is the Eker rat which establishes tumors that share many phenotypic characteristics with the cognate human disease [46, 48]. However, latencies up to one year, the prevalence of tumors (65%), the challenging monitoring of the tumor growth and difficulties in manipulating the cells for validation of potential effectors of proliferation are clear disadvantages of the Eker rat. Here, the Eker rat leiomyoma tumor-derived (ELT) cell lines [48], in particular ELT-3 cells, offer a suitable alternative approach. The establishment of a combination of ELT-3 cells and a lentiviral-mediated system for gene silencing could provide a suitable system for the investigation of basic mechanisms of uterine leiomyoma growth.

ELT-3 cells were shown to proliferate very rapidly, they did not reach cell cycle arrest due to confluence. Their doubling time was approximately 15 hours in maintenance medium. In basal medium, which was used in experiments to determine hormonal effects, doubling time was significantly increased but ELT-3 cells still maintain a proliferative potential. The morphology of ELT-3 cells was very heterogeneous ranging from a spindle to a more stellate form (like fibroblasts), or round and flat, or more epitheliod appearance (Figure 22). The proportion of morphologies depended on experimental conditions like density at separation, growth medium and treatment. In particular, if ELT-3 cells were overgrown, the morphology was coadunate. Their smooth muscle origin and the presence of steroid hormone receptors was analyzed using immunofluorescence staining, real-time PCR and Western Blot.

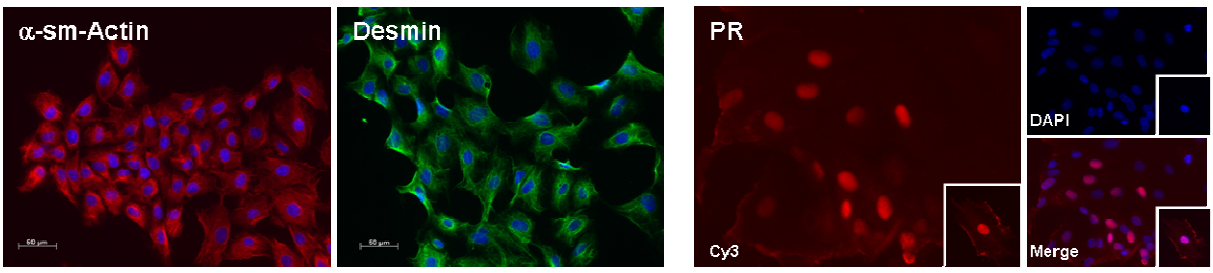

Figure 22. Characterizaton of ELT-3 cells via immunofluorescence staining for α-smooth muscle actin, desmin and PR protein. Nuclei were visualized by DAPI staining. Control staining without primary antibodies did not reveal specific signals (not shown).

Expression of the smooth muscle cell markers desmin and α-smooth muscle-actin was confirmed. The localization of progesterone receptor (PR) was shown to be nuclear in most of the cells (Figure 22). Real-time PCR analysis furthermore revealed the expression of the steroid hormone receptors estrogen receptor alpha (Esr1) and PR to be regulated by estradiol (Figure 23A, B). Western blot analysis confirmed these findings and furthermore, demonstrated that PR-A is the isoform which is predominantly present in ELT-3 cells (Figure 23C).

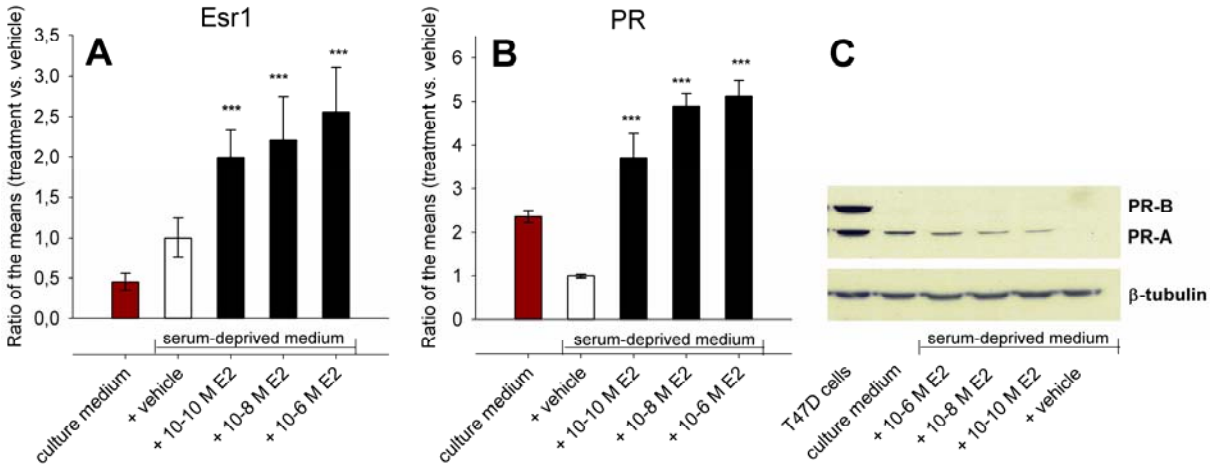

Figure 23. E2-induced expression of Esr1 and PR in ELT-3 cells. ELT-3 cells were cultured in maintenance medium or serum-deprived medium supplemented with vehicle or 100 pM, 10 nM and 1 µM estradiol for 48 hours. A-B) Esr1 (A) and PR (B) mRNA expression levels were analyzed via quantitative real-time PCR and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of two independent, time-separated experiments. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparison treatment vs. vehicle. C) E2-induced expression of PR isoforms in ELT-3 cells analyzed via Western blot. Examination of β-tubulin protein expression was used as a loading control. PR expression in T47D cells served as a positive control.

In vitro cell viability assays were established and performed to analyze hormone-dependent proliferation of ELT-3 cells. The influence of hormones on apoptosis rate of ELT-3 cell was furthermore determined via caspase 3/7 activity assays. Caspase 3 and caspase 7 are effector caspases in the apoptotic process and cause degradation of structural and nuclear proteins [171].

The E2-stimulated viability of ELT-3 cells was confirmed to be dose- and time-dependent. (Figure 24A). For an estradiol concentration of 100 pM and a time of treatment of seven days, the effects were maximal and therefore, were used for further experiments. Increasing concentrations of estradiol reduced caspase 3/7 activity in ELT-3 cells after 24 hours of incubation (Figure 24B). The inhibition of apoptosis was shown to be dose-dependent.

To confirm the stimulation of proliferation by estradiol pharmacologically, the properties of the ER modulator 4-hydroxytamoxifen (4-OHT) and the ER antagonist ZK 191703 on ELT-3 cell proliferation were tested.

The ER partial agonist 4-hydroxytamoxifen (4-OHT) displayed agonistic effects on ELT-3 cell proliferation (Figure 25A). In contrast, the ER destabilizer ZK 191703 inhibited ELT-3 cell proliferation per se. The E2-induced proliferation was inhibited by both 4-hydroxytamoxifen and ZK 191703 (Figure 25B). However, ZK 191703 was significantly more effective and repressed proliferation below baseline levels, i.e. proliferation in the presence of vehicle.

Moreover, ZK 191703 activated the apoptotic caspase 3/7 signaling pathways (data not shown).

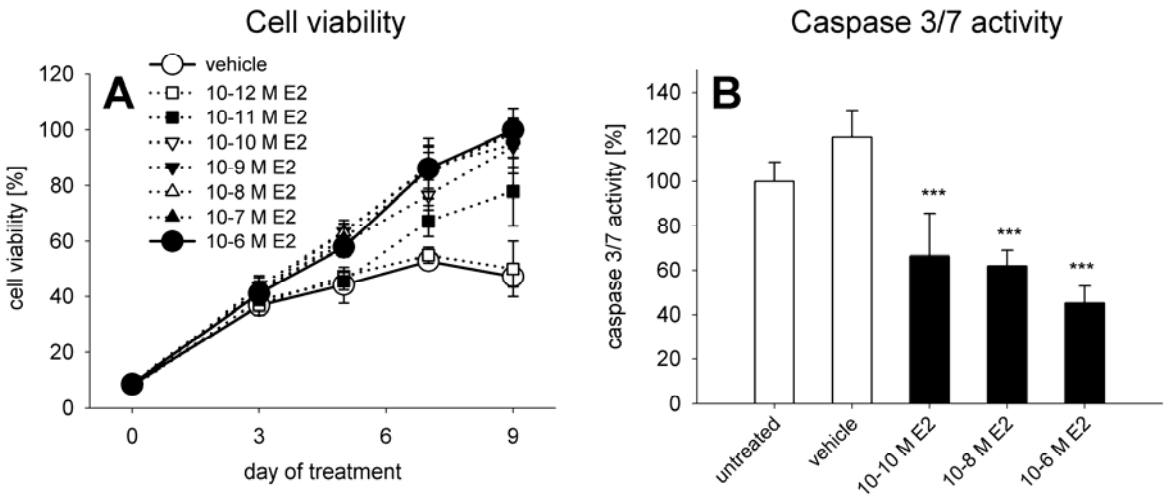

Figure 24. A) Kinetics of ELT-3 cell proliferation after treatment with estradiol. ELT-3 cells were cultured in basal medium (see 2.1.1) supplemented with increasing concentrations (10^{-12}-10^{-6} M) of estradiol or vehicle, and cell viability was analyzed on days 0, 3, 5, 7 and 9 using CellTiter-Glo assays. The graph represents at least two independent experiments and error bars denote CI (95%) of sextuplicate wells. B) E2-induced inhibition of ELT-3 cell apoptosis rate. ELT-3 cells were cultured in basal medium supplemented with vehicle, 100 pM, 10 nM and 1 µM estradiol. After 4 days of treatment, caspase 3/7 activity was analyzed using Caspase-Glo 3/7 assays. Treatment with vehicle served as a negative control. Caspase 3/7 activity in untreated cells was arbitrarily set as 100% and as a parameter for apoptosis rate the percent change of caspase 3/7 activity was determined for each treatment. The graph represents at least two independent experiments and error bars denote CI (95%) of sextuplicate wells. Statistical significance was assessed by Student's T-test (p-value < 0.001 ***) for the comparisons E2 vs. untreated.

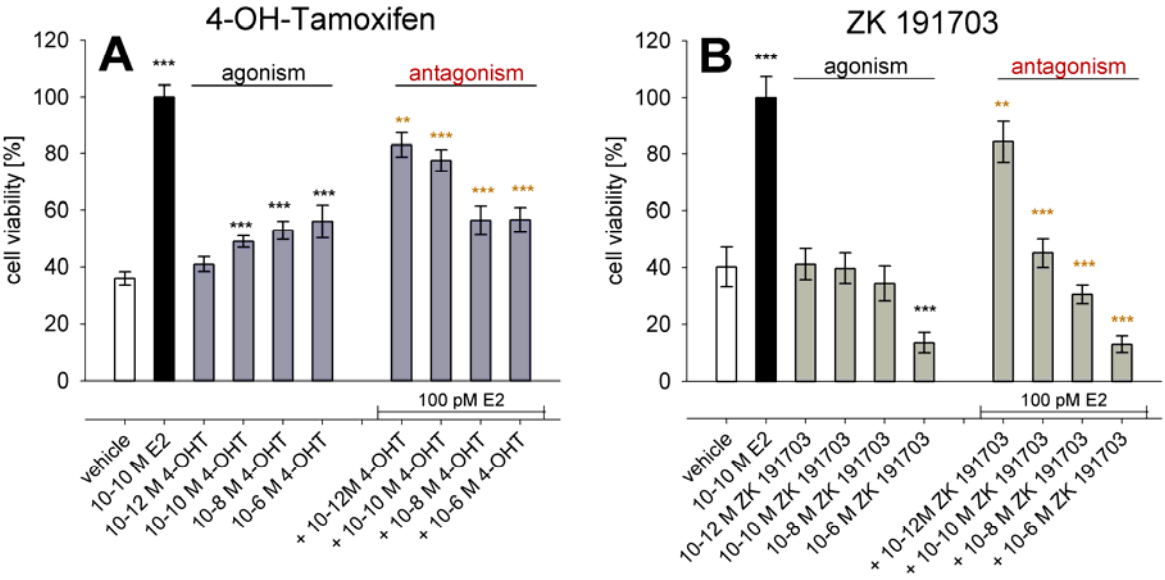

Figure 25. Effects of ER modulators on ELT-3 cell proliferation. Left part of diagram: Agonistic profile. ELT-3 cells were cultured in basal medium supplemented with increasing concentrations of 4-OH-tamoxifen (A) or ZK 191703 (B) for 7 days. Right part of diagram: Antagonistic profile. ELT-3 cells were cultured in basal medium supplemented with increasing concentrations of ER ligand (s.a.) and 100 pM estradiol for 7 days. Cell viability was obtained using CellTiter-Glo assays. Vehicle served as negative control and 100 pM estradiol as positive control. E2-induced proliferation was arbitrarily set as 100% and as a parameter for cell proliferation the percent change of cell viability was determined for each treatment. Each graph represents at least two independent experiments and error bars denote CI (95%) of sextuplicate wells. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons ER ligand/E2 vs. vehicle [agonism, black stars] or (ER ligand + E2) vs. E2 [antagonism, red stars].

ELT-3 cells have been shown to be tumorigenic in nude mice [48]. In the present study, an in vivo xenograft model was established by inoculating $5x10^6$ cells subcutaneously into the right hip of immunodeficient SCID mice.

Within 57 days post inoculation, solid subcutaneous tumors with a mean of 100 mm^2 area and low vascularization evolved (Figure 26A). The incidence for tumor establishment was markedly lower for mice which were ovariectomized three days before inoculation (20% vs. 100% in the presence of endogenous estradiol). Mice with endogenous estradiol levels formed significantly smaller tumors than those additionally substituted with exogenous estradiol (Figure 26B). Therefore, the growth of ELT-3 cell-induced tumors was demonstrated to be enhanced by high doses of estradiol.

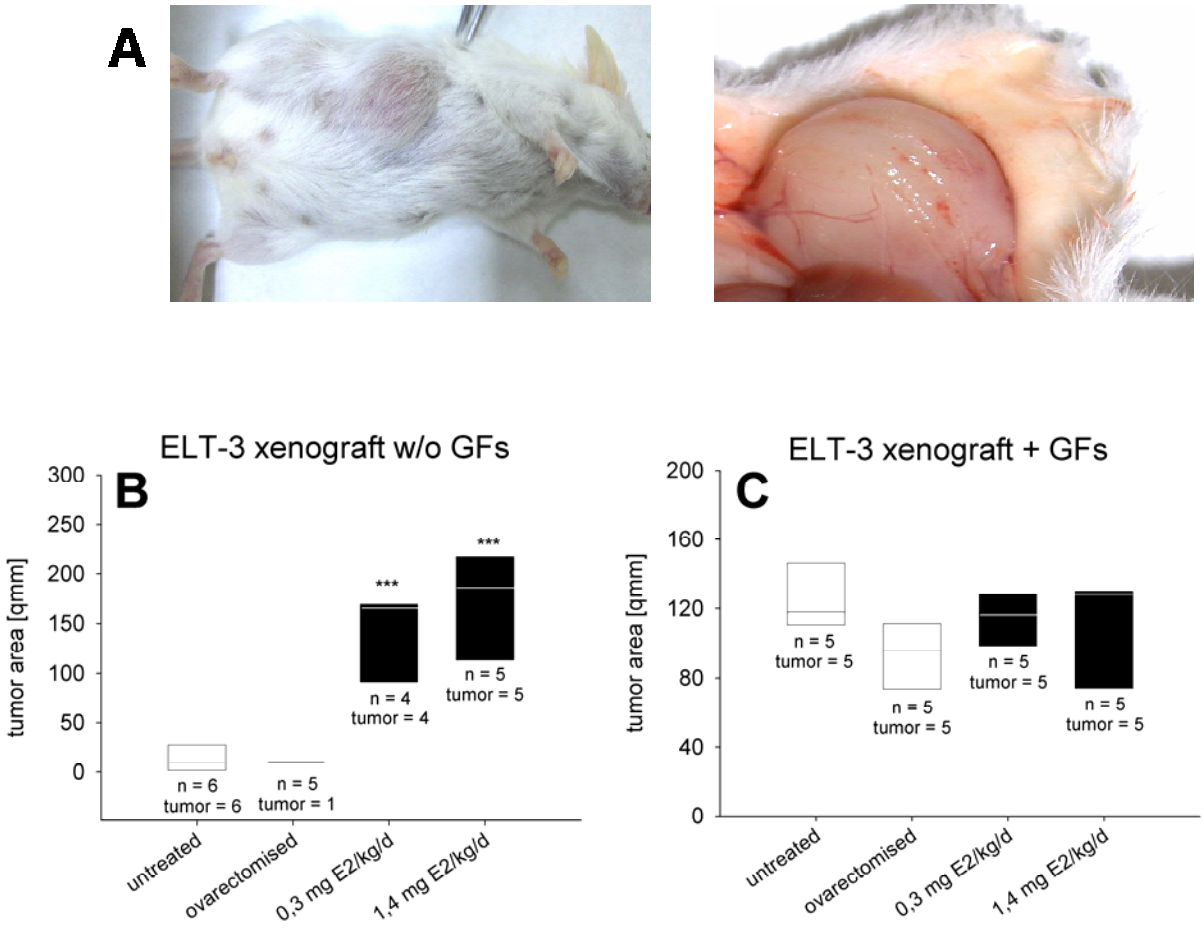

Figure 26. E2-stimulated growth of ELT-3 cell-induced xenografts in SCID mice. Mice were implanted with pellets of 17β-estradiol, ovariectomized or left untreated. After three days, ELT-3 cells ($5x10^6$ cells/100 µl) were inoculated subcutaneously (A) over the right hip of SCID mice either in serum-free RPMI 1640 medium (B) or in 1:1 (v/v) serum-free RPMI 1640 medium/MatriGel (C). Tumor area was examined 57 days post inoculation and results are presented as box plots. Statistical significance was assessed by Student's T-test (p-value < 0.001 ***) for the comparisons modulated E2-level vs. untreated. n = number of mice, tumor = number of established tumors 57 days post inoculation.

This hormone-responsiveness was lost if ELT-3 cells were inoculated in a mixture of medium and MatriGel (1:1). MatriGel is a gelatinous protein mixture secreted by mouse tumor cells that resembles a complex extracellular environment and contains structural proteins such as collagen and laminin, growth factors and numerous other proteins. The use of MatriGel facilitated the establishment of tumors. Compared to mice inoculated with ELT-3 cells in medium only, the incidence for tumors in the animals inoculated with cells in MatriGel was 100%, independent of the level of estradiol (Figure 26C). Even ovariectomized mice established tumors which were comparable in size to those of mice supplemented with exogenous estradiol. The increased concentration of growth factors in the extracellular space most likely led to a loss of tumor hormone-responsiveness.

In the following experiments, ELT-3 cells were inoculated in medium only. All mice were substituted with estradiol to shorten latencies and to obtain similar tumor sizes at treatment start.

If mice with established tumors were deprived of estradiol by ovarectomy or treated with the ER antagonist ZK 191703, E2-stimulated growth was inhibited. The efficacies of ovarectomy and ZK 191703 treatment were similar. ZK 191703 repressed the estradiol effect by approximately 73% (with a 95% one-sided confidence interval (36.4%, ∞) as assessed by Fieller test for one-sided confidence intervals for baseline-adjusted area under the curves (AUCs); Figure 27).

