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In vitro characterization of ZK 230211—A type III progesterone receptor antagonist with enhanced antiproliferative properties

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ABSTRACT

The progesterone receptor (PR) is a key regulator of female reproductive functions. Compounds with progesterone inhibiting effects (PR antagonists) have found numerous utilities in female reproductive health, ranging from contraception to potential treatment of progesterone-dependent diseases like uterine leiomyomas. Based on in vitro characteristics such as DNA binding activity and partial agonistic transcriptional behavior in the presence of protein kinase A activators (cyclic-AMP), three types of PR modulators with antagonistic properties have been defined. In this study, we analyzed the in vitro characteristics of the PR antagonist ZK 230211 in comparison to the classical antagonists onapristone and mifepristone. We focused on PR actions in genomic signaling pathways, including DNA binding activity, nuclear localization and association with the nuclear receptor corepressor (NCoR) as well as actions in non-genomic signaling, such as the activation of c-Src kinase signaling and cyclin D1 gene promoter activity. ZK 230211 represents a type of PR antagonist with increased inhibitory properties in comparison to mifepristone and onapristone. When liganded to the progesterone receptor, ZK 230211 induces a strong and persistent binding to its target response element (PRE) and increases NCoR recruitment in CV-1 cells. Furthermore, ZK 230211 displays less agonistic properties with regard to the association of PR isoform B and the cytoplasmic c-Src kinase in HeLa cells. It represses T47D cell cycle progression, in particular estradiol-induced S phase entry. In summary, our studies demonstrate ZK 230211 to be a type III progesterone receptor antagonist which is characterized by very strong DNA binding activity and strong antiproliferative effects in the cancer cell lines HeLa and T47D.

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1. Introduction

The progesterone receptor (PR) is a ligand-activated transcription factor which plays a key role in female reproduction [1]. In the absence of ligand, the PR is associated with cytoplasmic heat shock proteins and fixed in a transcriptionally inactive conformation. Upon ligand binding the conformational change of the ligand binding domain (LBD) results in the dissociation of heat shock protein complexes. The PR translocates into the nucleus where it binds as dimer to progesterone response elements (PREs) in promoters of progesterone target genes [2]. Hormones and antihormones induce related but distinct conformational changes, in particular concerning the position of the helix 12, located in the LBD [3,4]. Different displacements of the helix 12 lead to different cofactor association and subsequently, PR transcriptional control. Besides the inhibition of endogenous progesterone effects, PR antagonists have been shown to repress estrogen-dependent proliferation in the uterus and in the mammary gland [5,6]. Thus, PR antagonists are thought to provide new treatment options for estrogen- and progesterone-dependent gynecological disorders like endometriosis and uterine

Abbreviations: AR, androgen receptor; cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; BCA, bicinchoninic acid; bp, base pair; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle medium; ECL, enhanced chemiluminescence; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GR, glucocorticoid receptor; GST, glutathione S-transferase; ID, interaction domain; IPTG, isopropyl β-D-1-thiogalactopyranoside; LBD, ligand binding domain; Luc, luciferase; MEM, minimum essential medium; MMTV, mouse mammary tumor virus; NCoR, nuclear receptor corepressor; NR, nuclear receptor; OD, optical density; PA, progesterone receptor antagonist; PAA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PKA, protein kinase A; PMSF, phenylmethylsulphonyl fluoride; PR, progesterone receptor; PRE, progesterone response elements; PRM, progesterone receptor modulator; RBA, relative binding affinities; RT, room temperature; SDS, sodium dodecyl sulfate; SMRT, silencing mediator for retinoid and thyroid hormone receptor; TBS, Tris buffered saline.

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leiomyomas, and also for breast cancer [5,7–9]. In recent years, various types of antagonists have been developed and characterized. Similar to PR antagonists, selective progesterone receptor modulators (SPRMs) have been shown to be efficacious in the treatment of gynecological disorders. The term SPRM is assigned to substances with tissue-specific mixed agonistic/antagonistic properties. The most conclusive evidence for partial agonistic activity for SPRMs like J1042 and asoprisnil comes from the McPhail test. In contrast to PR antagonists like onapristone and mifepristone, which behave as pure antagonists in this assay, SPRMs display partial agonism.

PR modulators with antagonistic properties differ in their steroid receptor selectivity and their capability to modulate estrogenic effects [10]. Molecular analyses uncovered significant differences in PR-mediated mechanisms and endogenous activities [11,12]. Based on in vitro characteristics such as DNA binding activity and transcriptional behavior of the antagonist-occupied PR in the presence of protein kinase A activators (cyclic-AMP), three types of PR modulators have been defined [5,11]. Type I antagonists display pure antagonistic properties and prevent binding of the antagonist-occupied PR to the DNA (e.g. onapristone). Type II antagonists allow binding of the ligand-occupied receptor to the DNA, but reveal agonistic potential upon protein kinase A stimulation in vitro. Thereby, they exhibit partial PR agonistic activities in a tissue- and species-specific manner (e.g. mifepristone, asoprisnil) [13,14]. Type III PR antagonists induce PR binding to its PREs and display pure PR antagonistic activities without agonistic potential under diverse experimental settings.

In the present study, we analyzed the progesterone receptor antagonist ZK 230211 with regard to its effect on PR localization, non-genomic PR isoform B signaling, corepressor recruitment as well as its effects on T47D cell cycle regulation. ZK 230211 could be shown to be a type III progesterone receptor antagonist, in particular with strong antiproliferative properties in T47D cells.

2. Experimental

2.1. Receptor binding assay

The preparation of rabbit PR and rat AR, GR, and ER from cytosolic tissue fractions as well as the competition experiments were carried out as described previously [15]. The RBA values were determined in competition experiments using radioactively labeled standard compounds as reference. The reference compounds were progesterone for PR, R1881 for AR, dexamethasone for GR, and estradiol for ER.

PR agonist progesterone, AR agonist R1881, GR agonist dexamethasone and ER agonist estradiol (E2) were synthesized at Bayer Schering Pharma AG Research (Berlin, Germany).