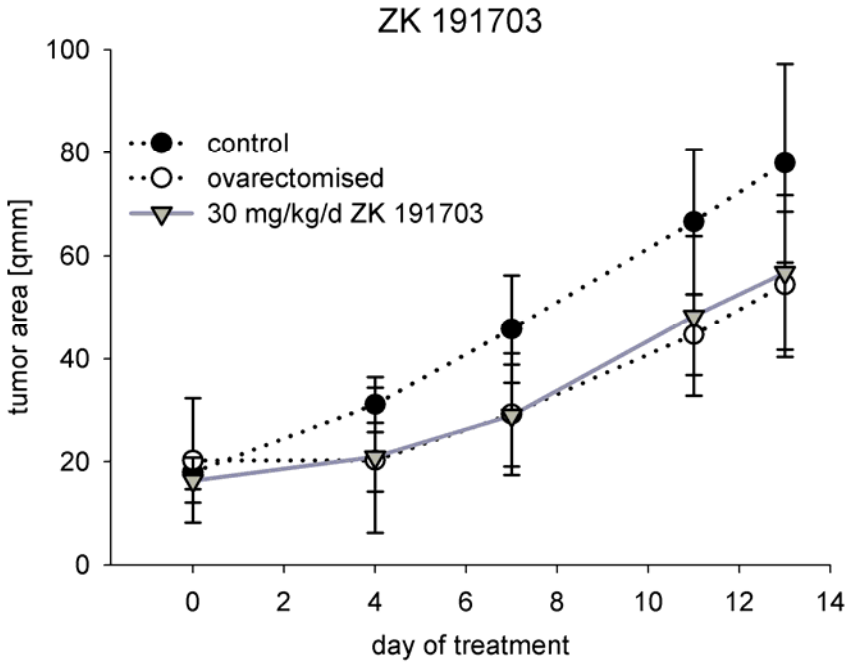

Figure 27. Inhibition of E2-stimulated growth of ELT-3 cell-induced xenografts in SCID mice. Mice were supplemented with 0.1 mg/kg estradiol-valerate on a weekly basis. ELT-3 cells ($5x10^6$ cells/100 µl serum-free RPMI 1640 medium) were inoculated subcutaneously three days after injection of the first estradiol-valerate depot. 39 days post inoculation, mice with tumors (area > 15 mm^2) were randomized and ovariectomized or treated with 30 mg/kg/d ER antagonist ZK 191703 or vehicle control for two weeks. Tumor growth was observed on days 0, 4, 7, 11 and 13 of treatment. The graphs represent two independent experiments and error bars denote CI (95%) of n = 10 mice per group. Statistical analyses was assessed by Fieller test for one-sided confidence intervals for baseline-adjusted AUCs (baseline = ovariectomized mice).

In other uterine leiomyoma model systems, it is difficult or even not possible to identify and confirm genes which are critical for proliferation and might affect the treatment of uterine leiomyomas. The ELT-3 cell model system potentially provides new approaches by manipulating leiomyoma-derived cells via transfection or viral transduction. To obtain cells stably expressing the preferred genetic modification, a lentiviral transduction system was used to insert shRNA sequences complementary to the gene of interest (see 2.1.11). As ELT-3 cell proliferation was observed to be highly sensitive to ER ligands, the estrogen receptor alpha (Esr1) was selected as candidate gene. Subsequently, the effects of Esr1 silencing were tested for a functional loss of the proliferative response to provide a proof of principle.

The selected sequence for silencing of the estrogen receptor alpha was shown to be efficacious, as Esr1 mRNA expression was reduced by 95% compared to wildtype cells or cells transduced with control shRNA (Figure 28A). The expression of the PR, an Esr1-downstream gene, was also significantly decreased on transcript and protein level demonstrating functional depletion of Esr1 (Figure 28B, C).

In proliferation assays, ELT-3 cells with silenced Esr1 expression demonstrated a loss of E2-induced cell proliferation (Figure 29A, black bars). The E2-independent cell proliferation was not affected (white bars). In ELT-3 cells transduced with randomized control shRNA, estradiol still stimulated proliferation, similar to the proliferation of untransduced wildtype cells.

In an in vivo xenograft experiment in immunodeficient SCID mice, only five of ten mice inoculated with ELT-3 cells expressing reduced levels of Esr1 developed tumors within 53 days post inoculation. Thus, the tumorigenic potential was reduced by 50%. Furthermore, silencing of Esr1 in ELT-3 cells caused a decrease in tumor size by around 76% with a 95% one-sided confidence interval (39.6%, ∞) as assessed by the Fieller test for one-sided confidence intervals for baseline-adjusted AUCs (Figure 29B). Expression of randomized control shRNA in ELT-3 cells did not affect tumor incidence, but even increased the tumor size by around 78% with a 95% one-sided confidence interval (-206.2%, ∞) as assessed by the Fieller test. In a direct comparison with an AUC quotient of around 13%, the growth of tumors induced by ELT-3 cells with silenced Esr1 expression was significantly lower than the growth of tumors induced by ELT -3 cells stably transduced with control shRNA.

In summary, the in vitro / in vivo ELT-3 cell model system for uterine leiomyomas exhibited general characteristics of the cognate human disease such as smooth muscle origin and hormone-responsiveness. In particular, the stimulating effects of estradiol on ELT-3 cell proliferation and tumor growth were confirmed. A lentiviral-mediated gene silencing system within ELT-3 cells was established to prove the impact of estrogen receptor alpha (Esr1) expression on ELT-3 cell proliferation. Hence, a new suitable model system which allows the elucidation of the mechanism of specific downstream genes involved in ELT-3 cell proliferation has been identified and established.

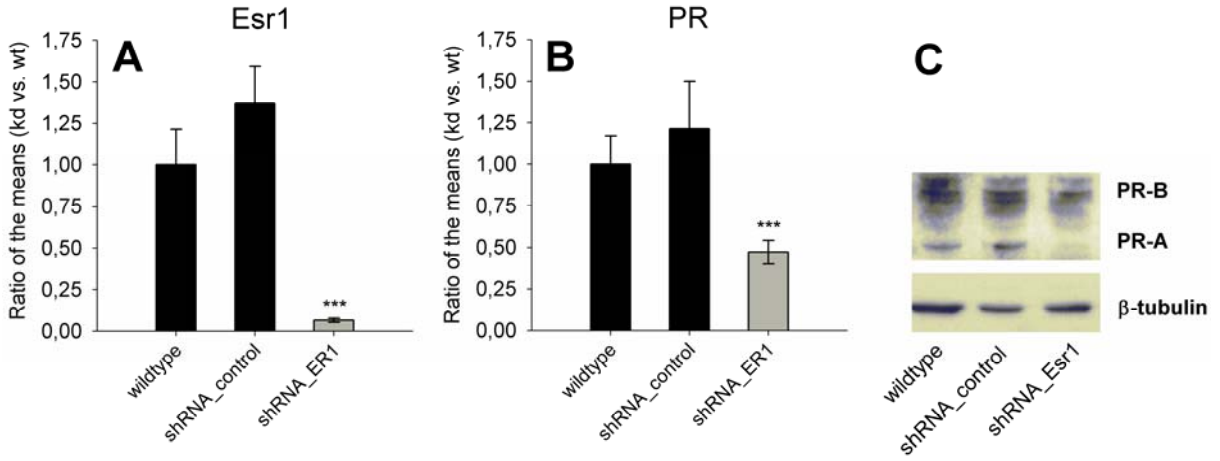

Figure 28. Expression of Esr1 and PR after Esr1 silencing in ELT-3 cells. ELT-3 cells were transduced with a lentiviral backbone (pGT396_hygro) containing shRNA complementary to Esr1 or randomized control shRNA and were selected by Hygromycin B treatment. Wildtype cells and polyclonal cells stably transduced with control-shRNA or Esr1-shRNA were analyzed for Esr1 (A) and PR (B) mRNA expression levels using quantitative real-time PCR. Expression levels were normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of stably transformed vs. wildtype cells and results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of two independent, time-separated experiments. Statistical significance was assessed by the Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparison stably transformed vs. wildtype ELT-3 cells. C) Expression of PR in wildtype cells and polyclonal cells stably transformed with randomized control shRNA or Esr1-shRNA was analyzed via Western Blot. Examination of β-tubulin protein expression was used as a loading control.

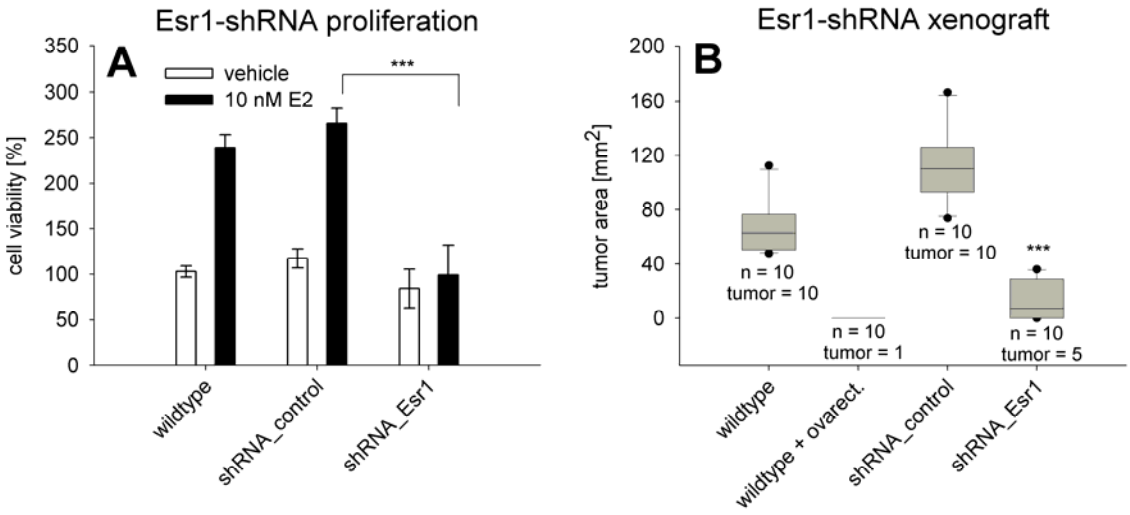

Figure 29. Loss of E2-induced proliferation and tumor growth after stable Esr1 silencing in ELT-3 cells. A) Wildtype cells and polyclonal cells stably transduced with randomized control shRNA or Esr1-shRNA were cultured in basal medium (see 2.1.1) supplemented with vehicle or 10 nM estradiol for 7 days. E2-independent proliferation of wildtype cells was arbitrarily set as 100% and as a parameter for cell proliferation the percent change of cell viability was determined for each cell line and E2-treatment. Each graph represents at least two independent experiments and error bars denote CI (95%) of quintuplicate wells. Statistical significance was assessed by the Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparison of ELT-3 cells stably transformed with Esr1-shRNA vs. control shRNA. B) Treatment groups were randomized and ovarecomized or supplemented with 0.1 mg/kg estradiol-valerate on a weekly basis. Wildtype cells and polyclonal cells stably transformed with control shRNA or Esr1-shRNA ($5x10^6$ cells/100 µl serum-free RPMI 1640 medium) were inoculated subcutaneously three days after injection of the first estradiol-valerate depot. Tumor area was examined 53 days post inoculation and results are presented as box plots. Statistical analyses and significance was assessed by the Fieller test for one-sided confidence intervals for baseline-adjusted AUCs (baseline = wildtype cells in ovariectomized mice) for the comparison of ELT-3 cells stably transformed with Esr1-shRNA vs. control shRNA. n = number of mice, tumor = number of established tumors 53 days post inoculation.

3.2.2 Proliferative and antiproliferative effects of PR modulators

To characterize the effects of PR modulators in the established ELT-3 cell leiomyoma model system, antiproliferative properties of the previously described PR modulators (3.1) were analyzed in single treatments as well as in the presence of 100 pM estradiol to investigate progesterone-independent effects as well as inhibitory effects on E2-stimulated proliferation.

R5020, the mixed antagonist mifepristone and the SPRM asoprisnil exhibited weak, but significant stimulating effects at high concentrations (Figure 30A, B, E). Treatment with onapristone or lonaprisan only did not change the proliferative response of ELT-3 cells (Figure 30C, D).

All PR modulators inhibited the E2-induced proliferation significantly and dose-dependently, albeit the pattern of inhibition was different. The type III antagonist lonaprisan displayed a highly significant antiproliferative effect ($p < 0.001$) at concentrations of 100 pM or higher and was the most effective PR antagonist analyzed (Figure 30D). Similar effects on E2-stimulated cell proliferation were observed for the PR agonist R5020.

The apoptosis rate of ELT-3 cells was not affected significantly after treatment with PR modulators (data not shown).

The type III PR antagonist lonaprisan was able to inhibit the E2-stimulated tumor growth in in vivo xenograft experiments with statistical significance. The efficacies of ovarectomy and lonaprisan treatment were similar. Lonaprisan inhibited the tumor size by around 89% (with a 95% one-sided confidence interval (36.4%, ∞) as assessed by Fieller test for one-sided confidence intervals for baseline-adjusted AUCs). In contrast, the type II antagonist mifepristone did not significantly antagonize the estrogenic effect and inhibited the tumor size only by around 16%, however, this was not conclusive (Figure 31).

In a direct comparison, the AUC for lonaprisan was around half of the estimated AUC for mifepristone (with a 95% confidence interval of (27.9%, 79.4%)). Tumors in mice treated with lonaprisan therefore grew significantly slower than tumors in mice treated with mifepristone.

Taken together, PR modulators affected ELT-3 cell proliferation themselves. They also inhibited E2-induced ELT-3 cell proliferation and tumor growth, although to different degrees. The pure antagonist lonaprisan exhibited the most effective antiproliferative properties in this functional model system.

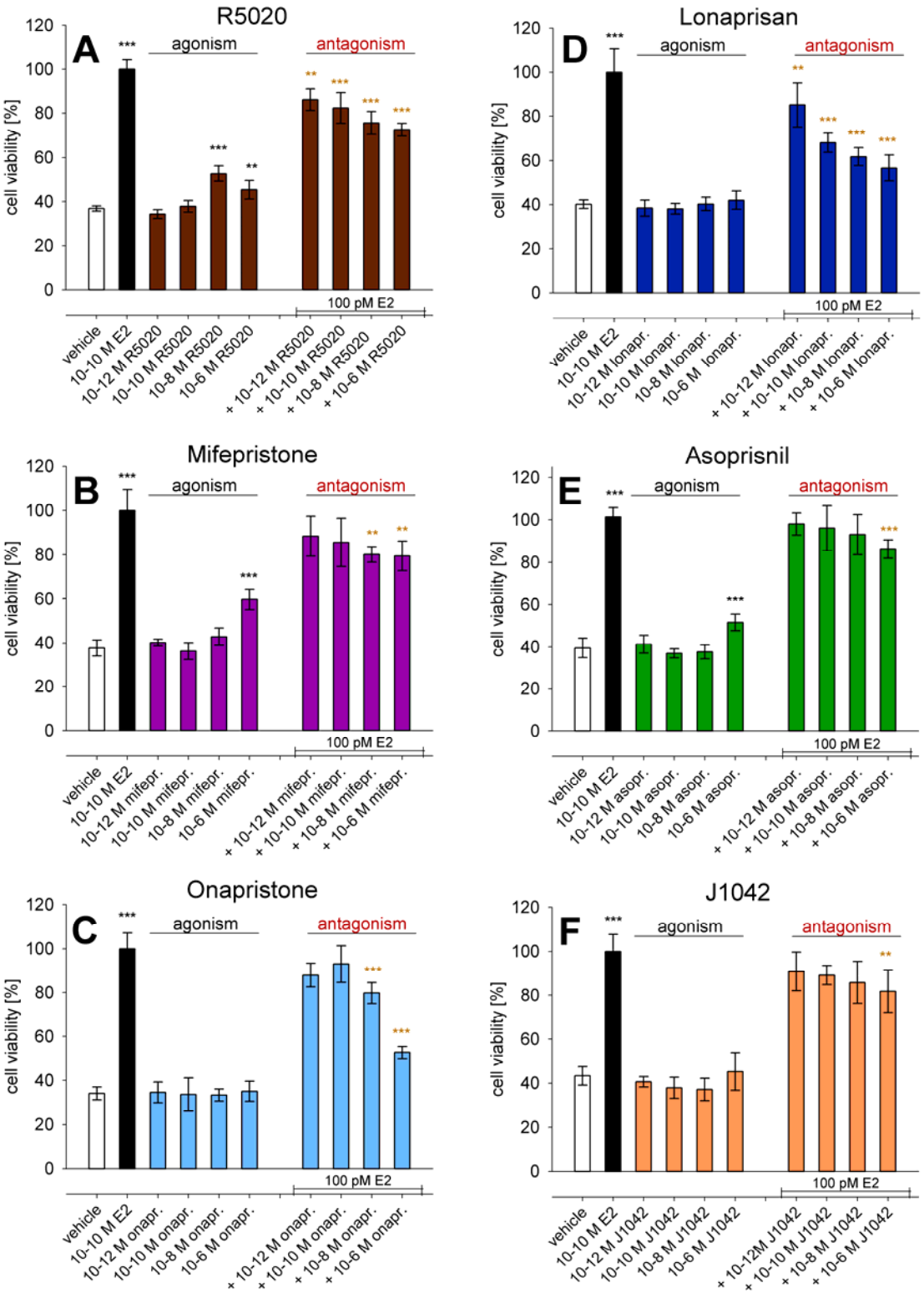

Figure 30. Analyses of PR modulator effects on ELT-3 cell proliferation. Left part of diagram: Agonistic profile. ELT-3 cells were treated with increasing concentrations of R5020 (A), mifepristone (B), onapristone (C), lonaprisan (D), asoprisnil (E) or J1042 (F) for 7 days. Right part of diagram: Antagonistic profile. ELT-3 cells were treated with increasing concentrations of PR ligand in the presence of 100 pM estradiol for 7 days. Cell viability was obtained using CellTiter-Glo assays. Vehicle served as negative control and 100 pM estradiol as positive control. E2-induced proliferation was arbitrarily set as 100% and as a parameter for cell proliferation the percent change of cell viability was determined for each treatment. Each graph represents at least two independent experiments and error bars denote CI (95%) of sextuplicate wells. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons PR ligand/E2 vs. vehicle [agonism, black stars] or (PR ligand + E2) vs. E2 [antagonism, red stars].

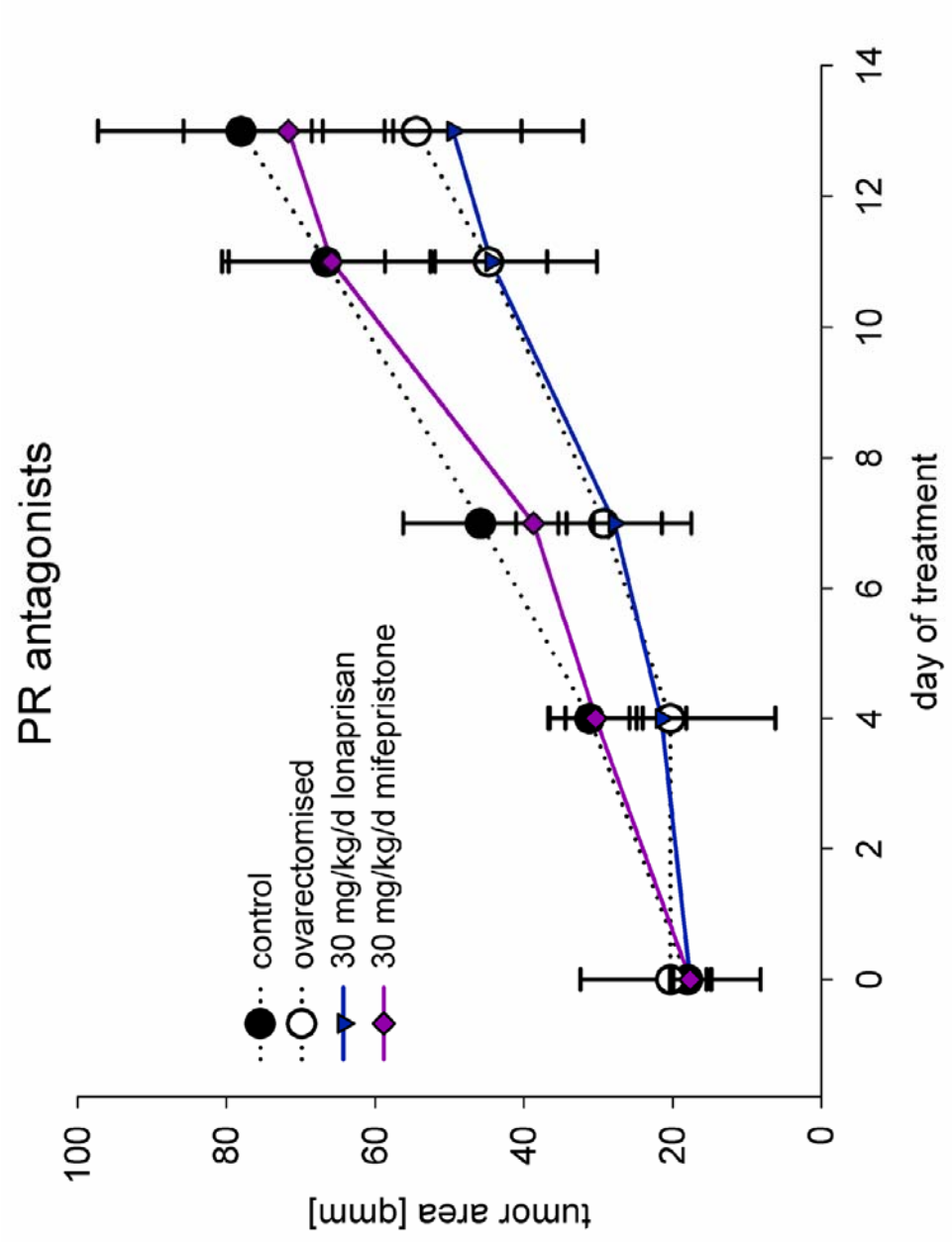

Figure 31. Inhibition of E2-stimulated growth of ELT-3 cell-induced xenografts in SCID mice. All treatment groups were supplemented with 0.1 mg/kg estradiol-valerate on a weekly basis. ELT-3 cells ($5x10^6$ cells/100 µl serum-free RPMI 1640 medium) were inoculated subcutaneously three days after injection of the first estradiol-valerate depot. 39 days post inoculation mice with tumors (area > 15 mm^2) were randomized and ovariectomized or treated with 30 mg/kg/d lonaprisan or mifepristone or solvent control for two weeks. Tumor growth was observed on days 0, 4, 7, 11 and 13 of treatment. The graph represents two independent experiments and error bars denote CI (95%) of n = 10 mice per group. Statistical analyses were performed using the Fieller test for one-sided confidence intervals for baseline-adjusted AUCs (baseline = ovariectomized mice).