2.2. Transactivation assay

To study the effects of hormones, in vitro assays were performed in different cellular backgrounds. The selection of the various cell lines was based on an optimization process for the different in vitro systems in terms of selectivity or signal-to-noise ratio and absence of interfering other nuclear hormone receptors, e.g. human neuroblastoma (SK-NM-C) cells were selected for transactivation assays of PR due to the absence of potentially interfering nuclear hormone receptors. Stably transfected SK-NM-C cells expressing either the human PR-A or PR-B and the mouse mammary tumor virus promoter linked to the LUC reporter gene [16,17] were maintained in MEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FBS and 100 U/ml penicillin, 0.1 mg/ml streptomycin, 4 mM L-glutamine, 1× non-essential amino acids and 1 mM sodium pyruvate (all from PAA Laboratories, Cölbe, Germany). Human breast adenocarcinoma (MVLN) cells with endogenous ERα and stably transfected with MMTV-LUC reporter gene which were kindly provided by M. Pons (Karlsruhe Research Center, Germany) were used for ER transactivation assays. Green monkey kidney fibroblast (CV-1) cells stably transfected with the rat AR and a MMTV-LUC reporter gene as described in Fuhrmann et al. [16] were used for AR transactivation assays. Mouse embryonic fibroblast (NIH 3T3) cells with endogenous GR and stably transfected with MMTV-CAT reporter gene which were a generous gift of A. Cato (Karlsruhe Research Center, Germany) were used for GR transactivation assays. Cells were maintained in DMEM (Invitrogen) containing 10% FBS. Fetal bovine serum (FBS) was purchased from BioWhittaker, Inc. (Walkersville, MD, USA). All cell lines were cultured at 37 °C with 5% CO_2.

Table 1
Relative binding affinities and transactivation profile of ZK 230211.

	RBA (%)			
	PR (rabbit)	AR (rat)	GR (rat)	ER (rat)
ZK 230211	140[a]	3.1[a]	22.2[a]	nc[b]
	Antagonist potency (IC_{50}, nM)			
	PR-A	PR-B	AR	GR	ERα
ZK 230211	0.0036	0.0025	54	16	ne
Mifepristone	0.028	0.025	10	2.2	nd
Onapristone	5.27	3.79	160	235	nd

nc = no competition, nd = not determined, ne = no effect.
The reference compounds were progesterone for PR, R1881 for AR, dexamethasone for GR, and estradiol for ER. The RBA values of respective compounds were arbitrarily designated as 100%. IC50 values for antagonistic potency were determined from full dose–response curves ranging from 10^{-12} to 10^{-6} M.
[a] 24 h incubation.
[b] 2 h incubation.

For the different transactivation assay systems, cells (10,000 cells per well) were seeded onto 96-well dishes and cultured in their respective medium supplemented with 3% charcoal-stripped FBS. After 48 h hormones were added and incubation was continued for 24 h. To determine agonistic activity, cells were cultured in the presence of ZK 230211, mifepristone or onapristone. LUC expression was given as a normalized response value relative to the maximal LUC expression produced by a reference agonist: R5020 for PR, R1881 for AR, dexamethasone for GR, and estradiol for ERα. As a negative control, cells were cultured in 1% ethanol (vehicle). For the determination of antagonistic activity, cells were treated with the respective reference compound and, additionally, with increasing amounts of ZK 230211, mifepristone or onapristone. As a negative control for reporter gene induction, cells were cultured in 1% ethanol. Medium was then removed and 160 μl of luminescence reporter gene assay system Steadylite HTS (PerkinElmer, Inc., Waltham, MA, USA) were added to each well. Plates were incubated for 15 min at room temperature to ensure complete cell lysis and luciferase reaction, and were read in a TopCount NXT (PerkinElmer, Inc.). Data was analyzed to obtain maximum efficacy, EC50 and IC50 values using Sigma Plot 8.0 software. Transactivation assays were carried out at least three times. In Table 1, data from one representative experiment are shown as the mean of triplicate values.

PR antagonists (ZK 230211, onapristone, mifepristone (RU486)) and standard PR agonist promegestone (R5020) were synthesized at Bayer Schering Pharma AG Research (Berlin, Germany).

2.3. Preparation of nuclear extracts

African green monkey kidney fibroblast (COS-1) cells were used for PR expression due to transfection efficiency and protein expression yield. Cells were obtained from LGC Promochem and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 4 mmol/l L-glutamine. COS-1

cells (1,000,000 per well) were seeded onto Ø10 cm-dishes in their growth medium and were allowed to attach overnight. After washing cells twice with PBS, serum-free medium was added and cells were transfected with 5 μg phPR1A expression plasmid in a 3-fold volume of Transfectam Reagent (Promega, Mannheim, Germany) for 3 h following the manufacturer's specifications. Medium was changed back to growth medium and cells were allowed to recover overnight. After starvation in medium containing 3% charcoal-stripped FBS for 24 h, cells were treated for 2 h with 10 nM PR antagonist (mifepristone, onapristone, ZK 230211) or vehicle (1% ethanol). Cells were then washed twice with PBS, scraped, collected and centrifuged at 4 °C. Pellets were disrupted by resuspension in lysis buffer (10 mM sodium phosphate (pH 7.4), 1 mM EDTA, 10% glycerine, 400 mM potassium chloride, 5 μM leupeptin, 0.5 μg/ml aprotenin, 0.1 benzamidine, 1.0 μg/ml pepstatin, 2.0 mM dithiothreitol, 0.2 mM PMSF; all from Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany). Supernatants were collected after ultracentrifugation and frozen at −80 °C. The protein concentration was determined according to the procedure of Bradford.

2.4. DNA binding assays

PRE-sense (5′-AGCTTAGAACACAGTGTTCTCTAGAG-3′) and PRE-antisense (5′-GATCCTCTAGAGAACACTGTGTTCTA-3′) sequences (Invitrogen, Karlsruhe, Germany) were annealed and labeled with [α-^{32}P]dATP (10 μCi/μl) using the Klenow fragment method as described in the Pharmacia Band Shift Kit (Pharmacia Biotech, Vienna, Austria). Band shift reaction was started in binding assay buffer (10 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 71 mM potassium chloride, 2.5% glycerine; all from Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) with 1 μg of poly (dI/dC) and 10 μg crude nuclear extract at 4 °C for 15 min. Radioactively labeled PRE sequences (40,000 cpm) were added. The reaction was proceeded at room temperature for 20 min. The bound DNA fragments were resolved on 5% polyacrylamide gels and dried gels were subjected to enhanced autoradiography as described previously [18]. Competition assays were performed by preincubation with cold competitor for 10 min prior to addition of labeled probe.