3.2.3 Gene expression profiles of ER and PR modulators

To elucidate the mechanism of the proliferative effects of estradiol and PR agonists as well as the antiproliferative effects of ER and PR modulators in ELT-3 cells, a gene expression profiling study was performed. In this study, the identification of convergent antiproliferative mechanisms for ZK 191703 and lonaprisan were one of the predominant objectives. Since microarray analyses and gene expression data in ELT-3 cells have not been reported by others, the selected time points ranged from 6 hours to 24 hours and 48 hours of treatment. ELT-3 cells were incubated with 10 nM ER agonist (estradiol, E2), PR agonist (R5020), ER antagonist (ZK 191703) and PR antagonist (lonaprisan), either alone or in combination with E2 or R5020 (Table 11).

Table 11. Treatment groups for the Affymetrix GeneChip analysis in ELT-3 cells.

Probe*	Treatment	Vehicle	10 nM E2	ER antagonist		10 nM P4 (R5020)	PR antagonist	
				Compound	c [nM]		Compound	c [nM]
1 / 11 / 21	-	-	-	-	-	-	-	-
2 / 12 / 22	+	+	-	-	-	-	-	-
3 / 13 / 23	+	+	+	-	-	-	-	-
4 / 14 / 24	+	+	-	ZK 191703	10	-	-	-
5 / 15 / 25	+	+	+	ZK 191703	10	-	-	-
6 / 16 / 26	+	+	-	-	-	+	-	-
7 / 17 / 27	+	+	+	-	-	+	-	-
8 / 18 / 28	+	+	-	-	-	-	Lonaprisan	10
9 / 19 / 29	+	+	+	-	-	-	Lonaprisan	10
10 / 20 / 30	+	+	-	-	-	+	Lonaprisan	10

1-10 = 6 hours of treatment, 11-20 = 24 hours of treatment, 21-30 = 48 hours of treatment; * = 5 replicates.

The initial simultaneous assessment of gene expression values in treated ELT-3 cells by Affymetrix GeneChip Rat230_2.0 arrays revealed uniform overall expression and distribution of transcripts indicating that expression profiles were consistent with established standards for gene expression analyses.

Analogous to the principle component analysis (PCA) shown in Figure 10, the PCA depicts the variance in gene expression profiles among samples. The principle component analysis of gene expression profiles in ELT-3 cells demonstrated that the time-dependent differences in gene expression profiles were larger than the treatment effects per se. However, for all time points samples from the respective treatment groups cluster together and the treatment groups clearly separate from each other (Figure 32A, B). Further analyses focused on the effects after 24 hours of treatment (Figure 33A, B) since this time point combines pronounced effects of expression levels with reduced effects on cell morphology caused by confluence of the cells.

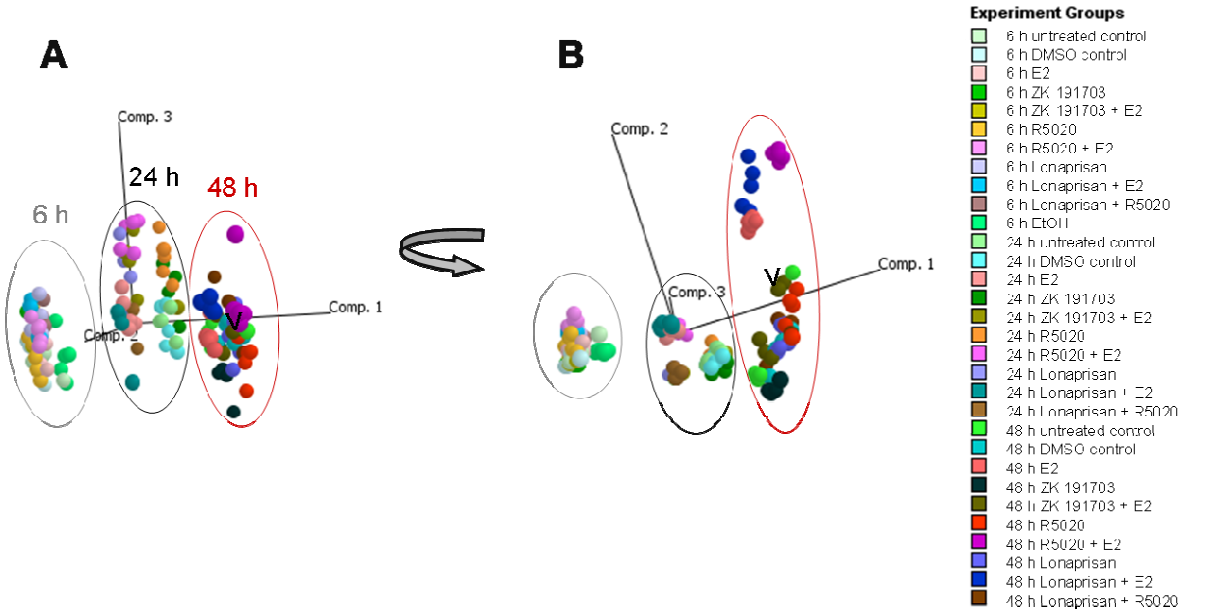

Figure 32. Principle Component Analysis (PCA) of ELT-3 cell expression profiles after ER/PR ligand treatment for 6, 24 and 48 hours. Samples are colored according to PR ligand treatment and the number of biological replicates was five for each treatment. Each plotted ball represents the expression profile of an individual sample based on the projection of the data on the first three principal components, accounting for most of the variability in the data (labeled axes). The PCA is shown for two different angular fields (A, B; 90°-rotation on component 1)

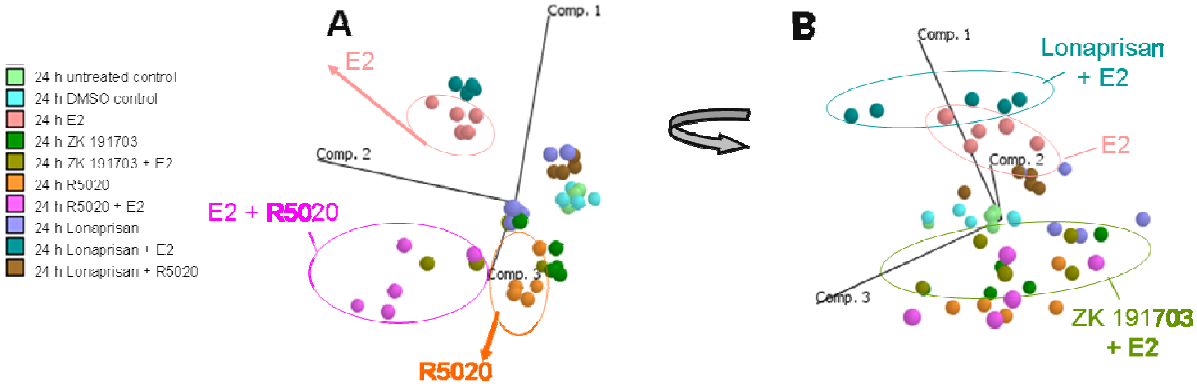

Figure 33. Principle Component Analysis (PCA) of ELT-3 cell expression profiles after ER/PR ligand treatment for 24 hours. Samples are colored according to PR ligand treatment and the number of biological replicates was five for each treatment. Each plotted ball represents the expression profile of an individual sample based on the projection of the data on the first three principal components, accounting for most of the variability in the data (labeled axes). The PCA is shown for two different angular fields (A, B; 90°-rotation on component 1)

All vehicle and all treatment samples formed distinct clusters. In single treatments, the highest variances to vehicle controls were observed for estradiol samples. R5020 samples displayed a moderate separation from vehicle samples. The smallest variances to vehicle controls were obtained for samples treated with ER and PR antagonist only. Samples treated with a combination of R5020 and lonaprisan also displayed a low variance to vehicle controls. The combined treatment of estradiol and R5020 resulted in an additive effect of single treatments, in vectorial illustration, and demonstrated the

largest variance to vehicle samples. The ER antagonist ZK191703 and the PR antagonist lonaprisan both affected E2-induced gene expression in combined treatments, although the effects of ZK 191703 were much stronger.

In summary, all analyzed ER and PR modulators influenced ELT-3 cell gene expression time-dependently. The effects of agonists, in particular in combined treatments, were the strongest. Estradiol-induced gene expression was modulated by the ER antagonist ZK 191703 and also by the PR antagonist lonaprisan.

3.2.4 Estradiol- and R5020-regulated gene transcripts

In an attempt to elucidate the proliferative properties of estradiol and R5020 in ELT-3 cells, genes were selected which were significantly regulated after ER agonist treatment, PR agonist treatment or a combination of both. Pairwise comparisons (treatment vs. vehicle) identified a total of 275 genes significantly regulated by estradiol and 77 genes significantly regulated by R5020. A Venn intersection analysis demonstrated 90 of the E2-regulated genes and 62 of the R5020-regulated genes to be also modulated in combined treatment, which affected a significantly higher number of genes (547 genes) than single treatments (Figure 34). Single treatments with estradiol and R5020 did not overlap in their effects on gene expression. Therefore, the gene expression profiles induced by estradiol and R5020 in ELT-3 cells were clearly different after 24 hours of treatment.

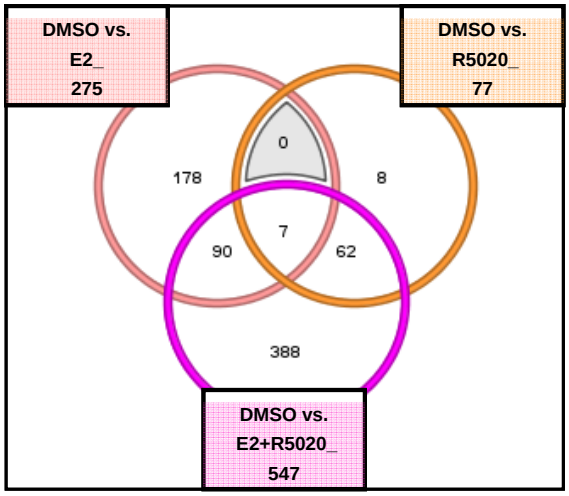

Figure 34. Venn intersection analyses of genes significantly regulated by E2, R5020 and R5020 in the presence of E2 (FC > 2, Volcano: FC > 5 and p-Value analogous to ST Q < 0.01 from T-test).

The pathways which contain protein products of genes significantly regulated by estradiol, R5020 or a combination of both are listed in Table 12. There was only one pathway, the ligand-dependent activation of the Esr1/SP pathway, which included a significant proportion of genes regulated after treatment with R5020.

In the presence of estradiol, R5020 modulated a significantly higher number of genes whose protein products are involved in extra-nuclear signaling pathways like TGF, WNT and cytoskeletal remodeling as well as regulation of activin A activity (Figure 35A). These pathways were complementary to the estradiol-regulated pathways. However, estradiol-regulated pathways like IGF-RI, ERBB and EGF signaling (Figure 35B) were not significantly influenced after combined treatment.

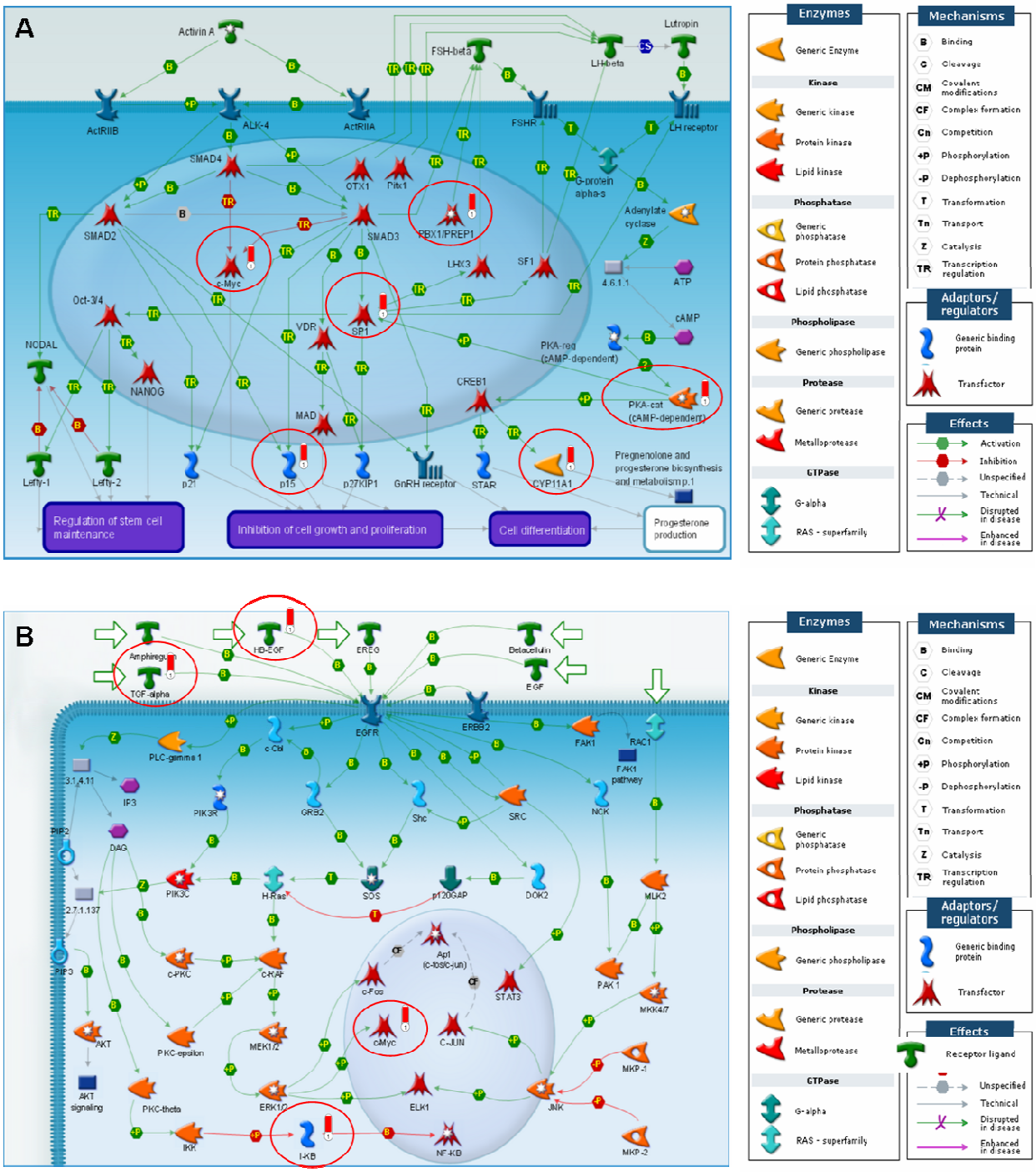

Figure 35. Influence of combined treatment of R5020 and estradiol on the activin A signaling in cell differentiation and proliferation (A) and influence of estradiol on EGF signaling pathway (B) as revealed by MetaCore pathway analyses. Red circles indicate significantly regulated genes (FC > 2, Volcano: FC > 5 and p-Value analogous to ST Q < 0.01 from T-test). Expression levels of genes that were inserted into pathways analysis are quantitative.

Table 12. Pathways significantly regulated by E2 and R5020 only as well as by R5020 in the presence of E2.

	E2	R5020	E2 + R5020
IGF-RI signaling	+	-	-
ERBB-family signaling	+	-	-
AKT signaling	+	-	-
Role of heterochromatin protein 1 (HP1) family in transcriptional silencing	+	-	+
MIF-mediated glucocorticoid regulation	+	-	-
dGTP metabolism	+	-	-
EGF signaling pathway	+	-	-
dCTP/dUTP metabolism	+	-	-
ECM remodeling	+	-	-
Ligand-dependent activation of the ESR1/SP pathway	-	+	-
TGF, WNT and cytoskeletal remodeling	-	-	+
Role of Activin A in cell differentiation and proliferation	-	-	+
Ephrins signaling	-	-	+
Reverse signaling by ephrin B	-	-	+
Rap1A regulation pathway	-	-	+
WNT signaling pathway / Response to extracellularr stimulus	-	-	+
Catecholamine metabolism	-	-	+
Regulation of G1/S transition	-	-	+

Number of regulated genes in this pathway signifcant ($p < 0.01$; +) or not significant ($p > 0.01$; -).

In order to identify PR downstream genes expressed after E2-induced sensitization to PR ligands as a result of increased PR expression, a profile distance search with the expression profile of the PR was performed within all time points examined. The analyses included all genes significantly regulated by R5020 in the presence of estradiol.

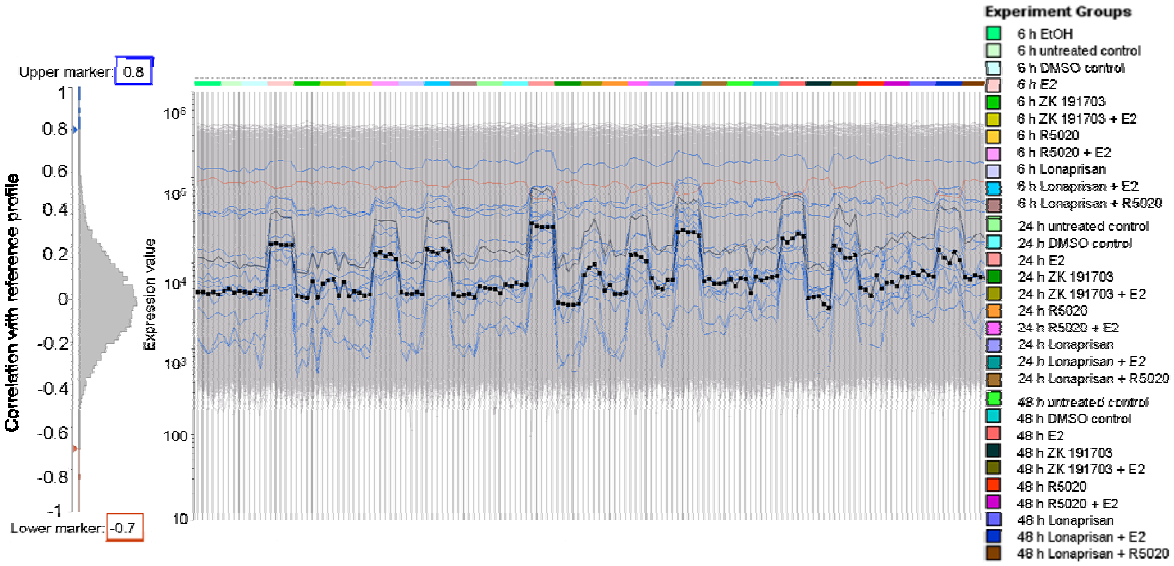

Figure 36. Profile distance search for genes whose expression pattern was correlated with the PR expression profile (correlation coefficient +/- 0.6, p-value < $2x10^{-16}$).

146 genes were correlated and 21 genes were anti-correlated to the PR expression profile (Figure 36). Genes with top correlation coefficients are listed in Table 19. The protein products are involved in diverse biological processes, including immune response (Cd200 antigen), cellular metabolism (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (Pfkfb3)), ion transport (ATPase Na^+/K^+ transporting polypeptide beta 3 (Atp1b3)), signal transduction from membrane (protein tyrosine phosphatase receptor type O (Ptpro)), transcription (E2F transcription factor 5 (E2f5), TSC22 domain family member 1 (Tsc22d1)), secretion of FSH (inhibin β subunit B (Inhbb)) and phospholipids binding (serum deprivation response protein (Sdpr)). In particular, E2F5 and Inhbb represent key factors modulating steroid hormone action. This underlines the importance of E2-induced PR expression.

Taken together, the proteins encoded by estradiol- and R5020-modulated genes were predominantly involved in growth factor signaling and ECM remodeling. In single treatments, the estradiol effects dominated the R5020 effects, but in combined treatments the R5020 effects were highly enhanced and contributed to the expression of relevant factors for steroid hormone responses in ELT-3 cells.

3.2.5 ZK 191703- and lonaprisan-counter-regulated gene transcripts

In order to elucidate the mechanisms of antiproliferative properties of the ER antagonist (ZK 191703) and the PR antagonist (lonaprisan) in ELT-3 cells, significantly regulated genes as well as counter-regulated genes were identified and further investigated by functional analyses.

Pairwise comparisons (treatment vs. vehicle) demonstrated significant regulation of 73 genes after treatment with the ER antagonist ZK 191703 only. 47 genes were significantly regulated after treatment with lonaprisan. In the presence of estradiol, 70 genes were regulated by ZK 191703 compared to vehicle. Analogous to the effects of R5020, the presence of estradiol highly increased the number of lonaprisan-regulated genes (386 genes).

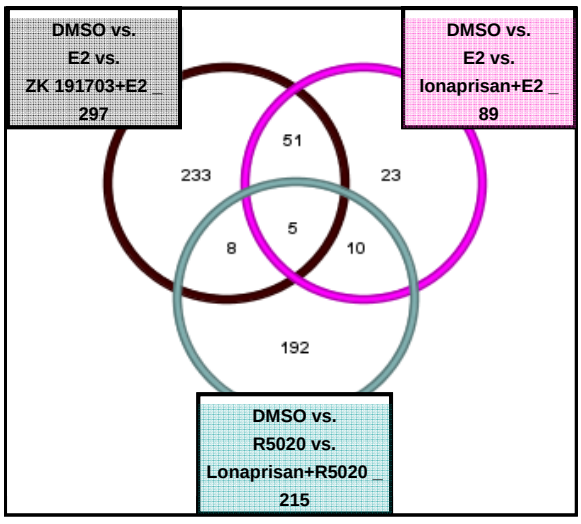

Figure 37. Venn intersection analyses of genes modulated by E2 or R5020 and significantly counter-regulated by ZK 191703 / lonaprisan (CR-score < -0.3).

Venn intersection analyses identified 297 genes which were modulated by estradiol and significantly counter-regulated by the ER antagonist ZK 191703. The PR antagonist lonaprisan counter-regulated 215 of R5020-modulated genes and 89 of estradiol-regulated genes, demonstrating a functional anti-estrogenic effect on a subset of ER downstream gene transcripts (Figure 37).

To visualize genes which were counter-regulated by lonaprisan (blue boxes) and which were not (white boxes), ANOVA analyses were performed and gene clusters were generated (Figure 38A, B).

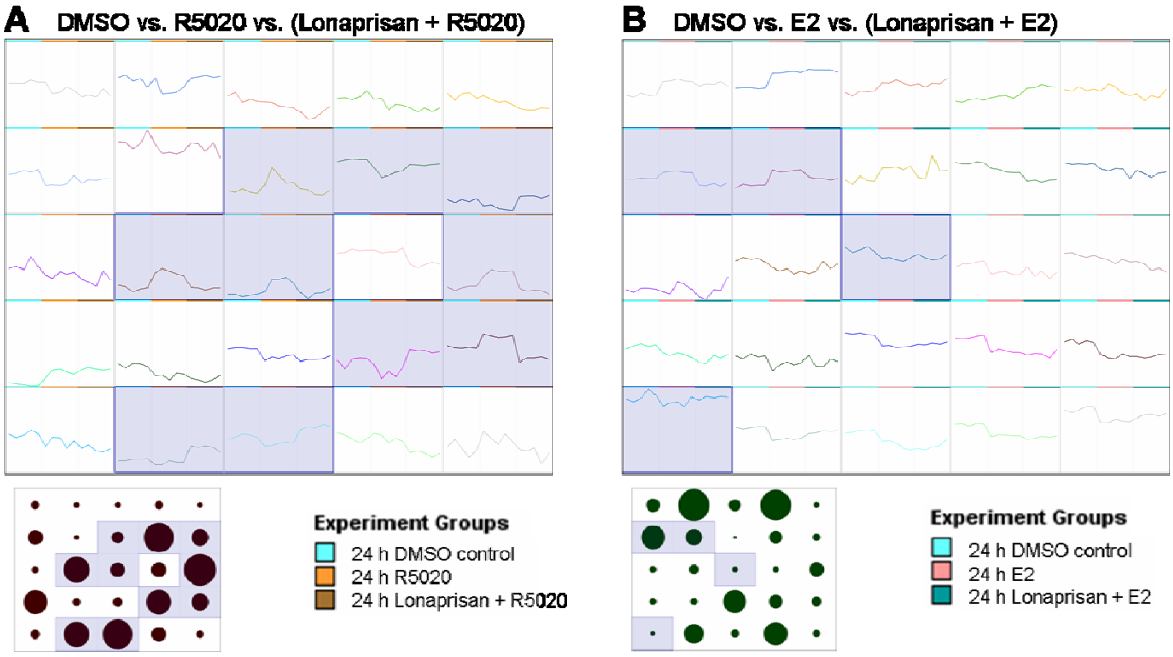

Figure 38. SOM clustering for PR antagonist lonaprisan counter-regulated genes. Cluster of genes induced by R5020 (A) or E2 (B) and counter-regulated by lonaprisan (p-value = 0.04, CR-score < -0.3) are shown in blue boxes. Cluster of genes not significantly counter-regulated by the PR modulator are shown in white boxes. The size of the dots corresponds to the number of genes.

Genes which might be important for lonaprisan- and ZK 191703-mediated antiproliferative activities on E2-induced ELT-3 cell proliferation are likely to be identified among the group of concomitantly counter-regulated genes. A categorical list of these genes is shown in Table 20. Gene ontology assessment and arrangement of genes into similar functional categories indicated that the protein products are mainly involved in hormone activity, membrane effects, cell cycle control and cell proliferation. In addition, genes which encode proteins in signal peptide processing (Pcsk5), transport (Slc25a30, Slc4a11, Slco5a1) and binding (Gpm6b, Btbd3, RGD1564376, RGD1563344) were included. 22 of the identified genes have not been annotated so far.

Four genes which were up-regulated by estradiol were selected for the confirmation of ER and PR antagonist-mediated counter-regulation using quantitative real-time PCR as an independent method. The genes chosen for validation were transforming growth factor alpha (TGFα), periplakin (Ppl), the

intermediate filament protein desmuslin (Dmn) and fibrinogen-like 2 (Fgl2). These genes encode factors in cell proliferation, membrane-bound receptor signaling, cell adhesion and coagulation, respectively.

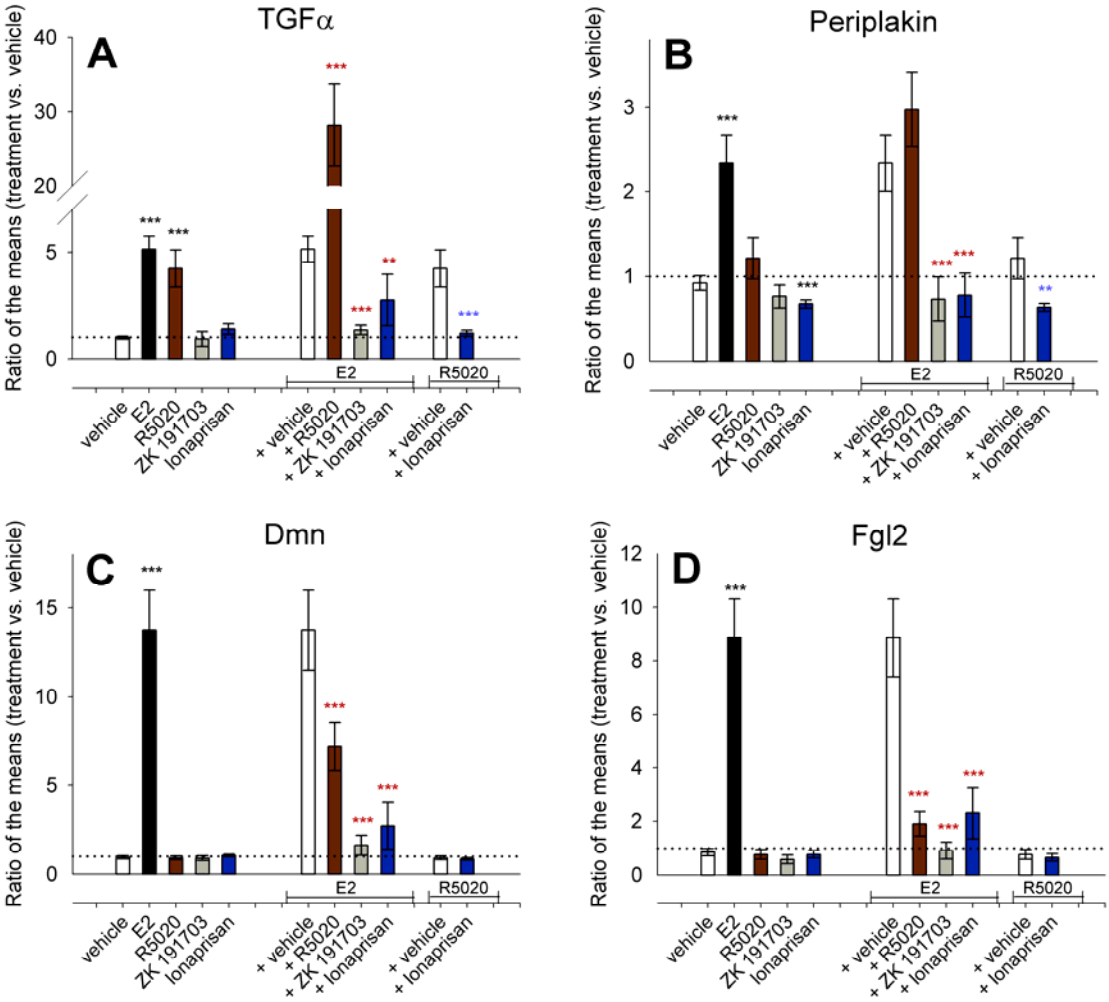

Figure 39. ER and PR antagonist counter-regulated genes, confirmed via quantitative real-time PCR. Left part of diagrams: Agonistic profile. ELT-3 cells were treated with vehicle or 10 nM of ER/PR ligand for 24 hours. Middle part of diagrams: Effect on E2-induced expression. ELT-3 cells were treated with vehicle or 10 nM of ER/PR ligand in the presence of 10 nM estradiol for 24 hours. Right part of diagrams: Effect on R5020-induced expression. ELT-3 cells were treated with vehicle or 10 nM lonaprisan in the presence of 10 nM R5020 for 24 hours. RNA expression levels of TGFα (A), Dmn (B), periplakin (C) and Fgl2 (D) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of three independent, time-separated experiments. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons ER/PR ligand vs. vehicle [agonism, black stars], (ER/PR ligand + E2) vs. E2 [ER antagonism, red stars] or (PR ligand + R5020) vs. R5020 [PR antagonism, blue stars].

TGFα transcript levels were up-regulated by estradiol and R5020, acting synergistically in combined treatment (Figure 39). Treatment with ZK 191703 or lonaprisan counter-regulated the hormonal effects. Periplakin was up-regulated by estradiol, but not by R5020 (Figure 39B). Whereas the PR antagonist lonaprisan revealed an inhibitory effect which was similar to the ER antagonist ZK 191703, R5020 did

not influence the E2-induced increase of Ppl transcript level. Desmuslin and fibrinogen-like 2 were both up-regulated solely by estradiol. The effect was counter-regulated by ZK 191703, lonaprisan and notably, also by the PR agonist R5020 (Figure 39C, D).

Transcript levels of inhibin β subunit B (Inhbb) and the predicted dehydrogenase/reductase (SDR family) member 7 (Dhrs7) were also examined by real-time PCR as these genes were differentially counter-regulated and might explain differences in the mechanisms of ER and PR antagonists.

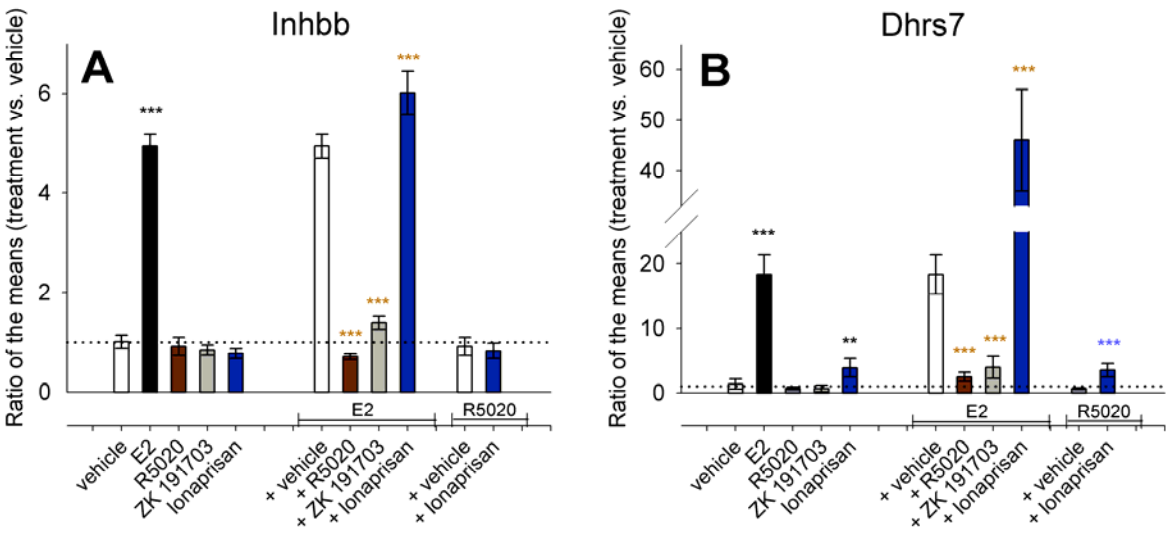

Figure 40. ER antagonist counter-regulated genes, confirmed via quantitative real-time PCR. Left part of diagrams: Agonistic profile. ELT-3 cells were treated with vehicle or 10 nM of ER/PR ligand for 24 hours. Middle part of diagrams: Effect on E2-induced expression. ELT-3 cells were treated with vehicle or 10 nM of ER/PR ligand in the presence of 10 nM estradiol for 24 hours. Right part of diagrams: Effect on R5020-induced expression. ELT-3 cells were treated with vehicle or 10 nM lonaprisan in the presence of 10 nM R5020 for 24 hours. RNA expression levels of Inhbb (A) and Dhrs7 (B) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of three independent, time-separated experiments. Statistical significance was assessed by Student's T-test (p-value < 0.005 **; p-value < 0.001 ***) for the comparisons ER/PR ligand vs. vehicle [agonism, black stars], (ER/PR ligand + E2) vs. E2 [ER antagonism, red stars] or (PR ligand + R5020) vs. R5020 [PR antagonism, blue stars].

The E2-induced expression of Inhbb and Dhrs7 was inhibited by ZK 191703 and interestingly, by R5020 (Figure 40A, B). Lonaprisan did not inhibit the estradiol effect but rather enhanced the E2-stimulated expression of Inhbb and Dhrs7. Dhrs7 gene expression was furthermore significantly increased after treatment with lonaprisan alone.

To determine whether the antiproliferative effects of lonaprisan and ZK 191703 in vivo (Figure 27, Figure 31) correlate with the regulation of genes in vitro, real-time PCR experiments were performed with tumor tissue from the xenograft experiments described above. The genes chosen for validation were TGFα, desmuslin, Inhbb and Dhrs7. Expression levels were obtained in pairwise comparisons to mice substituted with exogenous estradiol and treated with vehicle.

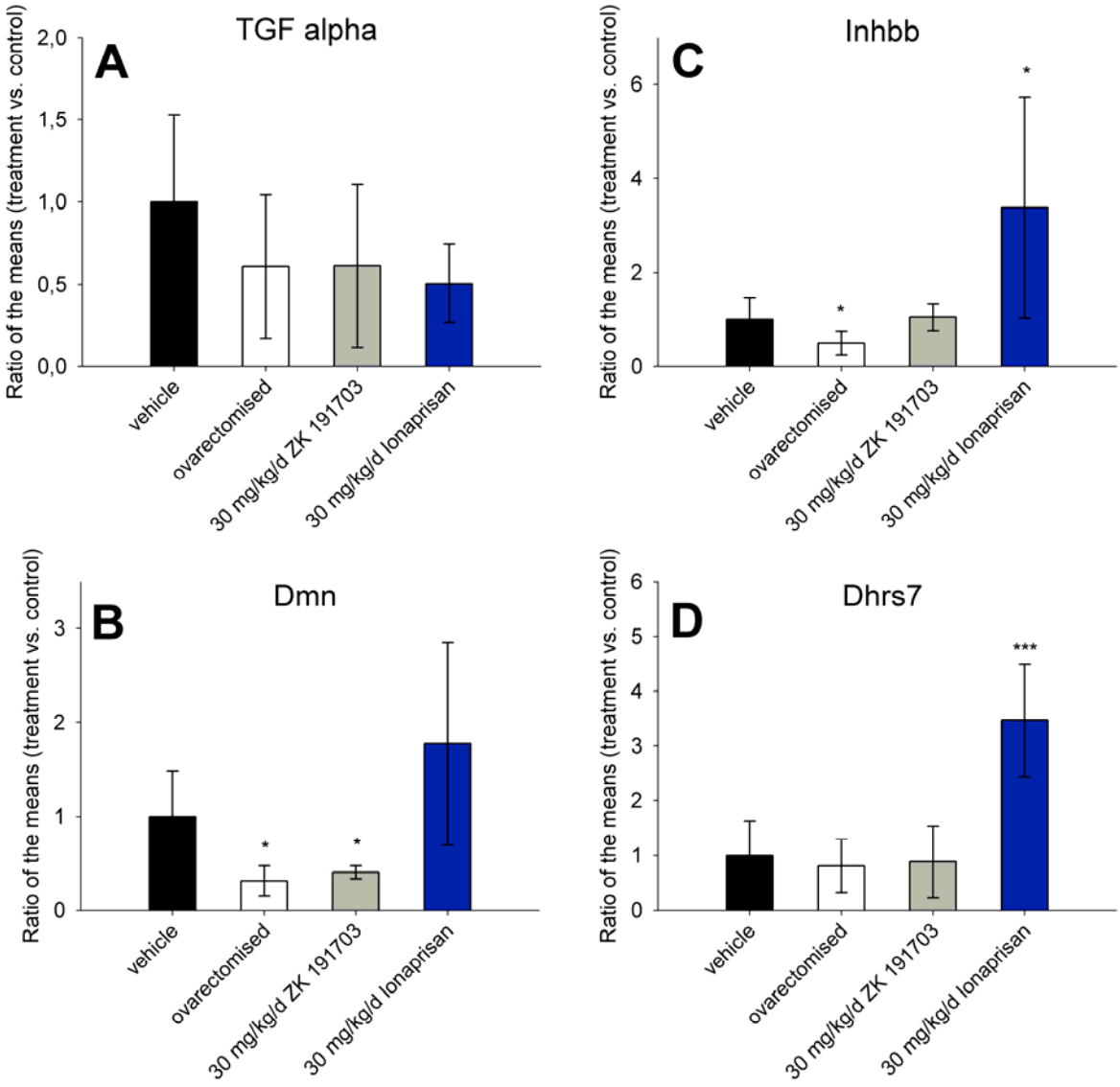

Figure 41. Gene expression in in vivo xenograft tumors after treatment with ER/PR antagonist, analyzed by using quantitative real-time PCR. All treatment groups were supplemented with 0.1 mg/kg estradiol-valerate on a weekly basis. ELT-3 cells ($5x10^6$ cells/100 µl serum-free RPMI 1640 medium) were inoculated subcutaneously three days after starting modulation of E2-level. 39 days post inoculation, mice with tumors (area > 15 mm^2) were randomized and ovariectomized or treated with 30 mg/kg/d ZK 191703 or lonaprisan or vehicle control. After two weeks, RNA was isolated from tumor tissues and expression levels of TGFα (A), Dmn (B), Inhbb (C) and Dhrs7 (D) were analyzed via TaqMan® gene expression assays and normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of treatment vs. vehicle. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of one experiment. Statistical significance was assessed by Student's T-test (p-value < 0.05 *, p-value < 0.005 **; p-value < 0.001 ***) for the comparisons ovarectomy vs. vehicle or ER/PR antagonist vs. vehicle.

TGFα mRNA levels tended to be decreased in tumors of mice which were ovarectomized or treated with ZK 191703 or lonaprisan (Figure 41A), very similar to in vitro expression profiles. E2-dependent desmuslin mRNA expression was significantly inhibited after ZK 191703-treatment or ovarectomy of mice (Figure 41B). The stimulating effects of lonaprisan on Inhbb and Dhrs7 gene expression in the presence of estradiol were confirmed to be statistically significant in vivo (Figure 41C, D). Treatment with ZK 191703 did not change the transcript levels of Inhbb and Dhrs7. Interestingly, tumor tissue of

ovariectomized mice showed significantly decreased Inhbb gene expression, confirming it as an Esr1 downstream gene.