2.5. Western blot

Human breast carcinoma (T47D) cells were used for PR localization analysis, because they show endogenous PR expression. Cells were obtained from LGC Promochem (Wesel, Germany; American Type Culture Collection (ATCC)) and maintained in phenol red-free RPMI 1640 (PAA Laboratories) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 4 mmol/l L-glutamine. T47D cells (250,000 per well) were seeded onto six-well plates in their growth medium and allowed to attach overnight. Medium was removed and media containing 5% charcoal-stripped serum as well as 100 nM R5020, PR antagonist (10 nM ZK 230211, 20 nM mifepristone, 200 nM onapristone) or vehicle (1% ethanol) were added in the presence of 100 pM estradiol. After 6 h and 48 h of treatment, cells were lysed, homogenized and fractioned with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) according to the manufacturer's recommendations. Lysates were frozen and stored at −80 °C. Amounts of protein were quantified colorimetrically using the BCA Protein Assay Reagent (Pierce). 20 μg of lysates were separated by 4–12% Bis–Tris–SDS-PAGE (Invitrogen, Karlsruhe, Germany) and transferred to Nitrocellulose membranes (Invitrogen). Membranes were blocked 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 1× Blocking Reagent (RotiBlock; Roth, Karlsruhe, Germany) at room temperature for 1 h. They were then probed with primary antibody (MS298-P0 anti-progesterone receptor; LabVision, Fremont; CA, USA) at 4 °C overnight. Horseradish peroxidase conjugated secondary antibody (NA931V anti-mouse-HRP; Amersham Biosciences, Little Chalfont, UK) was applied at room temperature for 3 h. All antibodies were diluted in TBS containing 0.05% Tween 20 and 1× Blocking Reagent. Protein levels were examined using an ECL Plus kit (Amersham Biosciences) and ECL Hyperfilm (Amersham Biosciences).

2.6. GST pulldown

Recombinant GST-mNCoR was produced in *E. coli* BL21 (Promega, Mannheim, Germany). The recombinant strain was grown to an optical density (OD) at 600 nm of 0.7–1.0 and was then induced with IPTG (isopropyl-β-D-thiogalactopyranosid) for 4 h. Cells were lysed by repeated freeze-thawing in lysis buffer (20 mM HEPES (pH 7.9), 60 mM KCl, 2 mM DTT, 1 mM EDTA, 4 mg/ml lysozyme; all from Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) and cleared by centrifugation. The fusion proteins were bound to glutathione-Sepharose 4B beads (GE Healthcare, Munich, Germany) and were washed with NETN buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl (pH 7.8), 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin; all from Sigma). Beads were then incubated at 4 °C for 2 h with ^{35}S-labeled PR protein, which was synthesized using a coupled in vitro transcription and translation system in accordance with the manufacturer's protocol (Promega. Mannheim, Germany). Finally, beads were extensively washed with NETN buffer and the bound material was analyzed by 12% SDS-PAGE, followed by autoradiography. In the case of hormone treatment, 100 nM of hormones were added during all incubation and washing steps.

2.7. Mammalian two-hybrid assay

For the analyses of genomic cofactor interactions, green monkey kidney fibroblast (CV-1) cells were selected due to transfection efficacy and high signal-to-noise ratio. The cells (5000 per well) were plated onto 96-well plates in phenol red-free DMEM containing 5% charcoal-treated FBS, 48 h before transfection with lipofectamine (Invitrogen, Karlsruhe, Germany). Cells were transfected with a total amount of 200 ng DNA (10 ng paaatkluc, 30 ng pCMX-GAL4/mNCoR and 160 ng pCMX-PR-LBD/VP16) in 1600 ng lipofectamine for 5 h. The transfection mix was replaced by medium containing the hormones to be tested (1 nM R5020, 100 nM mifepristone, 100 nM onapristone or 100 nM ZK 230211) or vehicle (0.1% DMSO). After 18 h treatment cells were analyzed to measure luciferase activity as described above (see Section 2.2). Data are represented as the mean of triplicate values which were obtained from a representative experiment. Independent experiments were repeated at least two times.

To monitor the non-genomic interaction of PR-B with c-Src kinase, human cervix carcinoma (HeLa) cells which were obtained from DSMZ (Braunschweig, Germany) were used. Cells were maintained in phenol red-free DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 4 mmol/l L-glutamine. For mammalian two-hybrid assay, cells (10,000 per well) were seeded onto 96-well dishes in phenol red-free DMEM containing 5% charcoal-stripped FBS overnight. Cells were transfected with FuGENE 6 following the manufacturer's specifications (Roche, Mannheim, Germany). A transfection mix (10 μl) containing 5 ng pCMV-AD/PR-B, 10 ng pCMV-BD/c-Src, and 100 ng pFR-Luc expression plasmid (Stratagene, La Jolla, CA, USA) in a 3-fold volume of FuGene 6 filled up with OptiMEM (Invitrogen) was added to the

medium and cells were permitted to recover overnight. Medium was removed and cells were treated for 24 h either with vehicle control (0.1% DMSO) or R5020 or PR antagonist (ZK 230211, onapristone, mifepristone) in increasing concentrations (10^{-11} to 10^{-6} M). To obtain luciferase expression, cells were subjected to the transactivation assays' procedure as described above (see Section 2.2). Statistical analysis was performed using SAS 9.1.3 service pack 4. Initially, the normalized data were summarized using simple descriptive statistics and were presented graphically as the mean of triplicate values which were obtained from a representative experiment. Independent experiments were repeated at least two times. In addition, a mixed linear model was used to determine an effect of treatment or concentration on the observed response values. Here, a correlation structure between measurements per treatment and concentration was estimated and involved into the analysis. Treatment groups were compared with the group of interest (e.g. R5020 positive control) using Dunnett's approach to adjust for multiple comparisons.

The plasmids pCMV-AD, pCMV-BD and pFR-Luc plasmids were purchased from Stratagene (La Jolla, CA, USA). The plasmid paaatkluc was kindly provided by A. Baniahmad (Justus-Liebig University of Giessen, Germany). PCMX-VP16 was a kind gift from R. Schüle (Gynecology department of the University Hospital of Freiburg, Germany). Deletion of the VP16 domain via digestion with HindIII and religation produced the pCMX plasmid which was used for the generation of pCMX-GAL4/mNCoR by first cloning the PCR-amplified GAL4-DNA binding domain from the pAS2 vector (Clontech, Mountain View, CA, USA) into the pCMX vector. Subsequently, the receptor binding domain (ID) of murine NCoR (aa 2005-2453) was transferred into the pCMX-GAL4 vector. PCMX-PR-LBD/VP16 was constructed by ligation of the PCR-amplified ligand binding domain of the PR isoform B into the BamHI site of the pCMX-VP16 vector. PCMV-BD/c-Src was generated by cloning the PCR-amplified fragment of the murine c-Src kinase (aa 1-250) into the EcoRI and XbaI site of the pCMV-BD vector.

2.8. Cyclin D1 promoter luciferase assay

To study the activation of cyclin D1 transcription by PR modulators, human osteosarcoma (U2OS) cells which were obtained from DSMZ (Braunschweig, Germany) were used. Cells were maintained in phenol red-free DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 4 mmol/l L-glutamine. For reporter gene assay, U2OS cells (10,000 per well) were plated onto 96-well dishes in their respective medium supplemented with 3% charcoal-stripped FBS. Cells were allowed to attach overnight and were then transfected with plasmids encoding firefly luciferase under the control of a 953 bp fragment of the human cyclin D1 promoter (pGL3-953CdLuc) and plasmids encoding pSG5-hPR-B [19]. 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 with vehicle (0.1% DMSO) as a negative control, R5020 or PR antagonist (ZK 230211, onapristone, mifepristone) in increasing concentrations (10^{-12} to 10^{-7} M) for 24 h. To obtain luciferase expression, cells were subjected to the transactivation assays' procedure as described above (see Section 2.2). Statistical analysis was performed as for the mammalian two-hybrid assay for non-genomic c-Src kinase interaction (see Section 2.7).

The pGL3-953CdLuc plasmid was kindly provided by M. Beato (Faculty of Molecular Biology and Cancer Research of the Philipps University of Marburg, Germany). The pSG5-PR-B plasmid was a kind gift from P. Chambon (Faculty of Medicine of the University of Strasbourg, France) [19].

2.9. Cell cycle analysis

Cell cycle analysis was performed in human breast carcinoma (T47D) cells, because T47D cells show endogenous functional PR and ERα expression. Additionally, publicly available data described hormonal sensitivity of T47D cells to cell cycle phases distribution. Cells were obtained from LGC Promochem (Wesel, Germany; American Type Culture Collection (ATCC)) and maintained in phenol red-free RPMI 1640 (PAA Laboratories) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 4 mmol/l L-glutamine. For cell cycle analysis, cells (200,000 per well) were plated in six-well plates in their growth medium and were allowed to attach overnight. Medium was removed, cells were washed twice with PBS, and medium with charcoal-stripped FBS was added. Cells were maintained under steroid hormone-free conditions for 2 days. 10 nM PR modulator (R5020, ZK 230211, mifepristone or onapristone) or vehicle (0.1% DMSO) were then added, either in the presence or in the absence of 100 pM estradiol. After 24 h of treatment cells were harvested by trypsinisation, pelleted by centrifugation, and washed once with PBS (PAA Laboratories, Cölbe, Germany). Media and washes were retained with the adherent cells. Cells were resuspended in 1 ml ethanol (70%) and pipetted several times to ensure a uniform single-cell suspension. The samples were stored at −20 °C until the day of analysis where they were centrifuged and pellets were washed once with PBS. After an additional centrifugation step, 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). The cells were dispersed by repeated pipetting 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 h. Cells were then analyzed in a fluorescence-activated cell sorting Caliber flow cytometer (FACS Calibur; BD Biosciences). The cell cycle profile for each sample was estimated using ModFit LT software (Verity Software House, Topsham, ME). A minimum of 10,000 gated cells was analyzed for each sample, and triplicate time-separated cultures were analyzed for each treatment.

3. Results

3.1. ZK 230211 acts as a very strong PR antagonist without agonistic transactivational activity in vitro

Relative binding affinities (RBA) of ZK 230211 to the progesterone receptor (PR), the glucocorticoid receptor (GR), the androgen receptor (AR), and the estrogen receptor (ER) were examined to determine receptor selectivity in correlation to Fuhrmann et al. [17]. ZK 230211 revealed a strong binding to the PR, considerably weaker binding to both GR and AR, and no binding to the ER (Table 1).

In order to determine the antiprogestogenic and progestogenic potency of ZK 230211 compared to the classical antagonists mifepristone and onapristone, in vitro transactivation assays were carried out in SK-N-MC cells stably transfected with human PR-A or PR-B (Table 1). ZK 230211 showed a ten times stronger antagonistic activity than the standard antagonist mifepristone ($IC_{50} = 2.5 \times 10^{-12}$ M vs. $IC_{50} = 2.5 \times 10^{-11}$ M) and a 1000-fold higher antagonistic activity than onapristone ($IC_{50} = 2.5 \times 10^{-12}$ M vs. $IC_{50} = 3.79 \times 10^{-9}$ M). Furthermore, ZK 230211 did not show any agonistic activity when tested at the highest concentration of 10^{-6} M (data not shown). Since ZK 230211 was shown to bind to the AR and to the GR, the effects on AR- and GR-mediated transcription were investigated in CV-1 or NIH3T3 Cells, respectively. ZK 230211 demonstrated similar activity at the AR when

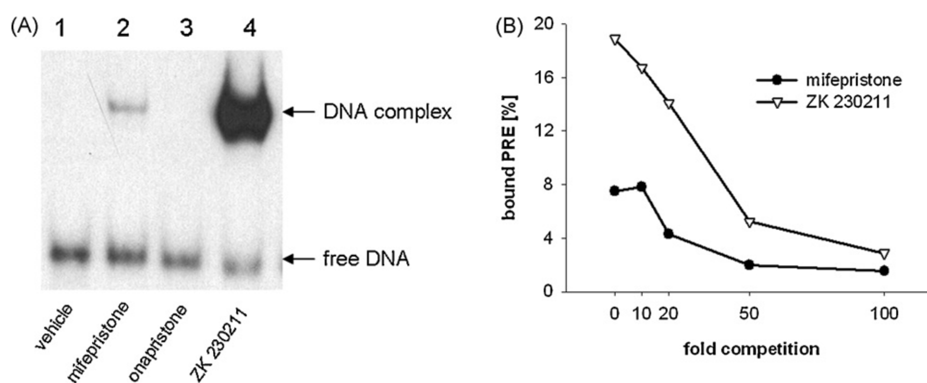

Fig. 1. Progesterone receptor bound to ZK 230211 can bind its response element with higher affinity than mifepristone- or onapristone-bound PR. For PR expression, the COS-1 cell expression system was used. (A) Extracted protein was pretreated with vehicle (lane 1), 10 nM mifepristone (lane 2), onapristone (lane 3) or ZK 230211 (lane 4), and was incubated with radiolabeled PRE. Samples were analyzed on 5% PAA gel and visualized by autoradiography. (B) PR was extracted, pretreated with 10 nM PR antagonist and incubated with radiolabeled PRE in the presence of increasing amounts of non-radiolabeled competitive PRE (0- to 100-fold molar concentration). Samples were analyzed on 5% PAA gel, visualized by autoradiography and quantified by ImageQuaNT.

compared to mifepristone. It exhibited antiandrogenic activity and only marginal androgenic activity (Table 1). In the GR transactivation assay, ZK 230211 displayed an about seven times lower antiglucocorticoid activity than mifepristone (Table 1) and no glucocorticoid activity (data not shown). To exclude any estrogenic or antiestrogenic activity, an ERα transactivation assay was carried out in MVLN cells. ZK 230211 showed neither estrogenic nor antiestrogenic activity (Table 1). In summary, ZK 230211 was highly steroid receptor-selective and exhibited only marginal endocrine side effects in the established steroid receptor specific cell systems.