Taken together, expression profiling of selected ER downstream genes in ELT-3 cells in vitro and in vivo confirmed common antagonistic effects of ZK 191703 and lonaprisan on a subset of genes and furthermore, demonstrated functional, non-competitive anti-estrogenic effects of lonaprisan and R5020. The effects of R5020 on ER downstream genes which were counter-regulated by the ER and the PR antagonist were partially agonistic and partially antagonistic, depending on the gene examined. Moreover, some genes which might be involved in the proliferative response of ELT-3 cells were regulated conversely by ZK 191703 and lonaprisan.

3.2.6 TGFα and inhibin β subunit B as potential downstream genes

In vitro analyses identified and confirmed E2-downstream genes to be significantly counter-regulated by both the ER antagonist ZK 191703 and the PR antagonist lonaprisan (Table 20). These genes might represent potential factors involved in ELT-3 cell proliferation. TGFα was selected as one such candidate target gene as it has been linked to proliferation [172]. Another factor with potential impact on ELT-3 cell proliferation is the inhibin β subunit B (Inhbb). Inhbb joins the α subunit, the β subunit B or the β subunit A to form inhibin B, activin B or activin AB, respectively. All Inhbb dimers have been linked to the modulation of pituitary FSH secretion [159, 173, 174]. Interestingly, Inhbb transcript levels were regulated conversely by ER and PR antagonists and might explain differences in their mechanisms.

In order to prove the functional relevance of these two selected downstream genes, analyses of their impact on ELT-3 cell proliferation were performed following silencing of each of the respective genes using the established lentiviral-mediated shRNA transduction system (see 2.1.11 and 3.2.1.). The efficacies of shRNA-mediated silencing of transforming growth factor (TGF) α- and inhibin β subunit B (Inhbb) mRNA expression were tested using real-time PCR.

Using TGFα-shRNA, TGFα mRNA expression was reduced by 60% compared to wildtype cells or cells transduced with randomized control shRNA (Figure 42A). In the in vitro proliferation assay, the polyclonal ELT-3 cell population with silenced TGFα expression demonstrated significantly reduced E2-stimulated cell proliferation (Figure 42B, black bars) whereas E2-independent cell proliferation remained unaffected (white bars). In ELT-3 cells transduced with control shRNA, estradiol still stimulated proliferation to similar extend as observed in untransduced wildtype cells.

In ELT-3 cells with stable Inhbb-shRNA expression, Inhbb transcription levels were significantly reduced by 93% compared to control cells (Figure 43A). Functionally, ELT-3 cells with reduced Inhbb gene expression showed an increased hormone-independent baseline proliferation (Figure 43B, white bars) whereas the E2-stimulated proliferation remained unaffected (black bars). The proliferation of ELT-3 cells transduced with randomized control shRNA was unchanged in the presence as well as in the absence of estradiol and therefore, was similar to wildtype cells.

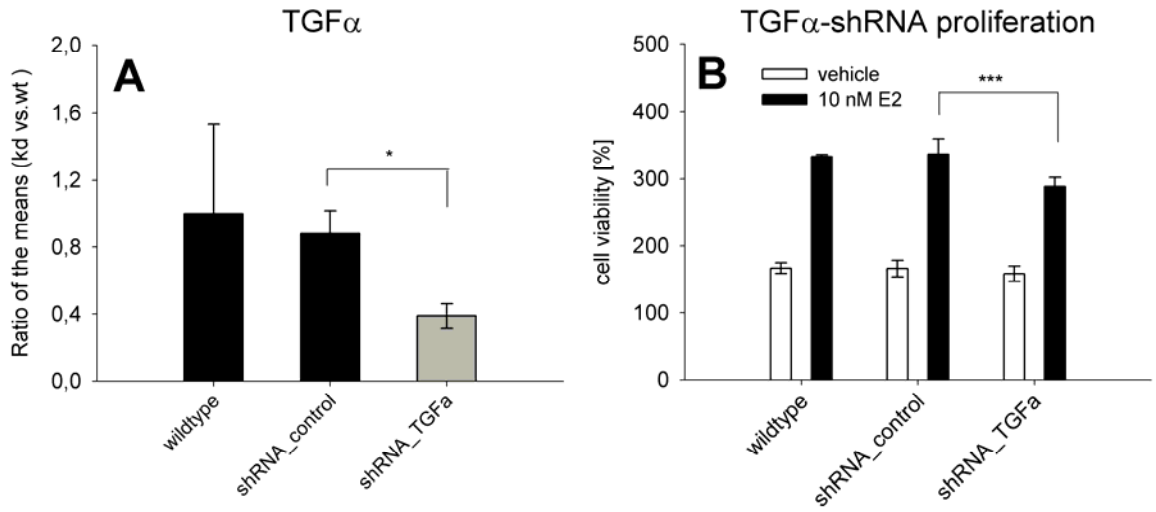

Figure 42. Effect of stable TGFα knock down on TGFα transcript levels and E2-induced proliferation. ELT-3 cells were transduced with shRNA complementary to TGFα or randomized control shRNA in a pGT396_hygro backbone and were selected by Hygromycin B. A) Cells were analyzed for TGFα expression levels using quantitative real-time PCR. Expression levels were normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of stably transformed vs. wildtype cells. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of two independent, time-separated experiments. B) Cells were cultured in basal medium supplemented with vehicle or 10 nM estradiol for 7 days. E2-independent proliferation of wildtype cells was arbitrarily set 100% and as a parameter for cell proliferation the percent change of cell viability was determined for each cell line and E2-treatment. Each graph represents at least two independent experiments and error bars denote CI (95%) of quintuplicate wells. Statistical significance was assessed by the Student's T-test (p-value < 0.05 *) for the comparisons of ELT-3 cells stably transformed with TGFα-shRNA vs. control shRNA.

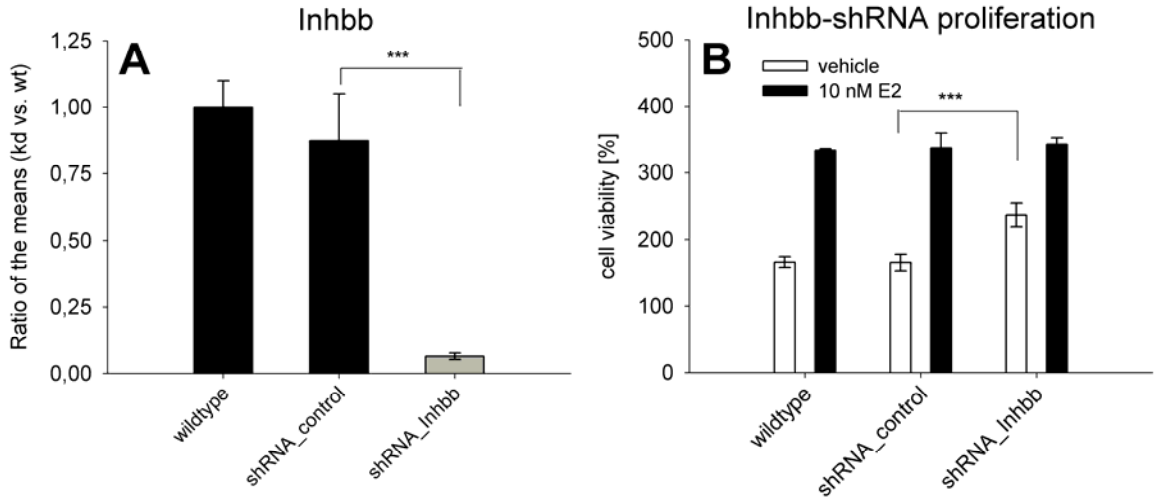

Figure 43. Effect of stable Inhbb knock down on Inhbb transcript levels and E2-induced proliferation. ELT-3 cells were transduced with shRNA complementary to Inhbb or control shRNA in a pGT396_hygro backbone and were selected by Hygromycin B. A) Cells were analyzed for Inhbb expression levels using quantitative real-time PCR. Expression levels were normalized to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A using the ΔΔCT-method. The fold changes were determined by pairwise comparisons of stably transformed vs. wildtype cells. Results were presented as ratio of the means (+/- CI, 95%) from duplicate wells of two independent, time-separated experiments. B) Cells were cultured in basal medium supplemented with vehicle or 10 nM estradiol for 7 days. E2-independent proliferation of wildtype cells was arbitrarily set 100% and as a parameter for cell proliferation the percent change of cell viability was determined for each cell line and E2-treatment. Each graph represents at least two independent experiments and error bars denote CI (95%) of quintuplicate wells. Statistical significance was assessed by the Student's T-test (p-value < 0.05 *) for the comparisons of ELT-3 cells stably transformed with Inhbb-shRNA vs. control shRNA.

In summary, TGFα and Inhbb expression levels, which were modulated by steroid hormones, displayed significant influence on ELT-3 cell proliferation. In particular, the PR antagonist lonaprisan was identified to repress the E2-induced increase in TGFα expression which might result in reduced E2-stimulated proliferation. Additionally, lonaprisan was shown to increase the expression of Inhbb, thereby inhibiting E2-independent proliferation. Taken together, lonaprisan represents a potent PR antagonist with antiproliferative properties in the ELT-3 cell model system for uterine leiomyomas. A part of lonaprisans mechanism seems to be based on the synergistic regulation of TGFα and Inhbb gene expression.

4. Discussion

In this thesis, a representative selection of PR modulators was analyzed for specific in vitro characteristics as well as their potential implication in the treatment of uterine leiomyomas. Currently, the traditional treatment of benign tumors in the uterine myometrium is surgical removal, in particular removal of the entire uterus (hysterectomy). There is no effective long-term medication available and a lack of well characterized, suitable and predictive in vitro and in vivo models makes research on novel treatment approaches challenging. Clinical studies have shown the impact of progesterone on conditions such as uterine leiomyomas [34, 133], and also endometriosis [130, 134] and breast cancer [128, 129]. Therefore, synthetic progesterone receptor modulators have been developed for the treatment of gynecological diseases, but the properties of PR modulators are diverse and many aspects of the molecular mechanisms are not resolved. In particular, progesterone-independent effects of PR modulators with antagonistic properties as well as inhibitory effects on estradiol action remain to be elucidated. Initially, a classification for PR antagonists into type I-III has been carried out several years ago [115, 126]. In this classification system, the mixed antagonist mifepristone, the classical antagonist onapristone and the pure antagonist lonaprisan are allocated to different subclasses based on PR antagonistic properties in vitro. Integration of tissue-selective PR modulators, currently known as selective progesterone modulators (SPRMs), into this classification model system assigned asoprisnil, J1042 and J912 to the category type II ligands [115]. Thereby, SPRMs are not differentiated from mixed antagonists like mifepristone, although they clearly display agonistic activities in a tissue-specific manner in vivo [116]. This demonstrates the prevalent problem for sufficient classification of PR ligands, especially in vitro with respect to their in vivo activities, if only antagonistic effects on PR agonist action are focused. The results of this thesis provide a new approach for PR modulator classification. Progesterone-independent properties were identified which clearly separate SPRMs from PR modulators from mixed and pure PR antagonistic activity in vitro. Unique activities of each PR modulator on estradiol action underline the importance of sufficient characterization to identify the most promising type of modulator for the treatment of uterine leiomyomas. Moreover, a new in vitro / in vivo model system for uterine leiomyomas was established to elucidate the mode of action of PR modulators in this gynecological indication.

4.1 Molecular mechanisms and classification systems for PR modulators

4.1.1 Antagonistic activity and ligand-induced PR interaction properties with cofactors

In order to characterize the genomic activity of PR modulators, cellular transactivation assays with steroidal PR agonists (R5020), SPRMs (asoprisnil, J1042, J912), mixed antagonists (mifepristone), classical PR antagonists (onapristone, ZK 137316) and pure antagonists (lonaprisan) as well as the non-steroidal tanaproget derivative PRA-910 were performed in a SK-NM-C cell-based assay system using the mammalian mammary tumor virus (MMTV) promoter. The MMTV promoter provides a well

characterized model system for hormonal regulation of transcription as it contains progesterone responsive elements (PREs) [175]. The results obtained demonstrate differences in the antagonistic potencies of PR modulators. In particular, lonaprisan and onapristone can be distinguished from other steroidal PR modulators as lonaprisan exhibits the highest whereas onapristone displays the lowest antagonistic potency. A discrimination between ligands with partial agonistic properties and PR antagonists is not possible as the effects on PR transcriptional activity were shown to be pure antagonistic for SPRMs, mixed, classical and pure PR antagonists (Figure 4). This is in disagreement with reports on partial agonistic activities of SPRMs in vivo [115, 116]. However, the non-steroidal PR modulator PRA-910 was shown to be a partial agonist with a transactivation profile distinct from agonists and antagonists. This is concordant with a unique activity profile of PRA-910 described by Zhang et al. [113].

Differences in the transcriptional activities of PR modulators might be attributed to different induced receptor conformations. It has been described that structural alterations are induced in the ligand-binding domain (LBD), primarily in the extreme C-terminus of the progesterone receptor [81] which is responsible for coactivator and corepressor interactions. Mammalian two-hybrid assays confirmed different cofactor interaction profiles for various PR ligands. The selected PR agonists, SPRMs and PR antagonists induce PR interactions with both corepressors and coactivators (Figure 5; data for PR-A and data for SRC family coactivators not shown). This supports the hypothesis that PR modulators induce receptor conformations with different affinities for cofactors [151] rather than conformations which completely prevent association with one type of cofactor. However, in the HeLa cell-based model system, association with corepressors is stronger for antagonist-bound PR while recruitment of SRC family coactivators is stronger for R5020-liganded receptor. Interestingly, the non-steroidal PR modulator PRA-910 does not induce PR interaction with any of the tested cofactors, neither with the coactivators nor with the corepressors. The completely distinct interaction profile indicates a receptor conformation which is different from conformations induced by steroidal PR ligands. All mammalian two-hybrid assays were performed in HeLa cells because this human cell line lacks endogenous PR expression and was shown to exhibit very low endogenous expression levels of cofactors which were utilized for the interaction studies (see 3.1.2.1).

The steroidal PR antagonists mifepristone, onapristone, ZK 137316 and lonaprisan differ in their induced interactions of PR with the corepressor NCoR interaction domain (ID), although the differences are marginal. The strong onapristone-induced interaction of PR with the SMRT-ID (Figure 5C) might be the result of higher concentrations of receptor available for the interaction because onapristone-bound PR has been described to display very low or absent binding activity to endogenous PREs [126]. The strong interaction with SMRT is furthermore restricted to PR isoform B (data for PR-A not shown). However, association of onapristone-bound PR-B is stronger with the SMRT fragment (ID1) than with the NCoR fragment (ID1-3), suggesting a specific cofactor interaction profile induced by onapristone.

Whereas PR interactions with full-length coactivators (SRC-1, SRC-2, SRC-3) are not profoundly different for steroidal PR modulators, the recruitment of truncated forms (SRC-1-NR-D, SRC-3-NR-D) is exclusive to agonistic ligands (Table 7). In particular, the LX-H10 peptide, which contains the LxxLL-

motif of coactivators [149, 150], can serve as an initial indication that PR modulators with PR agonistic activities in vivo also exhibit agonist-like properties in vitro. J1042- and asoprisnil-induced interactions of PR with the LX-H10 peptide are similar to R5020 whereas mixed as well as pure antagonists do not recruit the LX-H10 peptide (Figure 6; data for PR-A not shown). The potency of SPRMs recruiting activity is reduced compared to agonist R5020 and furthermore the efficacy of J912 is weaker. These observations reflect the partial agonistic activity of the respective PR ligand in endometrial transformation in the rabbit (Mc Phail test) [139], serving as a reference assay for PR modulator classification in vivo. Thus, the LX-H10 peptide interaction profile of SPRMs correlates with their agonistic properties in vivo, although the interaction model is rather artificial. The LX-H10 peptide has been identified in a phage peptide library of $(X)_7$LxxLL$(X)_7$ peptides as it was useful in determining receptor selectivity in HepG2 cells [150]. In the performed mammalian two-hybrid assays in HeLa cells, a PR isoform-selective recruitment of LX-H10 peptide was not confirmed. However, a tendency to a more potent recruitment to PR-B rather than to PR-A was observed (data not shown).

In addition to the cofactor-mediated genomic actions, the PR isoform B has been described to exhibit non-genomic activities. In particular, PR agonists like R5020 have been shown to promote fast changes in the activity of kinase pathways such as c-Src kinase, PI3K, AKT, PKA, ERK1/2 and MAPK signaling [105, 153]. The rapid effects of a PR ligand on these signaling pathways are independent of transcription and contribute to the effects on hormone-dependent cell proliferation [154]. Mammalian two-hybrid experiments with c-Src kinase identified different PR-B interaction profiles with this non-genomic cofactor for the ligands analyzed. All PR modulators were shown to induce PR-B interaction with c-Src kinase in the absence of the sex steroids progesterone and estradiol (Figure 7), although to different degrees. The lonaprisan-occupied PR recruits c-Src kinase with the lowest efficacy and subsequently activates the downstream cyclin D1 promotor less than the other tested PR modulators. The efficacy and potency of mifepristone-induced non-genomic activity is comparable to R5020 (Figure 7A, Figure 8A). This observation is concordant with previous reports on strong, agonist-like c-Src kinase downstream MAPK activation and cyclin D1 expression induced by mifepristone [100]. Interestingly, the classical antagonist onapristone also displays strong induction of PR-B/c-Src interaction. Its markedly weaker potency might be the result of lower receptor binding activity of onapristone. The differences observed in non-genomic PR interaction profiles support the hypothesis that respective ligand-induced receptor conformations modulate the association with cytoplasmic factors such as c-Src kinase in addition to the interaction with genomic cofactors. The results obtained from analyses with SH3 domain-deletion mutants of c-Src kinase (data not shown) furthermore confirm the hypothesis that the SH3 domain is necessary for interaction of PR-B with c-Src kinase [153], independent of the ligand bound. The exact mechanism of cytoplasmic PR-dependent c-Src kinase signaling is discussed controversially [102, 104, 153] and remains to be elucidated. The biological relevance of PR modulator-induced c-Src kinase signaling in humans is furthermore undetermined. The presence of ovarian hormones in clinical studies with women in reproductive age facilitates only the investigation of the antagonistic potential of PR modulators. Progesterone-independent, pure PR modulator-induced effects on non-genomic signaling pathways can not be assessed in these experimental settings. However, in the presence of hormones, PR modulators clearly exhibit antiproliferative effects [176].

In summary, the protein protein interaction studies indicate that various PR modulators can be differentiated by distinct cofactor recruitment profiles, although they can not be distinguished by cellular transactivation assays. Therefore, mammalian two-hybrid assays might be useful for the characterization of PR modulators in future drug development.

4.1.2 Unique gene expression profiles

The differences in cofactor recruitment profiles directly reflect on distinct gene expression profiles for the PR modulators analyzed. Global gene expression analyses were performed in T47D cells which serve as a very suitable in vitro model for PR ligand action. They express high levels of functional PR-B and -A under basal conditions which allows analysis of progestin action in the absence of estradiol. Furthermore, reference studies of other global PR ligand expression profiling studies in T47D cells have been published [155-158] and offered a thorough basis for the experimental design (Figure 9). All PR modulators, even the pure antagonist lonaprisan, exert individual progesterone-independent effects on T47D cell gene expression, although to different degrees (Table 14). As expected, the effect of R5020 on PR target gene expression is by far the strongest (1981 regulated genes). The non-steroidal PR modulator PRA-910 exhibits a very prominent influence on T47D cell gene expression (645 regulated genes) which is different from R5020 and also from PR antagonists and SPRMs. This supports previous reports from Bray et al. on a unique gene regulation profile of PRA-910 [156]. The decline in the transcriptional activity of asoprisnil (80 regulated genes), J1042 (70 regulated genes), J912 (30 regulated genes), mifepristone (25 regulated genes) and onapristone (3 regulated genes) seems to be correlated with increasing antagonistic properties in in vivo assays [115, 116]. In particular, the overall low transcriptional effect of onapristone-bound PR supports the previous theory that onapristone prevents PR binding to DNA and thus minimally influences PR target gene expression [126]. Interestingly, ZK 137316 and the pure and very potent PR antagonist lonaprisan were observed to regulate a relatively high number of genes with statistical significance (62 and 89 regulated genes, respectively). However, based on the results obtained, lonaprisan-bound PR predominantly down-regulates genes (82 %) compared to vehicle control. Since previous experiments demonstrated that lonaprisan-bound PR exhibits strong DNA-binding activity and strong corepressor recruitment, the inhibitory effects of lonaprisan-bound PR could be explained by preventing agonist-bound PR to bind to PREs. The PR antagonist ZK 137316 and the SPRM J912 also display strong down-regulation of genes in the expression analyses whereas mifepristone shows only a trend to down-regulation. The strongest agonistic activities on T47D cell gene expression as indicated by a predominant up-regulation of target genes were observed for PRA-910 (74% up-regulated) and, as expected, for the PR agonist R5020 (62% up-regulated). This is consistent with previous reports on a massive up-regulation of PR downstream genes by PR ligands with agonistic properties in T47D cells [157, 158]. Furthermore, the study results confirm progesterone target genes such as periplakin (PPL) [155], hydroxysteroid (11-beta) dehydrogenase 2 (HSD11B2) [158], transforming growth factor beta-stimulated protein TSC-22 (TSC22D1), HSP90-binding immunophilin (FKBP51) and Na+/K+ -ATPase alpha 1 subunit (ATP1A1) which have been described to be regulated by PR agonists [157].