3.2. ZK 230211-occupied progesterone receptor showed strong binding to its response element

Previous studies using the conventional electrophoretic mobility shift assays (EMSA) have shown that onapristone failed to stimulate PR binding to progesterone response elements (PREs) in vitro [20]. To determine whether ZK 230211 recruits PR to its PREs, EMSAs with human PR bound to ZK 230211, mifepristone or onapristone were performed. A prominent induction of PRE binding activity was observed for ZK 230211 (Fig. 1A). Mifepristone also stimulated PR binding to its response element, although with a 2.5-fold lower binding activity (Fig. 1B). No binding of the onapristone-liganded PR was detected under these conditions. Competition with unlabeled PRE demonstrated specificity of PRE binding data (Fig. 1B).

3.3. ZK 230211-bound progesterone receptor revealed enhanced nuclear localization in T47D cells

The classical mechanism for PR signaling is the dimerization of the steroid receptor upon ligand binding, followed by translocation into the nucleus where it can bind to its PREs. Besides the stimulation of PRE binding, the active transport from the cytoplasm into the nucleus might differ depending on the ligand-induced receptor conformation. To determine the effects of the three PR antagonists on nuclear localization, nuclear extracts and cytosolic fractions of T47D cells were analyzed for endogenous PR protein levels using Western blot. After binding ZK 230211, the PR displayed enhanced nuclear and decreased cytosolic localization within 6 h of treatment (Fig. 2A and B). This effect increased after 48 h. The other tested PR antagonists did not show such a significant nuclear accumulation of the PR. The nuclear localization of R5020-bound PR increased after 6 h of treatment, but decreased within the next 42 h, most likely via PR destabilization mechanisms. The antagonists mifepristone and onapristone only marginally enhanced nuclear localization of the PR in this experimental setting, irrespective of the time points examined.

3.4. ZK 230211 promoted the interaction of the progesterone receptor with the corepressor NCoR

The observation that ZK 230211 is a more potent antagonist in transactivation assays compared to mifepristone and a stronger promoter of PRE binding would suggest a more active recruitment of corepressors which are known to keep the receptor in a transcriptionally inactive state [21], e.g. the nuclear receptor corepressor (NCoR). Therefore, it was of interest to test the ability of PR to associate with the corepressor NCoR in the presence of ZK 230211. In order to analyze the interaction between NCoR and the liganded receptor, the mammalian two-hybrid system in CV-1 cells was employed. In this system, a construct expressing the ligand binding domains (LBD) of the human PR fused to the strong activation domain of VP16 in the pCMX plasmid and a construct

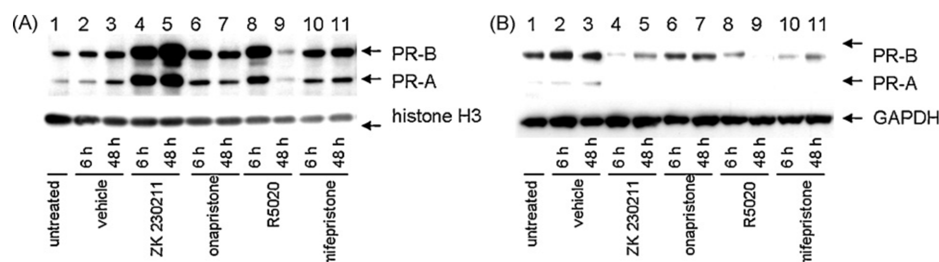

Fig. 2. The nuclear translocation of PR isoforms is enhanced after ZK 230211 binding. T47D cells were treated with vehicle (lanes 2 and 3), 10 nM ZK 230211 (lanes 4 and 5), 200 nM onapristone (lanes 6 and 7), 100 nM R5020 (lanes 8 and 9) or 20 nM mifepristone (lanes 10 and 11). After 6 h (lanes 2, 4, 6, 8, 10) and 48 h (lanes 3, 5, 7, 9, 11), cells were lysed and nuclear extracts (A) as well cytosolic fractions (B) were analyzed for PR-A and PR-B protein content using Western Blot. Loading controls: Histone H3 expression for nuclear extracts and GAPDH expression for cytosolic fractions.