In the principle component analysis (PCA) which projects all regulated probe sets on the Affymetrix GeneChips for each PR modulator on the three principle components of variability, unique gene expression modulation properties were identified for each PR modulator in the absence of R5020. Vehicle controls, PR antagonists (onapristone, mifepristone, ZK 137316, lonaprisan) and interestingly J912 exert the largest differences to R5020 samples and therefore to PR agonists in general (Figure 10A). The formation of subclusters furthermore refers to slight differences in the gene expression profiles induced by PR antagonists (Figure 10B). In particular, lonaprisan- and ZK 137316-treated samples cluster apart from the other antagonists. Moreover, J1042- and asoprisnil-treated samples significantly separate from all other PR modulators. PRA-910-treated samples cluster between SPRM- and R5020-treated samples, indicating a distinction from SPRMs and also from PR agonists [156]. Based on the results obtained from expression analyses of combined treatments with R5020, the majority of agonist-induced effects are counter-regulated by the respective PR modulator (Figure 13). However, the maximal counter-regulation is restricted to the individual effects which the respective PR modulator exhibits in the absence of R5020. Non-counter-regulated genes were identified to be either regulated by the PR modulator per se or to be regulated synergistically by R5020 and the PR modulator (Figure 14).

Lists of significantly regulated genes identified exclusive effects of each type (I-III) of PR antagonist as well as of asoprisnil and J1042. A specific effect of the type I antagonist onapristone is the down-regulation of the jumonji domain containing 2B (JMJD2B) gene, which was observed to be regulated in the opposite direction by the agonist R5020 (Table 17; Figure 15B). Like the Jumonji family members JMJD2A and JMJD2C, JMJD2B contains a JmjN domain. The protein encoded by this gene has been reported to be important for transcriptional control [160, 161]. Besides the putative function of JMJD2B in the regulation of PR target gene transcription, the mechanism of onapristone-mediated down-regulation of JMJD2B gene expression might be of interest. Genes which were identified to be specifically regulated by the type II antagonist mifepristone encode, amongst others, factors which are predominantly involved in β-adrenergic receptor signaling (Table 9). The β-adrenergic receptor-dependent regulation of cyclic-AMP (cAMP), a second messenger, and the enhanced partial agonistic properties of mifepristone, which have been described by Sartorius et al. [177] with regard to transcriptional activities in the presence of elevated intracellular cAMP levels in T47D cells, suggest a cross-talk of mifepristone-bound PR with β-adrenergic signaling. The molecular mechanism is not clear so far but R5020 and 8-bromo-cAMP have been demonstrated to induce transcription synergistically through independent pathways [177]. The type III antagonist lonaprisan exhibits specific effects which mostly result in an inhibition of gene expression (Table 16). An example for a gene which was identified to be down-regulated in the microarray analysis by lonaprisan, but not by onapristone or mifepristone, is the inhibin β subunit B (Inhbb). However, Inhbb was shown to be also regulated by ZK 137316 and J912 in subsequent real-time PCR experiments (Figure 15A). Therefore, the regulation of Inhbb might be predictive for specific common antagonistic properties of ZK 137316 and J912 which are most similar to lonaprisan in their global gene expression profiles (Figure 10, Table 14). Furthermore, modulation of Inhbb gene expression might be important for lonaprisans' mode of action in vivo as Inhbb has been linked to the modulation of pituitary FSH secretion. Other specific lonaprisan-regulated

genes, which were apparent from pathway analyses, are important for the control of cell cycle progression, in particular G1/S transition (Figure 16). In addition, based on 29 unique genes which are significantly changed in expression level by asoprisnil and J1042, but not by J912, mixed, classical or pure antagonists, novel stratification markers for SPRMs were identified (Figure 14; Table 15). The biological functions of the proteins encoded by the exclusively SPRM-regulated genes are diverse including the regulation of hormone activity, membrane associated signaling events and transcription. Thus, there is not one SPRM-regulated signaling pathway which differentiates SPRMs from PR antagonists, but rather a complex interaction of pathways discriminating SPRMs. Based on real-time PCR results, in particular the member 3 of subfamily J of potassium inwardly-rectifying channels (KCNJ3) is exclusively regulated by the SPRMs asoprisnil and J1042. J912 also displays moderate induction of KCNJ3 transcription which confirms its previous designation as a `weak` SPRM. In combination with other KCNJ subunits, KCNJ3 forms heterotetrameric potassium current channels which are activated via G protein-coupled receptors [178]. Selective potassium influx could represent a signaling pathway activated by PR ligands with special in vivo properties as exhibited by SPRMs. Further investigation of the underlying mechanism of KCNJ3-induction and the relevance on SPRMs function might be of interest.

The differences in gene expression profiles observed for various PR modulators suggest different induced receptor conformations and subsequently, different mode of actions. PR ligand-induced gene expression profiles included distinct expression levels of cyclin D1 and E2F1 which are important cell cycle regulators. The cell cycle can be divided in two periods: the mitotic (M) phase during which the cell splits into two cells and the interphase during which the cell grows and duplicates its DNA. The first phase within the interphase, the gap1 (G1) phase, is marked by synthesis of various enzymes which are required in S phase. The S phase starts when DNA synthesis commences, leading to a duplicate set of chromosomes. Thereafter, the production of proteins which are required during mitosis begins with the gap2 (G2) phase and lasts until the cell enters mitosis. The gap0 (G0) phase is a quiescent state and indicates non-proliferative cells. G0 phase is generally entered from G1 phase. Cyclin D1 functions as a regulatory subunit of the cyclin-dependent kinases CDK4 and CDK6 whose activity is required for cell cycle G1/S transition [179]. E2F1 is a transcription factor of the E2F family which has been reported to play a crucial role in hormonal regulation of the proliferative response of breast cancer cells [165] [180]. Based on the results obtained from real-time PCR analyses, PR agonists, SPRMs and various types of PR antagonists show profound differences in their efficacies to modulate cyclin D1 and E2F1 expression in combined treatments with R5020 and also themselves. The observed increase in cyclin D1 and E2F1 expression levels after R5020 treatment and the inhibition of PR agonist-induced expression by mifepristone are consistent with previous reports from Musgrove et al. and Maas et al. [162, 181]. Similar antagonistic effects on R5020 stimulating action were identified for lonaprisan (Figure 18). The SPRMs asoprisnil and J1042 do not inhibit the R5020-induced effects on cyclin D1 expression, but antagonize agonist-induced expression of E2F1. Gene- and ligand-specific properties were also obtained in expression analyses after single treatments. Lonaprisan significantly down-regulates cyclin D1 and E2F1 whereas the SPRMs asoprisnil and J1042 display significant induction of cyclin D1 gene transcription. Onapristone does not significantly regulate any of the genes examined.

Taken together, all compounds tested display unique gene expression profiles under standardized conditions in the absence as well as in the presence of PR agonists like R5020. In particular, the non-steroidal PR modulator PRA-910 as well as the SPRMs asoprisnil and J1042 differ from mixed and pure antagonists and also from J912. A gene expression fingerprint is likely suitable to identify SPRM-like as well as pure antagonistic activities in the development of novel PR modulators.

4.1.3 Inhibition of estradiol-induced cell cycle progression and cell proliferation

Consistent with different gene expression patterns of relevant cell cycle regulators, the PR modulators analyzed display different efficacies to modulate T47D cell cycle progression and cell proliferation. The effects of R5020 and PRA-910 on cell cycle progression are similar to those reported for estradiol, namely a reduced proportion of cells in G0/G1 phase and an increased proportion of cells in S phase [182] (Figure 19A, B). Furthermore, the proportion of cells in G2/M phase is strongly increased after R5020 and after PRA-910 treatment (Figure 19C). The SPRMs asoprisnil and J1042 display similar, partial agonistic effects, although to a minor degree when compared to R5020 and PRA-910 (Figure 19A-C). PR antagonists, in contrast, do not increase S and G2/M phases (Figure 19A-C). In particular, lonaprisan and ZK 137316 demonstrated antagonistic activity as they reduce S phase entry of cells. In combined treatments with estradiol, which were performed to investigate potential functional inhibitory effects on estradiol action, the strong PR antagonists inhibit the E2-induced increase in cells synthesizing DNA and passing G2/M phase whereas R5020, PRA-910 and SPRMs display either no or synergistical effects (Figure 19D-F).

However, proliferation assays demonstrated that all PR ligands inhibit E2-stimulated T47D cell proliferation in long-term exposure experiments (Figure 21). This biphasic regulation of cell cycle progression has been described previously and is supposed to be evoked by p21 expression and other cell cycle regulating factors [168, 183]. The p21 gene encodes a potent cyclin-dependent kinase (CDK) inhibitor which binds to and represses the activity of cyclin-CDK complexes [184, 185]. A rise in p21 protein levels has been described to start when cyclin protein levels are declining [183]. Concordant with this hypothesis, R5020 was observed to induce a transient increase of cyclin D1 transcription after 8 hours and an increase of p21 transcription after 24 hours in real-time PCR analyses (Figure 18A; Figure 20B, D). The pure antagonist lonaprisan also evokes an increase of p21 transcript levels after 24 hours of treatment (Figure 20B, D), but represses early transcription of cyclin D1 and E2F1 (Figure 18A, C). Concomitantly, lonaprisan causes a reduction of CDK6 expression. The activity of the protein encoded by the CDK6 gene has been described to appear first in mid-G1 phase and to promote cell cycle progression [186]. Synergism of reduced cyclin D1 and E2F1 transcript levels after short-term exposure as well as reduced CDK6 and increased p21 expression levels after long-term treatment might explain the overall inhibitory effects of lonaprisan demonstrated in cell cycle and proliferation analyses. However, reduced cyclin D1 gene expression in T47D cells after treatment with lonaprisan, ZK 137316 and J912 is to some extent contrary to their induction of cyclin D1 promotor activity which was observed in reportergene assays in U2OS cells (Figure 8). Furthermore, the previously described effects of mifepristone to decrease cyclin D1 expression, increase p21 expression, and subsequently

inhibit S phase entry [169] could not be reproduced in this study. This discrepancies might be caused by different treatment durations and distinct cellular backgrounds. Different pretreatments such as insulin-free, serum-free and growth factor-depleted conditions might influence the effects of hormones on cell cycle progression [164, 187-189]. Nevertheless, the effects of different PR antagonists were shown to be distinct under identical experimental conditions.

In summary, the antiproliferative potential was shown to be different for various types of PR modulators. In particular, the receptor conformation induced by lonaprisan strongly inhibits cell cycle progression and stimulatory effects of estradiol. These antiproliferative properties might be useful for the treatment of gynecological disorders.

4.1.4 Classification of PR modulators

The obtained in vitro cofactor interaction profiles and gene expression profiles demonstrated new molecular determinants which could refine previous PR modulator classification systems [115, 116], in particular progesterone-independent effects as well as inhibitory effects on estradiol action. The analyses confirm the existing systems to a large degree, but also show difficulties for a strict classification by emphasizing the unique properties of each PR modulator (Table 13).

Table 13. In vitro activities for the classification of PR modulators.

	Previous classification		DNA binding	In vitro transactivation (MMTV promoter)		Mammalian two-hybrid (LX-H10-interaction)	Gene expression in T47D cells (8 hours of treatment)			
	Character	Type		Standard	+ cAMP		KCNJ3	AMIGO2	PTHLH	Global (PCA cluster)
R5020	agonist	n.d.	+	+++	+++	+++	↔	↓↓↓	↓↓	A
PRA-910	partial agonist	n.d.	+	+	n.e.	+++	↔	↓↓↓	↓↓	B
Asoprisnil	SPRM	II	+	-	+	+++	↑↑↑	↓↓	↓↓	C
J1042	SPRM	II	+	-	+	+++	↑↑	↓↓	↓↓	C
J912	SPRM	II	+	-	+	+	↑	↓	↔	D
Mifepristone	antagonist	II	+	-	+	-	↔	↔	↔	D
ZK 137316	antagonist	II	+	-	n.e.	-	↔	↔	↔	E
Lonaprisan	antagonist	III	+++	-	-	-	↔	↔	↔	E
Onapristone	antagonist	I	-	-	-	-	↔	↔	↔	D

+++ = strong effect; + = effect; - = no effect; ↑ = up-regulated; ↓ = down-regulated; ↔ = no change; A - E = type of cluster in the PCA; PCA = principle component analysis; n.d. = not designated; n.e. = not evidenced

PRA-910 as a non-steroidal scaffold clearly separates from the other steroidal PR modulators in its identified in vitro properties. It induces no PR/cofactor interaction, neither with the coactivators nor with the corepressors tested, except the LX-H10 peptide. Furthermore, PRA-910 clearly demonstrates a distinct gene expression profile in T47D cells (Figure 10), confirming previously reported differences to steroidal agonists and antagonists [156]. The pronounced partial agonistic activity in transactivation assays (Figure 4B, D) as well as the correlation with R5020 effects in T47D cell cycle analyses (Figure

18A, C, Figure 19) are consistent with the reported agonistic activity in vitro [113], but somehow contradictory to its almost antagonistic activity in vivo [112, 114, 190].

The steroidal PR antagonist onapristone differs from the other PR modulators, in particular because of very low regulation of transcription (Table 14, Figure 11, Figure 12). The results from the global gene expression analysis confirm the previous hypothesis that onapristone prevents binding of the PR to DNA [125, 126]. The absence of active PR target gene regulation directly transfers into its observed inability to modulate PR-mediated expression of genes relevant for cell cycle control (Figure 18 – Figure 20). Moreover, onapristone-bound PR does not influence any of the E2-induced actions on T47D cell cycle progression, suggesting a mode of action that is different from the other types of PR antagonists. The reduced potency of onapristone-bound PR in transactivation assays and interaction studies with cytoplasmic factors like c-Src kinase (Figure 4C, Figure 7A) is most likely due to its weaker PR binding activity when compared to other PR modulators.

Lonaprisan represents a type of antagonist which inhibits PR target gene expression and hormone-dependent cell proliferation most efficaciously. The predominant down-regulation of genes in the global gene expression analysis (Figure 11) and the minimal activation of cytoplasmic non-genomic signaling pathways which was obtained from mammalian two-hybrid assays (Figure 7A, Figure 8A) might be caused by strong binding to PREs and accumulation of the lonaprisan-bound PR in the nucleus. In particular, the lonaprisan-induced expression pattern of cell cycle regulators might be responsible for its high antiproliferative effects. Lonaprisan clusters apart from other PR modulators in the global gene expression analyses (Figure 10). Highest similarities of lonaprisan and ZK 137316-induced gene expression profiles are to some extent contradictory to the previously described classification of ZK 137316 as type II antagonist [115] and rather suggests a classification of ZK 137316 as a type III antagonist.

The type II antagonist mifepristone differentiates from pure PR antagonists like lonaprisan and also from SPRMs in non-genomic interaction studies as well as gene expression analyses. It displays slight agonistic potential in vitro (Figure 7A, Figure 8A, Figure 10), which is consistent with its species- and tissue-specific partial agonistic activity which has been reported from in vivo analyses [122]. However, the degree of agonistic properties is much lower for mifepristone than for the SPRMs asoprisnil and J1042, especially in the gene expression profiling studies and in the cell cycle analyses (Figure 19A-C), indicating profound differences in the mode of action for these two classes of PR modulators.

The SPRMs asoprisnil and J1042 display high similarities. Cofactor interaction and gene expression analyses demonstrated significant differences from mixed, classical and pure antagonists, and also from J912. In particular, the protein protein interaction experiments with the LX-H10 peptide served as an initial indication that SPRMs exhibit properties which are similar to PR agonists (Figure 7). Moreover, J1042 and asoprisnil show the strongest overlap with agonist-induced gene expression profiles in T47D cells (Figure 12) and display agonist-like effects on T47D cell cycle progression, although to a minor degree than R5020 (Figure 19). The tissue-selective partial agonistic effects were shown to be profoundly distinct from the properties of the mixed antagonist mifepristone [115]. These differences are

consistent with reports on various agonistic potentials of mifpristone and SPRMs in vivo which have been reported by Elger et al. and Schubert et al. [116, 136]. J912 exhibits not only 'SPRM-like', but also 'pure antagonist-like' effects, in particular in the gene expression and cell cycle analyses (Figure 10, Figure 14, Figure 19). This refers to more antagonistic characteristics of J912 than displayed by asoprisnil and J1042 and is somehow contradictory to its previous classification as a `weak` SPRM [135]. However, the results confirm previous reports on ambivalent effects of J912 in vivo [115, 116]. This underlines the difficulties to strictly classify PR modulators like J912 as the classification of J912 depends on the cell- and species-specific activities surveyed (Table 13).

Taken together, the presented data approve distinct considerations of steroidal and non-steroidal PR modulators and support the concept of classifying steroidal PR modulators into pure agonists (R5020), selective progesterone receptor modulators (J1042 and asoprisnil), mixed antagonists (mifepristone), and pure antagonists (lonaprisan). However, the data also point to transition states between SPRMs and antagonists (e.g. J912), or mixed and pure antagonists (e.g. ZK 137316; Table 13). As described by Elger et al. [116], PR modulators seem to be capable to induce a continuum of differential PR signaling effects (Figure 44 A), but this model system should be refined by grades (Figure 44B) to consider the profound differences a PR modulator can display depending on the in vitro / in vivo assay surveyed.

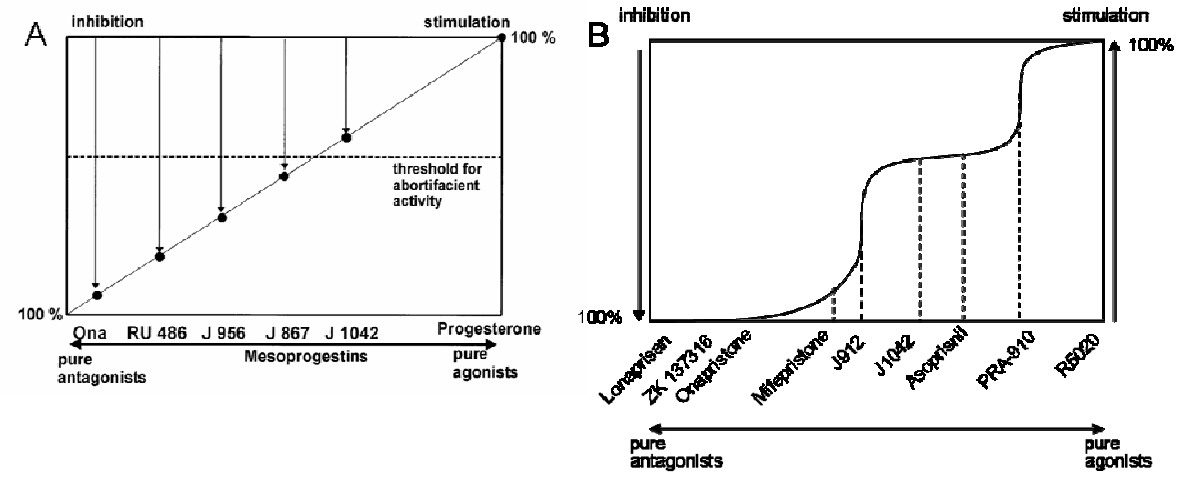

Figure 44. Continuum of PR ligands. The line schematically describes the maximum possible activation or inhibition of PR, respectively, at the level of the in vitro / in vivo assay surveyed. (A) Model system from Elger et al. 2000 [116]. (B) New model system based on cofactor interaction and gene expression data.