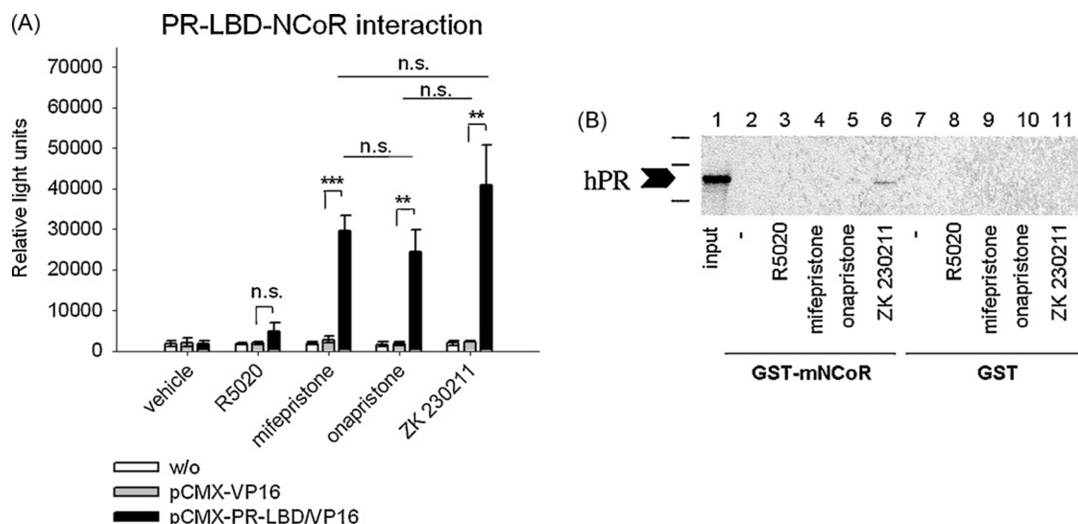

Fig. 3. ZK 230211-bound PR has higher affinity to the corepressor NCoR than PR bound to mifepristone or onapristone. (A) CV-1 cells were transfected with pCMX-PR-LBD/VP16, pCMX-GAL4/NCoR and interaction sensitive reporter (paaatluc) expression plasmids. As a negative control, cells were transfected with pCMX-VP16, pCMX-GAL4/NCoR and paaatluc expression plasmids. After 18 h of treatment with PR modulators (1 nM R5020, 100 nM mifepristone, 100 nM onapristone or 100 nM ZK 230211) or vehicle (0.1% DMSO), cells were lysed and luciferase activity was determined according to a standard assay. Statistical significance was assessed by Student's *T*-test ($^{**}p < 0.005$, $^{***}p < 0.001$, n.s. = not significant) for comparisons pCMX-VP16 vs. pCMX-PR-LBD/VP16 or respective treatments. (B) Radiolabeled PR was incubated with vehicle (lanes 2 and 7), 100 nM R5020 (lanes 3 and 8), 100 nM mifepristone (lanes 4 and 9), 100 nM onapristone (lanes 5 and 10) or 100 nM ZK 230211 (lanes 6 and 11). A pulldown with GST-mNCoR (lanes 2–6) or GST as a control (lanes 7–11) was performed and bound PR was analyzed by 12% SDS-PAGE and visualized by autoradiography.

expressing the receptor binding domain (ID) of NCoR fused to the GAL4-DNA binding domain in the pCMX plasmid was used. The ability of the PR-LBD/VP16 fusion to activate transcription from a GAL4-responsive reporter plasmid was taken as a readout for the interaction between PR and the corepressor. Generally, the antagonist-bound PR-LBD displayed a strong and significant interaction with the NCoR-ID (Fig. 3A). The vehicle control did not show any significant interaction. Compared to the NCoR-recruitment induced by antagonists, the R5020-induced association was lower (1.7-fold for R5020 vs. 12.8-fold for ZK 230211; Fig. 3A). Additionally, the interaction between the PR-LBD and the NCoR-ID were dose-dependent for all PR ligands tested (data not shown).

To examine the direct interaction of full-length PR with NCoR without interfering cellular side effects and other cofactor binding, an in vitro pulldown between recombinant GST-mNCoR (or GST alone as a negative control) and radioactively labeled PR protein was performed. The association of ZK 230211-occupied PR with GST-mNCoR was clearly visible and stronger than the interactions induced by the other PR antagonists tested (Fig. 3B). The PR conformation induced by mifepristone, onapristone and R5020 was not capable to recruit NCoR to the PR in a detectable degree under these experimental conditions. Control lanes with GST did not show interactions after treatment with PR modulators at all (Fig. 3B). These data suggest that the ZK 230211-bound PR strongly interacts with the corepressor NCoR in pulldown experiments, distinguishing it from the other antagonists tested.

3.5. ZK 230211-bound progesterone receptor induced low rapid non-genomic effects on intracellular signaling pathways

Non-genomic activation of c-Src/p21ras/Erk and PI3K/Akt-dependent pathways has been implicated in the stimulation of human breast cancer cell cycle progression and proliferation by progestins [22–26]. Moreover, trigger of DNA synthesis and cell proliferation in human mammary cancer-derived cells is a cooperative effect of estrogen and progesterone non-transcriptional actions [27]. It has been reported that in addition to the synthetic progesterone analogue R5020 the PR antagonist mifepristone also stimulates a rapid activation of the c-Src/ras/raf/MEK-1/MAPK signaling pathway in breast cancer cells in a PR-dependent manner [22,23,28]. To analyze the ligand-induced association with c-Src kinase for ZK 230211 in comparison to mifepristone and onapristone, mammalian two-hybrid assays were performed in HeLa cells transfected with the full-length PR isoform B in the NFκB activation domain plasmid pCMV-AD, the c-Src kinase fragment in the GAL4-DNA binding domain plasmid pCMV-BD, and a GAL4-responsive luciferase reporter (pFR-Luc). The interaction between PR-B and c-Src-kinase induced by the respective ligand was assessed by measuring the ability of the VP16/PR-B fusion to activate transcription from a GAL4-responsive reporter plasmid. Mifepristone- and onapristone-liganded PR both showed a strong association with c-Src kinase (efficacy = 90–100%) whereas ZK 230211 only induced interaction of PR and c-Src with reduced efficacy (ZK 230211 max. efficacy = 33%; Fig. 4A). The potency of mifepristone-induced interaction was comparable to what was demonstrated by the agonist R5020. Moreover, the mifepristone-induced profile displayed no statistical significant difference to the R5020-induced one (Dunnett's approach) whereas the onapristone-induced profile did. The potency of association induced by onapristone was about 30-fold lower ($EC_{50} = 1.2 \times 10^{-10}$ M vs. $EC_{50} = 3.9 \times 10^{-9}$ M; Fig. 4A). A comparison of onapristone and ZK 230211 effects furthermore revealed significant differences for these two profiles (Dunnett's approach). So, we identified differences for all three antagonists with ZK 230211 resulting in the lowest overall effect. In addition, a lack of the SH3 domain in c-Src kinase expression plasmids abolished all ligand-induced recruitments to PR (Fig. 4B).

To determine whether differences in PR-B-c-Src-interaction influence the expression of respective downstream genes, a cyclin D1 reporter gene assay was performed in a U2OS cell system optimized for analysis of cyclin D1 promoter activity. Cyclin D1 expression does not rely on the direct transcriptional activity of liganded PR as the cyclin D1 promoter is characterized by the lack of consensus PREs [29]. Consistent with c-Src kinase interaction, the cyclin D1 promoter was activated in response to R5020 treatment (Fig. 4A and B). PR bound to ZK 230211 displayed the lowest efficacy of induction of cyclin D1 promoter activity in comparison to the other tested PR ligands (efficacy = 47%; Fig. 4C). The non-genomic transcriptional activation by mifepristone was similar to R5020 (Fig. 4C), whereas the onapristone-liganded PR displayed