4.2. Elucidation of PR modulator effects in a uterine leiomyoma model system

4.2.1 The ELT-3 cell model system for uterine leiomyomas

The Eker rat leiomyoma tumor-derived cell line ELT-3 was confirmed to provide a suitable model system for the exploration of the role of steroid hormones and hormone receptors in myometrial tumorigenesis and subsequently, the characterization of PR modulator effects under disease relevant conditions. Consistent with previous reports on ELT-3 cell characteristics [48, 191, 192], these cells showed uterine leiomyoma characteristics which correlate well with the human condition, in particular smooth muscle cell marker and steroid hormone receptor (estrogen receptor alpha and progesterone receptor) expression as well as responsiveness to ovarian hormones (Figure 22, Figure 23). The latter highlights their suitability compared to immortalized human uterine leiomyoma cells and myometrial cells as the responsiveness to ovarian hormones in these model systems is not characterized or even not retained [193-195].

ELT-3 cells have previously been reported to induce tumors when grafted into nude mice [48]. The xenograft experiments, which were performed in this thesis, identified the suitability of severe combined immunodeficient (SCID) mice for ELT-3 cell transplantation. One advantage of SCID mice over nude mice in heterologous xenograft analyses is that they have no residual B cell function. The complete lack of humoral immunity in SCID mice might be responsible for the previously described differences in the establishment and growth of other tumors types grafted in SCID and nude mice [196, 197]. The observed tumor latency for the ELT-3 cell-induced xenograft model of about six weeks in intact SCID mice is not significantly different from the tumor latency of about two to ten weeks which has been reported in intact nude mice. However, based on the results obtained, latencies are more consistent in the SCID mouse model and a tumor incidence of 100% was achieved compared to an incidence of 50% observed in nude mice [48]. The rigidity and the low peripheral vascular system identified in subcutaneously formed tumors in SCID mice (Figure 26A) correlates with prominent collagenous extracellular matrix (ECM) and formations of a 'vascular capsule' which have been described in the corresponding human disease [198, 199].

Previous reports on hormone-responsive proliferation of ELT-3 cells, in particular in response to estradiol [192], were confirmed in in vitro cell viability assays and in in vivo xenografts. Although there is little known about the response of human leiomyoma cell lines to estradiol in vitro, the obtained 2.3-fold increase in the proliferation rate of ELT-3 cells is similar to the about 2-fold increase which has been reported in the estrogen-responsive MCF-7 breast carcinoma cell line and the PEO4 ovarian adenocarcinoma cell line [200, 201]. Subsequent apoptosis assays identified a dose-dependent decline in caspase 3/7 activity (Figure 24B) which supports previous observations that estradiol contributes to the survival of leiomyoma cells through down-regulation of anti-apoptotic proteins [202-204]. Based on the results obtained from the established xenograft model in SCID mice, the absolute concentration of estradiol modulates the growth rate of tumors which already exist, but do not influence the latency of tumor development (Figure 26B). However, prolonged latencies and reduced tumor incidences in the

experiments with ovariectomized, non-estradiol supplemented mice suggest that the overall absence of estradiol decreases the likelihood of tumor formation. Tumors induced by ELT-3 cells which were inoculated in a mixture of medium and MatriGel establish and grow independently of the estradiol level (Figure 26C). MatriGel is known to provide a complex extracellular environment which contains structural proteins such as collagen and laminin, growth factors and numerous other proteins. Signaling pathways induced by high levels of exogenous growth promoting factors might substitute the hormone responsive signaling pathways. For example, a crucial role of local growth factors in regulating uterine leiomyoma growth has been demonstrated for epidermal growth factor (EGF) [205-207]. Endogenous secretion of autocrine/paracrine growth factors like EGF might furthermore explain the decreased, but consistent growth of established tumors, even in the absence of estradiol (Figure 27).

The previously reported ability of estrogen receptor (ER) modulators to antagonize the effects of estradiol on ELT-3 cell proliferation and tumor growth [31, 49, 192, 208] was confirmed by the results obtained from analyses after ZK 191703 and 4-hydroxytamoxifen (4-OHT) treatment. In particular, pure antagonistic activities of the ER destabilizer ZK 191703 were demonstrated (Figure 25B, Figure 27). 4-hydroxytamoxifen antagonizes the estradiol-induced proliferation of ELT-3 cells, but acts as a weak partial agonist in the absence of estradiol (Figure 25A). This is consistent with its agonistic activity which has been described in other uterine compartments such as the endometrium [209, 210]. However, the results are somehow contradictory to previous reports on pure antagonistic properties of 4-OHT in ELT-3 cells [192]. Discrepancies in the responsiveness to ER modulators like 4-OHT might be the result of other experimental conditions such as medium composition.

In an approach to establish a new system for the confirmation of genes which might be involved in ELT-3 cell proliferation, the ability of a replication-deficient lentiviral vector to efficiently infect rat leiomyoma cells was demonstrated. Decreased expression levels of the estrogen receptor alpha (Esr1), which was chosen as the first tool target gene, were obtained after lentiviral-mediated short hairpin (sh) RNA transduction (Figure 28A). The subsequent decline in estradiol-responsive cell proliferation and tumor growth as well as the reduced ability of respective ELT-3 cells to form tumors in SCID mice (Figure 29A, B) confirm the functional consequences of Esr1 depletion. The obtained results are furthermore consistent with previous reports on adenovirus-mediated expression of truncated estrogen receptor constructs (Esr1-536) in benign rat pituitary prolactinoma cells which have been described to exhibit decreased cell proliferation and reduced tumor growth in nude mice [211]. However, the observed inhibitory effect of Esr1 silencing on proliferation might partially be attributed to the concomitant lack of PR (Figure 28B, C) which is expressed downstream of Esr1 in ELT-3 cells (Figure 23B, C).

In summary, the ELT-3 cell-based model system for uterine leiomyomas reconstitutes properties which have been described for human leiomyomas. Thus, it provides a suitable in vitro / in vivo model system for the analyses of potential treatment approaches. Furthermore, the lentiviral delivery of short hairpin RNA offers an option for silencing of genes which might be involved in leiomyoma cell proliferation. It can be used for the confirmation of identified steroid hormone receptor downstream genes.

4.2.2 Hormone-regulated downstream genes in leiomyoma-derived cells

Estradiol and progesterone both have been shown to be major mediators of myometrial and leiomyoma cell proliferation. However, the most conclusive evidence comes from clinical observations, making a distinction of the relative importance of estradiol versus progesterone difficult. Based on the results obtained from gene expression analyses in ELT-3 cells, neither a pure estradiol [11-13] nor a pure progesterone hypothesis [16-18, 20] seems to be sufficient. The influence of estradiol on ELT-3 cell gene expression is much more prominent than the influence of R5020 in single treatments. However, the effects on gene expression are strongest when ELT-3 cells were treated with a combination of estradiol and R5020 (Figure 33, Figure 34), suggesting additive or synergistic effects of the two steroid hormone receptors. The increase in regulated genes seems to be mainly attributable to the transcriptional activity of PR, because R5020-regulated genes are proportionally higher represented in combined treatments (81%) than genes regulated by estradiol (33%). Full PR signaling activation in the presence of estradiol might be caused by the observed and previously reported estradiol-dependent induction of PR expression (Figure 23B, C) [12, 212]. Consistent with this concept, profile distance search analyses identified that the expression profiles of a large number of genes are significantly correlated or anti-correlated with progesterone receptor expression levels (Table 19). In particular, the correlation of genes which encode key regulators in hormonal response (e.g. inhibin β subunit B), cell proliferation (e.g. E2f5) and differentiation (e.g. Tsc22d1) confirm a large influence of PR action on proliferative processes in rat leiomyoma cells.

Besides synergistic activities in combined treatments, the gene expression analyses demonstrated transdominant suppression of ER transcriptional activity for the R5020-bound PR in a gene-specific manner (Figure 39, Figure 40). Repression of ER-mediated transcription by PR ligands has previously been reported in rat leiomyoma cells [191], and also in breast cancer and endometrial cells [213-215]. The hypothesis that PR agonists and antagonists repress ER transcriptional activity by different mechanisms [214, 216] is furthermore confirmed by the predominantly converse regulation of estradiol-induced genes by R5020 and lonaprisan (Figure 39, Figure 40).

Pathway analyses demonstrated that R5020-mediated regulation of specific estradiol-induced genes directly reflects on the modulation of estradiol-influenced pathways. In particular, the estrogenic effects on ERBB, epidermal growth factor (EGF) and insulin-like growth factor (IGF) signaling are suppressed by elevated PR signaling in combined treatments. A modulation of EGF and IGF-I signaling pathways by estradiol and progesterone has previously been described in cultured human leiomyoma cells [172]. Interestingly, these pathways have also been reported to be modulated in response to TGFβ-treatment in leiomyoma and myometrial smooth muscle (LSMC and MSMC) cells [217]. The TGFβ superfamily is of particular interest with regard to uterine leiomyomas, because some members (TGFβ1-3) are not only capable to promote mitogenesis, but also stimulate the synthesis of many extracelluar matrix components leading to fibrosis [218, 219]. The identified coherence of downstream pathways and the reported amplification of leiomyoma cell proliferation after cotreatment with estradiol and TGFβ [220] suggests synergistic effects for estradiol and TGFβ signaling. Growth factor signaling therefore plays an important role in the consideration of hormone-responsive ELT-3 cell proliferation.

Cell viability assays and in vivo xenograft analyes demonstrated that ligand-induced PR signaling evokes inhibitory effects on estradiol-stimulated proliferation in ELT-3 cells. In particular, the presence of the pure PR antagonist lonaprisan causes strong antiproliferative effects (Figure 30, Figure 31). The highly antagonistic properties of lonaprisan in the in vitro / in vivo ELT-3 cell model support its classification as type III antagonist (see 4.1) in a disease relevant system. Moreover, the observed antiproliferative potentials of PR modulators generally confirm previous reports on their efficacy in clinical trials [33, 34]. Slightly stimulatory effects of R5020, mifepristone and asoprisnil in the absence of estradiol were only observed in non-physiological high concentrations. Nevertheless, these agonistic activities differentiate PR agonists, SPRMs and mixed antagonists from the pure antagonist lonaprisan in the uterine leiomyoma model system.

Common inhibitory effects of lonaprisan and the ER antagonist ZK 191703 on estradiol-induced gene transcription offer a suitable possibility to elucidate potentially proliferative ER-downstream pathways in ELT-3 cells. Among the diverse common counter-regulated genes which were identified by intersection anaylses (Figure 38, Table 19), the modulation of transforming growth factor α (TGFα) (Figure 39A) seemed to be most plausible to affect leiomyoma growth. TGFα expression has been linked to proliferation previously [172]. The mature TGFα peptide shares approximately 30% sequence homology with EGF and has been reported to induce similar biological effects by activating the tyrosine kinase component of the EGF receptor [221]. Based on gene expression silencing experiments, TGFα expression levels were confirmed to influence estradiol-dependent proliferation of leiomyoma-derived cells (Figure 42B). This is consistent with the described theory that the mitotic effects of sex steroids are partly mediated by the local production of peptide growth factors such as TGFα and EGF [222-226]. In addition, previous studies have demonstrated that the expression of TGFα is up-regulated by EGF or TGFα itself [221, 227, 228], suggesting TGFα to be under positive auto-regulatory control. Such an amplification mechanism for TGFα triggered by estradiol has been proposed for different tissues such as skin and breast epithelial cells [228, 229] and could be applicable for uterine leiomyomas, too. Disruption of this feed-forward cycle might be an attractive approach for the inhibition of cell proliferation.

In addition, analyses of gene transcripts which were identified to be divergently counter-regulated by ZK 191703 and lonaprisan promise to be appropriate for the investigation of differences between ER and PR antagonist-mediated antiproliferative mechanisms. The converse regulation of the inhibin β subunit B (Inhbb) seemed to constitute the most conclusive biological relevance. The inhibin β subunit B (Inhbb) forms homodimers (activin B) and heterodimers with the β subunit A (activin AB) or the distantly related polypeptide chain inhibin α (inhibin B). Inhibins and activins belong to the TGFβ superfamily of growth factors [219] and have been linked to pituitary FSH secretion [159, 173, 174]. Because the significant reduction of Inhbb transcript levels in the performed gene silencing experiments (Figure 43A) was shown to result in increased hormone-independent proliferation of ELT-3 cells (Figure 43B), reports on the growth-retarding effects of activin A in endometrial cancer [230] and myometrial cells [231] might be transferable to activins and inhibins which consist of the Inhbb subunit. Moreover, interactions of activin, ER and PR signaling in progesterone target tissues have been reported

previously [230, 232, 233]. The estradiol-induced Inhbb expression, which was observed in the gene expression analyses, might initiate a feedback that maintains the cells in an estradiol-responsive and hormone-sensitized state.

TGFα and Inhbb signaling pathways are importantly influenced by the PR antagonist lonaprisan. The real-time PCR analyses demonstrated that lonaprisan treatment suppresses the estradiol-induced expression of the proliferative factor TGFα and enhances the stimulatory estradiol effect on expression of the antiproliferative factor Inhbb. In gene silencing experiments, TGFα and Inhbb expression influence ELT-3 cell proliferation only partially when considered separately. However, simultaneous interference with both pathways likely provides a basis for the inhibitory effect of lonaprisan which represents a potent PR antagonist with antiproliferative properties in the ELT-3 cell model system for uterine leiomyomas.

In summary, the gene expression and gene silencing experiments in ELT-3 cells indicate a complex interplay between estradiol, progesterone, TGFα and TGFβ signaling which likely modulates ELT-3 cell proliferation. A change in the expression levels of the transforming growth factor family members TGFα and Inhbb significantly influences ELT-3 cell proliferation. The strong antiproliferative effect of the PR antagonist lonaprisan is at least partially elicited by the regulation of these pathways.

To summarize the obtained results from mechanistic and functional analyses of PR modulators:

Profound differences were observed between the in vitro activities of agonists, SPRMs, mixed and pure antagonists, especially in their global gene expression profiles in T47D cells. The progesterone-independent modulation of gene expression varies and is unique for each PR modulator. A gene expression fingerprint is likely suitable to identify SPRM-like as well as pure antagonistic activities. The observed classification differences directly translate into distinct functional effects in cell cycle progression and proliferation analyses, in particuar with regard to inhibitory effects on estradiol action. Lonaprisan displays the highest antiproliferative activity in T47D cells as well as in the novel established Eker rat tumor-derived (ELT-3) cell-based in vitro / in vivo model system for uterine leiomyomas. The ELT-3 model system reveals important properties of human leiomyomas. Gene expression profiling identified downstream genes of hormone and antihormone action. In particular, transforming growth factor (TGF) α and inhibin β subunit B (Inhbb) were demonstrated to influence cell proliferation using the novel established combination of lentiviral-mediated gene silencing and the previously described ELT-3 cell line. The lonaprisan-induced direction of TGFα and Inhbb gene expression might explain its highly antiproliferative effects in leiomyoma-derived cells.

5. Conclusions

Protein protein interaction assays and global gene expression profiling studies demonstrated profound differences between PR agonists, SPRMs, mixed and pure PR antagonists. The identified in vitro properties refer to distinct progesterone-independent gene expression inhibition by different types of antagonists. SPRMs clearly demonstrate agonistic behaviour in gene expression profiling and protein protein interaction. The non-steroidal partial agonist PRA-910 has a very strong effect on gene expression and displays highest similarity to the agonist R5020. Taken together, the progesterone-independent effects on gene expression vary and are unique for each PR modulator. Importantly, these differences were shown to directly translate into distinct antiproliferative effects in cell cycle progression analyses. Furthermore, PR modulators differ in their inhibitory effects on stimulatory estradiol action. PR modulators with the strongest antiproliferative effects in the established in vitro / in vivo system for uterine leiomyomas promise to be the most active type for the treatment of these tumors.

The ELT-3 cell model reveals important characteristics of the corresponding human disease. The newly established combination of ELT-3 cells and a lentiviral delivery of gene silencing provides a suitable system for the investigation of basic mechanisms of uterine leiomyoma growth. As demonstrated in the global gene expression profiling study, the combination of estradiol and progesterone evokes the strongest influence on hormone downstream signaling cascades. This suggests that both steroid hormones act synergistically in the etiology and pathogenesis of the tumors. Future analyses of downstream genes using the established lentiviral-mediated gene silencing system in ELT-3 cells can provide the basis for a more detailed analysis of the mechanisms of uterine leiomyoma development and growth.

Finally, the combination of gene expression profiling and functional gene silencing analyses in ELT-3 cells demonstrated that the antiproliferative effect of the strong PR antagonist lonaprisan is at least partially elicited by the regulation of TGF signaling.

In summary, the analyses which were performed on a relevant selection of PR modulators in mechanistic and functional model systems further elucidate the mode of PR modulator action as well as the mechanism of hormonal uterine leiomyoma growth modulation. In particular, the results obtained from analyses of PR modulators with high antiprolferative effects could reinforce drug development for this gynecological indication.

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Appendix

A1. Tables of gene expression data

Table 14. PR modulator-regulated genes assessed by Affymetrix GeneChip analysis in T47D cells.

DMSO vs.	R5020	PRA-910	Asoprisnil	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
R5020	**1981**	593	62	53	20	12	1	34	44
PRA-910	1388 / 52	**645**	37	37	7	4	1	18	27
Asoprisnil	1919 / 18	608 / 43	**80**	38	14	9	0	17	21
J1042	1928 / 17	608 / 33	42 / 32	**70**	6	8	0	7	11
J912	1961 / 10	638 / 23	66 / 16	64 / 24	**30**	11	0	23	23
Mifepristone	1969 / 13	641 / 21	71 / 16	62 / 17	19 / 14	**25**	0	16	15
Onapristone	1979 / 2	644 / 2	80 / 3	70 / 3	30 / 3	25 / 3	**3**	0	0
ZK 137316	1947 / 28	627 / 44	63 / 45	63 / 55	7 / 39	9 / 46	3 / 62	**62**	48
Lonaprisan	1937 / 45	618 / 62	59 / 68	59 / 78	7 / 66	10 / 71	3 / 89	14 / 41	**89**

Cells were treated with vehicle or 10 nM of PR ligand (100 nM PRA-910) for 8 hours. The entire number of significantly regulated genes (treatment vs. vehicle) is presented in the diagonal (e.g. 30 genes regulated by J912). Genes identified in corresponding sets of column and row are shown above the diagonal (e.g. 11 J912-regulated genes were also observed in mifepristone-treated cells). Genes exclusively identified in one of the two sets (column / row) are presented below the diagonal (e.g. 19 J912-regulated genes were not identified in mifepristone-treated cells, 14 mifepristone-regulated genes were not observed in J912-treated cells). Cells were analyzed in quintuplicate (tenfold for vehicle control), time-separated experiments. Statistical significance was assessed by pairwise comparisons of treatment versus vehicle (FC > 2, Volcano: FC > 2.5 - 5 and p-Value analogous to ST Q < 0.01 from T-test).

Table 15. Categorical list of genes significantly regulated by J867 and J1042, but not by J912 and antagonists.

Identifier	Gene Name	Gene Symbol	Gene ID	Fold change								
				R5020	PRA-910	Asoprisnil	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
Hormone activity												
230746_s_at	stanniocalcin 1	STC1	6781	-3,4	n.c.	-3,4	-2,4	n.c.	n.c.	n.c.	n.c.	n.c.
211756_at	parathyroid hormone-like hormone	*PTHLH*	5744	n.c.	-4,4	-3,2	-2,8	n.c.	n.c.	n.c.	n.c.	n.c.
Membrane effects												
Cell adhesion / cytoskeletal interactions												
208353_x_at	ankyrin 1	ANK1	286	n.c.	n.c.	2,2	2,1	n.c.	n.c.	n.c.	n.c.	n.c.
222108_at	adhesion molecule with Ig-like domain 2	*AMIGO2*	347902	-31,8	-9,9	-2,9	-2,6	n.c.	n.c.	n.c.	n.c.	n.c.
Protein transporter												
213413_at	stoned B-like factor	STON1	11037	-8,7	-3,0	-2,1	-2,1	n.c.	n.c.	n.c.	n.c.	n.c.
Ion channels												
233059_at	potassium inwardly-rectifying channel, subfamily J, member 3	*KCNJ3*	3760	n.d.	n.d.	4,7	5,1	n.d.	n.d.	n.d.	n.d.	n.d.
Membrane organization												
214255_at	ATPase, Class V, type 10A	ATP10A	57194	11,5	6,0	2,6	2,5	n.c.	n.c.	n.c.	n.c.	n.c.
227834_at	taxilin beta	TXLNB	167838	-4,5	n.c.	-2,5	-2,3	n.c.	n.c.	n.c.	n.c.	n.c.
Signal transduction from membrane												
221245_s_at	frizzled homolog 5 (Drosophila)	FZD5	7855	3,3	2,9	2,3	2,3	n.c.	n.c.	n.c.	n.c.	n.c.
214724_at	DIX domain containing 1	DIXDC1	85458	2,7	n.c.	2,3	2,2	n.c.	n.c.	-1,2	n.c.	n.c.
203108_at	G protein-coupled receptor, family C, group 5, member A	*GPRC5A*	9052	n.c.	n.c.	2,2	2,3	n.c.	n.c.	-1,5	n.c.	n.c.
215306_at	luteinizing hormone/choriogonadotropin receptor	LHCGR	3973	-7,4	-4,5	-2,2	-2,7	n.c.	n.c.	n.c.	n.c.	n.c.
231120_x_at	protein kinase (cAMP-dependent, catalytic) inhibitor beta	PKIB	5570	-5,7	-3,0	-2,4	-2,2	n.c.	n.c.	n.c.	n.c.	n.c.
223843_at	scavenger receptor class A, member 3	SCARA3	51435	-5,1	-2,1	-2,1	-2,2	n.c.	n.c.	n.c.	n.c.	n.c.
Ca^{2+} binding proteins												
1553392_at	EF-hand calcium binding domain 3	EFCAB3	146779	4,7	3,9	2,5	2,1	n.c.	n.c.	-1,5	n.c.	n.c.
Nucleic acid and protein processing												
DNA replication/transciption/translation												
206045_s_at	nucleolar protein 4	NOL4	8715	-10,3	-6,4	-2,2	-2,6	n.c.	n.c.	n.c.	n.c.	n.c.
Chaperones/protein folding												
203810_at	DnaJ (Hsp40) homolog, subfamily B, member 4	DNAJB4	11080	4,4	1,7	2,4	2,1	n.c.	n.c.	n.c.	n.c.	n.c.
Transcription factors												
228854_at	zinc finger and BTB domain containing 16	*ZBTB16*	7704	119,9	108,8	7,1	5,9	n.d.	n.d.	n.d.	n.d.	n.d.
213293_s_at	tripartite motif-containing 22	TRIM22	10346	62,9	21,5	4,5	3,4	n.d.	n.d.	n.d.	n.d.	n.d.
209211_at	kruppel-like factor 5 (intestinal)	KLF5	688	2,7	2,3	2,7	2,4	n.c.	n.c.	n.c.	n.c.	n.c.
229228_at	cAMP responsive element binding protein 5	CREB5	9586	-4,4	-2,8	-2,8	-2,0	n.c.	n.c.	n.c.	n.c.	n.c.
Cell cycle												
41644_at	SAM and SH3 domain containing 1	SASH1	23328	3,7	4,5	2,0	2,3	n.c.	n.c.	n.c.	n.c.	n.c.
Metabolism												
227361_at	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	HS3ST3B1	9953	5,7	4,3	2,6	2,2	n.c.	n.c.	n.c.	n.c.	n.c.
Defense response												
243311_at	defensin, beta 32	*DEFB32*	400830	6,9	4,1	6,6	3,6	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown function												
226614_s_at	chromosome 8 open reading frame 13	C8orf13	83648	n.c.	-2,0	2,1	2,0	n.c.	n.c.	n.c.	n.c.	n.c.

Genes were organized by primary function according to Gene Ontology (GO). Statistical significance was assessed by pairwise comparisons of treatment versus vehicle (FC > 2, Volcano: FC > 2.5 - 5 and p-Value analogous to ST Q < 0.01 from T-test). Italic genes were validated independently by TaqMan® quantitative real-time PCR. n.c. = no change (FC < 2.0), n.d. = not detected.

Table 16. Categorical list of genes significantly regulated by lonaprisan, but not by onapristone and mifepristone.

Identifier	Gene Name	Gene Symbol	Gene ID	Fold change								
				R5020	PRA-910	Asoprisnil	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
Hormone activity												
205258_at	inhibin, beta B (activin AB beta polypeptide)	*INHBB*	3625	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0	-2,1
Membrane effects												
Cell adhesion / cytoskeletal interactions												
230559_x_at	FYVE, RhoGEF and PH domain containing 4	FGD4	121512	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	2,2	2,2
225685_at	CDC42 effector protein (Rho GTPase binding) 3	CDC42EP3	10602	2,3	2,4	n.c.	n.c.	-2,1	-2,0	n.c.	-2,4	-2,3
205534_at	BH-protocadherin 7 (brain-heart)	PCDH7	5099	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,2	-2,2
214598_at	claudin 8	CLDN8	9073	2,1	2,4	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0	-2,7
G-protein coupled receptor protein signaling												
218326_s_at	leucine-rich repeat-containing G protein-coupled receptor 4	LGR4	55366	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	2,2	2,2
203586_s_at	ADP-ribosylation factor 4-like	ARL4D	379	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0	-2,0
Signal transduction from membrane												
1558695_at	Pleckstrin homology domain containing, family A, member 5	PLEKHA5	54477	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	2,3
219155_at	phosphatidylinositol transfer protein, cytoplasmic 1	PITPNC1	26207	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	2,0	2,1
226034_at	Dual specificity phosphatase 4	DUSP4	1846	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,6
Ion transport												
205542_at	six transmembrane epithelial antigen of the prostate 1	STEAP1	26872	4,2	3,2	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0	-2,1
Endothelial marker												
238455_at	plexin domain containing 2	PLXDC2	84898	n.c.	n.c.	-2,5	-2,5	n.c.	n.c.	n.c.	n.c.	-2,1
Endocytosis												
226992_at	nitric oxide synthase trafficker	NOSTRIN	115677	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,2
Junctional complexes												
229578_at	junctophilin 2	JPH2	57158	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,2
Cell proliferation												
203469_s_at	cyclin-dependent kinase (CDC2-like) 10	CDK10	8558	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0	-3,0
Apoptosis												
211367_s_at	caspase 1, apoptosis-related cysteine peptidase	CASP1	834	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,1	-2,4
218856_at	tumor necrosis factor receptor superfamily, member 21	TNFRSF21	27242	3,0	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0
Nucleic acid and protein processing												
Chromatin structure												
205967_at	histone cluster 1, H4c	HIST1H4C	8364	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,3
Transcription												
230636_s_at	Kruppel-like factor 9	KLF9	687	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	2,0
227210_at	Scm-like with four mbt domains 2	SFMBT2	57713	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,3	-2,3
Protein modification												
238505_at	ADP-ribosylarginine hydrolase	ADPRH	141	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	2,1
Catabolic / metabolic processes												
211138_s_at	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	KMO	8564	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,5	-3,0
230892_at	2-deoxyribose-5-phosphate aldolase homolog (C. elegans)	DERA	51071	n.c.	n.c.	n.c.	n.c.	-2,0	n.c.	n.c.	n.c.	-2,2
1553434_at	cytochrome P450, family 4, subfamily Z, polypeptide 2 pseudogene	CYP4Z2P	163720	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,1	-2,1
Multicellular organismal development												
201324_at	epithelial membrane protein 1	EMP1	2012	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,2	-2,2
1559661_at	odz, odd Oz/ten-m homolog 3 (Drosophila)	ODZ3	55714	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,1
228184_at	dispatched homolog 1 (Drosophila)	DISP1	84976	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0
Transport activity												
1553147_at	RAN binding protein 3-like	RANBP3L	202151	3,1	3,7	2,1	n.c.	n.c.	n.c.	n.c.	-3,1	-3,0
219229_at	solute carrier organic anion transporter family, member 3A1	SLCO3A1	28232	n.c.	-3,2	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	-2,6
Binding activity												
213629_x_at	metallothionein 1F	MT1F	4494	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	2,2	2,6
220622_at	leucine rich repeat containing 31	LRRC31	79782	3,2	3,6	n.c.	n.c.	n.c.	n.c.	n.c.	-2,0	-2,6

Table 17. Categorical list of genes significantly regulated by onapristone, but not by mifepristone and lonaprisan.

Identifier	Gene Name	Gene Symbol	Gene ID	Fold change								
				R5020	PRA-910	Asoprisnil	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
Nucleic acid processing												
212492_s_at	jumonji domain containing 2B	*JMJD2B*	23030	2,8	2,7	n.c.	n.c.	n.c.	n.c.	-2,1	n.c.	n.c.

Table 18. Categorical list of genes significantly regulated by mifepristone, but not by onapristone and lonaprisan.

Identifier	Gene Name	Gene Symbol	Gene ID	Fold change								
				R5020	PRA-910	Asoprisnil	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
Angiogenesis												
205608_s_at	angiopoietin 1	ANGPT1	284	n.c.	n.c.	n.c.	n.c.	-2,0	-2,2	n.c.	-2,1	n.c.
Protein processing												
226553_at	transmembrane protease, serine 2	TMPRSS2	7113	n.d.	n.c.	n.c.	2,3	n.c.	2,6	n.c.	2,0	n.c.
215898_at	tubulin tyrosine ligase-like family, member 5	TTLL5	23093	n.d.	n.c.	n.d.	n.c.	n.c.	2,5	n.d.	n.c.	n.d.
Unknown function												
228865_at	chromosome 1 open reading frame 116	C1orf116	79098	n.d.	n.c.	4,1	4,2	n.c.	2,4	n.d.	n.c.	n.c.
223019_at	family with sequence similarity 129, member B	FAM129B	64855	n.c.	n.c.	n.c.	2,0	n.c.	2,0	n.c.	n.c.	n.c.

Table 16-18: Genes were organized by primary function according to Gene Ontology (GO). Statistical significance was assessed by pairwise comparisons of treatment versus vehicle control (FC > 2, Volcano: FC > 2.5 - 5 and p-Value analogous to ST Q < 0.01 from T-test). Italic genes were validated independently by TaqMan® quantitative real-time PCR. n.c. = no change (FC < 2.0).

Table 19. Top list of genes which expression profiles correlated with PR gene expression profiles.

Identifier	Gene Name	Gene Symbol	Gene ID	Fold change								Correlation
				E2	R5020	ZK 191703	Lonaprisan	E2 + R5020	E2 + ZK 191703	E2 + Lonaprisan	R5020 + Lonaprisan	
Reference profile												
1387563_at	progesterone receptor	Pgr	25154	4,8	-1,2	-1,7	n.c.	2,0	1,5	4,1	n.c.	1,0
Correlated expression profiles												
1369670_at	Cd200 antigen	Cd200	24560	6,7	-2,1	-3,1	n.c.	3,4	2,0	1,6	-1,3	0,9
1369794_a_at	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Pfkfb3	117276	3,6	-1,5	-1,9	n.c.	2,2	n.c.	5,7	n.c.	0,8
1398300_at	ATPase, Na+/K+ transporting, beta 3 polypeptide	Atp1b3	25390	1,3	n.c.	-1,3	n.c.	n.c.	n.c.	1,2	n.c.	0,8
1368412_a_at	protein tyrosine phosphatase, receptor type, O	Ptpro	50677	2,7	n.c.	-1,2	n.c.	1,8	n.c.	2,4	n.c.	0,8
1379356_at	Similar to RIKEN cDNA C230093N12 (predicted)	RGD1310037	365903	1,7	n.c.	n.c.	n.c.	n.c.	n.c.	2,0	n.c.	0,8
1388154_at	E2F transcription factor 5	E2f5	116651	3,2	n.c.	-1,2	n.c.	2,4	1,5	1,7	n.c.	0,8
1377163_at	inhibin beta-B	Inhbb	25196	6,5	1,2	n.c.	n.c.	n.c.	1,6	7,0	n.c.	0,8
1382452_at	serum deprivation response protein	Sdpr	316384	2,2	-1,2	-1,3	-1,2	1,2	n.c.	2,2	-1,2	0,8
Anti-correlated expression profiles												
1398759_at	transforming growth factor beta 1 induced transcript 4	Tsc22d1	498545	-1,6	n.c.	n.c.	n.c.	-1,2	n.c.	-1,3	n.c.	-0,8

Genes with top correlation coefficients > + 0.8 or < -0.8 in profile distance search with PR expression profile.

Table 20. Categorical list of E2-induced genes significantly counter-regulated by ZK 191703 and lonaprisan.

Identifier	Gene Name	Gene Symbol	Gene ID	Fold change						
				E2	R5020	ZK 191703	Lonaprisan	E2 + R5020	E2 + ZK 191703	E2 + Lonaprisan
Hormone activity										
Steroid hormone receptor signaling										
1380781_at	progesterone receptor	Pgr	25154	5,7	n.c.	-1,7	1,3	2,6	1,5	4,4
Prolactin signaling										
1370471_at	prolactin-like protein B	Prl6a1	24657	3,2	n.c.	-1,3	n.c.	-1,4	n.c.	n.c.
Oxidoreductase activity										
1368102_at	hydroxysteroid 11-beta dehydrogenase 2	Hsd11b2	25117	2,9	3,6	1,3	-1,4	6,7	1,6	-1,4
Membrane effects										
Cell adhesion / cytoskeletal interaction										
1379340_at	laminin, gamma 2	Lamc2	192362	4,1	1,5	n.c.	n.c.	3,4	1,2	2,1
1372658_at	desmuslin	*Dmn*	308709	4,7	n.c.	-1,3	-1,3	3,3	n.c.	1,2
1391187_at	Periplakin (predicted)	*Ppl*	302934	2,6	1,4	0,9	0,9	3,9	0,9	0,7
1373977_at	kinesin family member 5C (predicted)	Kif5c	311024	3,9	n.c.	1,4	1,6	2,6	1,5	2,9
G-protein coupled receptor protein signaling										
1387241_at	G-protein coupled receptor 88	Gpr88	64443	8,7	-	-	-	2,6	2,5	2,3
1387146_a_at	endothelin receptor type B	Ednrb	50672	2,4	1,2	n.c.	-1,4	2,0	1,4	1,4
1387389_at	receptor (calcitonin) activity modifying protein 3	Ramp3	56820	3,9	-1,4	-1,7	1,2	1,6	1,2	3,0
1387908_at	RAS, dexamethasone-induced 1	Rasd1	64455	3,6	n.c.	-1,4	-1,4	1,6	n.c.	1,3
Ca2+ binding activity										
1370517_at	neuronal pentraxin 1	Nptx1	497675	10,4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cell cycle / cell proliferation										
Growth factor activity										
1381449_s_at	transforming growth factor alpha	*Tgfa*	24827	3,4	3,5	n.d.	n.d.	19,2	n.d.	n.d.
Transcription factor activity										
1388154_at	E2F transcription factor 5	E2f5	116651	3,2	n.c.	n.c.	n.c.	2,4	1,5	1,7
1368308_at	myelocytomatosis viral oncogene homolog (avian)	Myc	24577	2,8	n.d.	n.d.	1,6	2,3	n.d.	n.d.
Kinase activity										
1367802_at	serum/glucocorticoid regulated kinase	Sgk	29517	2,3	1,5	n.c.	-1,4	2,5	n.c.	1,3
beta-catenin binding activity										
1390429_at	axin2	Axin2	29134	7,0	-1,4	n.c.	1,3	2,1	1,3	3,8
Apoptosis										
1368294_at	deoxyribonuclease I-like 3	Dnase1l3	116687	1,9	-1,3	-1,3	-1,4	n.c.	n.c.	-1,4
Bone formation										
growth factor activity										
1368945_at	bone morphogenetic protein 2	Bmp2	29373	2,6	n.c.	-2,0	n.c.	n.c.	1,6	1,6
collagenase activity										
1388204_at	matrix metallopeptidase 13	Mmp13	171052	2,2	-1,3	1,2	1,5	-1,4	n.c.	-1,4
Coagulation										
1392894_at	fibrinogen-like 2	*Fgl2*	84586	6,7	n.d.	n.d.	n.d.	2,3	n.d.	n.d.
Immune response										
1387180_at	interleukin 1 receptor, type II	Il1r2	117022	13,8	n.d.	n.d.	n.d.	n.d.	n.d.	3,1
1369670_at	Cd200 antigen	Cd200	24560	6,7	-2,0	-3,3	n.c.	3,4	2,0	1,6
Catabolic / metabolic processes										
1393848_at	ribonucleotide reductase M2	Rrm2	362720	2,3	n.c.	n.c.	1,5	n.c.	n.c.	1,5
1384112_at	5' nucleotidase, ecto	Nt5e	58813	3,0	-	-	-	-	-	1,7
1368413_at	amiloride binding protein 1	Abp1	65029	4,7	-1,4	-2,5	1,2	1,7	1,5	3,4

Genes were organized by primary function according to Gene Ontology (GO). Statistical significance was assessed by counter-regulation scores (CR-score < -0.3). Italic genes were validated independently by TaqMan® quantitative real-time PCR. n.c. = no change (FC = 1.0 +/- 0.1), n.d. = not detected.

A2. List of publications

This thesis has been performed from August 2005 until July 2008 in the laboratories of Bayer Schering Pharma AG, GDD-TRG Women's Healthcare, Gynecological Therapy 1.

Parts of this thesis were submitted for publication or already presented:

Afhüppe, W., Sommer, A., Müller, J., Schwede, W., Fuhrmann, U., and Möller, C.
Global gene expression profiling of progesterone receptor modulators in T47D cells provides a new classification system.
J Steroid Biochem Mol Biol 2009, **113**: 105-115.

Afhüppe, W., Beekman, J., Otto, C., Hoffmann J., Fuhrmann, U., and Möller, C.
In vitro characterization of lonaprisan – a type III progesterone receptor antagonist with enhanced antiproliferative properties.
Submitted to Steroids

Poster:

Bone, W., Doecke, W.-D., Kreft, B., Merz, C., Möller, C., Müller, J., **Oehr, W.**, Pohlenz, H.-D., Prinz, F., Schröder, J., Seidel, H., Sommer, A., Zollner, T.:
A strategy for the discovery of novel targets for the treatment of uterine fibroids.
GDD conference Bayer Schering Pharma AG, February 2008, Berlin, Germany

Bone, W., Fritsch, M., Gottwald, U., Hauff, P., Kreft, B., Merz, C., Möller, C., **Oehr, W.**, Prinz, F., Reinhardt, M., Römer, W., Sacher, F., Schröder, J., Zollner, T.:
Development of in vitro and in vivo models for uterine fibroids.
GDD conference Bayer Schering Pharma AG, February 2008, Berlin, Germany

Oehr, W., Otto, C., Fuhrmann, U., Möller, C.:
Ligand-induced interactions of progesterone receptor in genomic and non-genomic pathways.
51.Annual Meeting of the German Society of Endocrinology (DGE), March 2007, Salzburg, Austria

Oehr, W., Möller, C., Fuhrmann, U.:
Ligand-induced interactions of PR isoforms and cofactors.
Bregenz Summer School on Endocrinology, July 2006, Bregenz, Austria

Previous publications:

Mittag, J.*, **Oehr, W.***, Heuer, H., Hamalainen, T., Brachvogel, B., Poschl, E., and Bauer, K.
Expression and thyroid hormone regulation of annexins in the anterior pituitary.
J Endocrinol 2007, **195**: 385-392.
***** (J Mittag and W Oehr contributed equally to this work)

Previous poster:

Aichinger, E., **Oehr, W.**, Douglas, C., Mladek, C., Hauser, M.-T.:
Characterization of the putative Chitinase POM1/AtCTL1 from Arabidopsis thaliana.
Joint Annual Meeting of the ÖGBM, ÖGGGT, ÖGBT and ANGT, September 2005, Vienna, Austria

A3. Curriculum vitae

Wiebke Afhüppe, geb. Oehr

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Professional Experience	since 07/2008	Biomarker Expert Women's Healthcare, Clinical Biomarkers, Bayer Schering Pharma AG, Berlin
Education	since 08/2005	PhD Student, Gottfried Wilhelm Leibniz University of Hanover; PhD Thesis ("Progesterone receptor modulators and uterine leiomyomas – implications and mode of action"), Bayer Schering Pharma AG, TRG Women's Healthcare, Gynecological Therapy 1
	05/2004-11/2004	Diploma Thesis, Max Planck Institute for Experimental Endocrinology, Hanover; Supervisor: Prof. Dr. Karl Bauer
	10/1999-11/2004	Studies in Biochemistry, Gottfried Wilhelm Leibniz University of Hanover; Final German Biochemistry Licentiate Degree with a Grade of 1.0 (Scale from 1 (=best) to 6)
	08/1992-07/1999	Secondary School, Burgdorf; Diploma for University Admission ("Abitur") with a Grade of 1.2 (Scale from 1 (=best) to 6)
Scholarships	08/2005-06/2008	Scholarship by the Bayer Schering Pharma AG; PhD Thesis
	12/2004-04/2005	Scholarship by the Max Planck Society; Research Project, Max Planck Institute for Experimental Endocrinology, Hanover
Memberships	since 10/2007	Member of the "Deutsche Gesellschaft für Endokrinologie" (DGE)
	since 03/2003	Member of the "Gesellschaft für Biochemie und Molekularbiologie" (GBM)
Qualifications		Qualification in Latin
Languages		German (native speaker) English (fluent)

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