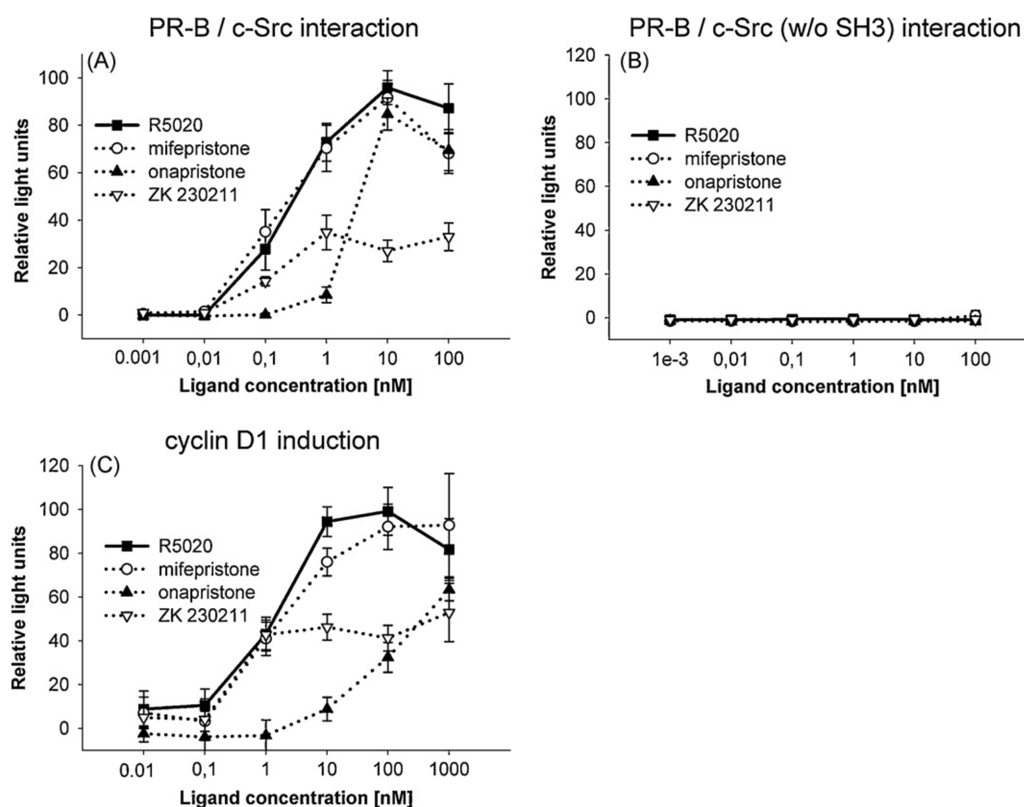

Fig. 4. Reduced induction of non-genomic c-Src-interaction and cyclin D1 gene promoter activity for the PR-B liganded with ZK 230211. (A) Interaction with c-Src kinase. HeLa cells were transfected with pCMV-BD/c-Src and pCMV-AD/PR-B expression plasmids and an interaction sensitive reporter (pFR-Luc). After 18 h of treatment with R5020, mifepristone, onapristone or ZK 230211 in increasing concentrations (10^{-12} to 10^{-7} M), cells were lysed and luciferase activity was determined. (B) Cyclin D1 reporter gene assay. U2OS cells were transfected with pSG5-PR-B expression plasmid and pGL3-953Cdluc reporter gene plasmid. After 18 h of treatment with R5020, mifepristone, onapristone or ZK 230211 in increasing concentrations (10^{-11} to 10^{-6} M), cells were lysed and luciferase activity was determined according to a standard assay. Statistically, a correlation structure between measurements per treatment and concentration was estimated and involved into the analysis. Treatment groups were compared with the group of interest (e.g. R5020 positive control) using Dunnett's approach to adjust for multiple comparisons.

a markedly weaker potency ($EC_{50} = 1.3 \times 10^{-7}$ M) and comparable efficacy as ZK 230211-bound PR at the highest dose utilized (notice that the dose–response curve of onapristone did not reach plateau). Statistical analysis (Dunnett's approach) furthermore revealed significant differences between onapristone and ZK 230211 induced profiles. In summary, the reduced cyclin D1 promoter activity in combination with the results in PR-B/c-Src kinase interaction studies demonstrate that ZK 230211 is an antagonist which shows different and less activation of this non-genomic signaling pathway compared to the other PR antagonists.

3.6. Inhibiting effects of ZK 230211-liganded progesterone receptor on cell cycle progression in T47D cells

Motivated by the observed differences in c-Src kinase recruitment and the detected differences in cyclin D1 promoter activation, the further experiments focused on the consequences of PR antagonists' action on cell cycle progression. PR antagonists are known to exhibit antiproliferative properties on endometrial and breast cancer cells [5,30], but their properties differ depending on experimental conditions and the time point examined [6,31,32]. In the presented studies, the effects of R5020, mifepristone, onapristone and ZK 230211 on T47D cell cycle phases were analyzed under insulin-free conditions after 24 h of treatment, either alone or in combined treatment with estradiol(E2).

The agonist R5020 revealed stimulatory effects. It enhanced S phase entry and induced a G2/M phase arrest and subsequently, reduced the proportion of cells in G0/G1 phase (Fig. 5A–C). The PR antagonists mifepristone and onapristone did not affect cell cycle phases themselves under the experimental conditions utilized. The strongest antagonistic effects were observed for ZK 230211 which significantly repressed cell cycle progression when applied in the absence of estradiol (Fig. 5B).

The E2-induced increase in S and G2/M phase was inhibited neither by R5020 nor by the classical antagonist onapristone (Fig. 5D–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. ZK 230211 inhibited both the E2-induced increase in the proportion of cells passing S phase and G2/M phase. Therefore, ZK 230211 maintained cells in G0/G1 phase and diminished the E2-induced shift in cell cycle phases which underlines an enhanced antiproliferative capacity in the T47D breast cancer cell line.

4. Discussion

In this study, we characterized molecular determinants of the steroidal PR antagonist ZK 230211. ZK 230211 was shown to be a very strong antagonist with increased potency, favorable selectivity and lacking agonistic properties in the tested in vitro systems.

4.1. Corepressor recruitment

Differences in DNA binding and transcriptional activities of PR evoked by the three antagonists onapristone, mifepristone and ZK 230211 might generally be attributed to induction of different receptor conformations. Structural alterations of PR are induced in the ligand binding pocket, primarily in the C-terminus [3], in

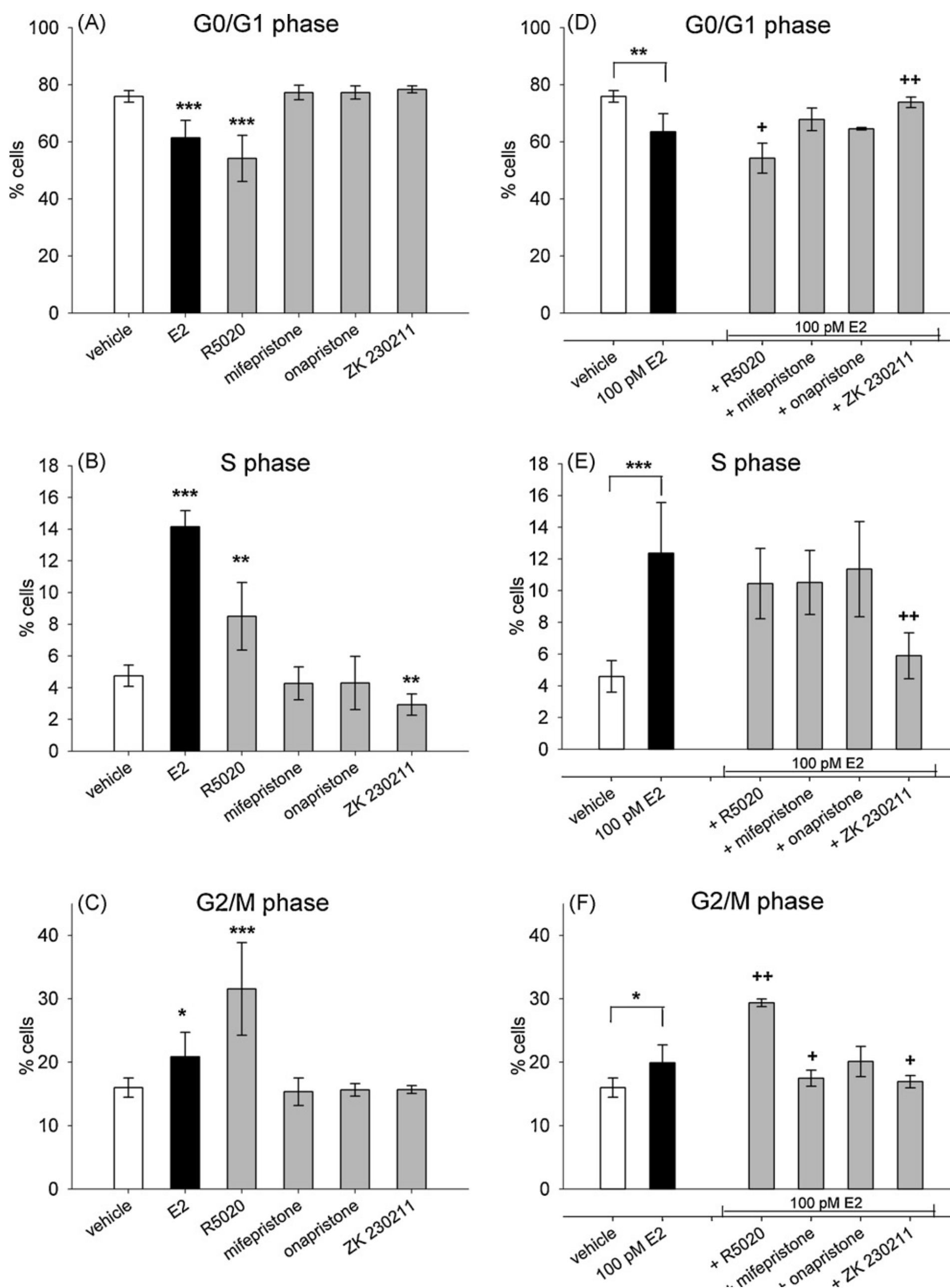

Fig. 5. ZK 230211 reverses estradiol-induced S phase entry in T47D cells. Cell cycle phases were visualized using propidium iodide staining and FACS analysis. (A–C) Cell cycle phases after 24 h of treatment with vehicle, 10 nM estradiol or 10 nM of PR ligand. (D–F) Cell cycle phases after 24 h of treatment with vehicle or 10 nM PR ligand in the presence of 100 pM estradiol. Statistical significance was assessed by Student's *T*-test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$) for the comparisons vehicle vs. treatment (stars) or E2 vs. treatment + E2 (crosses).

particular the activation function 2 (AF-2). In some cases, the DNA binding and the AF-1 domains are affected as well. In crystal structures, it has already been shown that the position of helix 12 in the ligand binding domain of PR is dependent on the agonist or antagonist bound [33]. The turn of the helix 12 is responsible for the formation of a surface that preferentially allows binding of peptides containing the NR box motifs (LXXLL, where L = leucine and X = any amino acid) present in coactivators [34,35] or those containing CoRNR boxes present in corepressors [36,37]. The ability of the antagonist-occupied receptor to bind to coactivators or corepressors is directly correlated with its transcriptional activity and target gene expression from distinct promoters [38,39]. For example overexpression of NCoR leads to a suppression of mifepristones' partial agonistic activities, whereas overexpression of the coactivators L7/SPA and SRC-1 enhanced partial agonistic properties [21,40,41]. In contrast, the activity profile of onapristone is not affected by increased corepressor or coactivator expression [40,41], indicating a unique receptor conformation distinct from that induced by mifepristone [4]. In the present study, the LBD of the PR was used in a mammalian two-hybrid assays to describe the influence of the different antagonist-induced conformations on the ability of the LBD to bind to the corepressor NCoR. The results demonstrate a stronger NCoR interaction for the antagonist-bound LBD when compared to the agonist-bound LBD (Fig. 3A). These findings support the concept of differences between antagonist- and agonist-occupied PR described above [21,41]. However, similar recruitment of NCoR to the PR-LBD for the three antagonists is somewhat contrary to the observation in GST-pulldown experiments utilizing full-length

Table 2
In vitro activities of type I (onapristone), type II (mifepristone) and type III (ZK 230211) PR antagonists.

	Binding to DNA	Nuclear localization	In vitro transactivation activity (MMTV promoter)		Interaction with NCoR	Interaction with c-Src	Cyclin D1 promoter activity	Inhibition of S phase entry		Inhibition of G2/M phase	
			Standard	Plus cAMP				Alone	Plus E2	Alone	Plus E2
Onapristone	–	+	–	–	++	++	++	–	–	–	–
Mifepristone	+	+	–	+	+	+++	+++	–	–	–	+
ZK 230211	+++	+++	–	–	+++	+	+	+	+	–	+

+++ = very strong effect, ++ = strong effect, + = effect, – = no effect.

PR-B. Here, ZK 230211 induces a different PR conformation that is more affine for NCoR than induced by onapristone or mifepristone (Fig. 3B). The recruitment under mammalian two-hybrid conditions is likely influenced by the cellular environment (e.g. presence of other secondary cofactors) and the use of the truncated PR-LBD (instead of full-length constructs, which displayed lacking transfection efficacy in this setting and resulted in a low signal-to-noise ratio). As the DNA binding domain and the N-terminus of the progesterone receptor can influence cofactor binding properties [42,43], and cellular squelching and transduction pathway alterations might be present in transformed cellular systems, the GST-pulldown experiments comprising the full-length proteins is more suitable to determine the specific direct binding affinity of these two proteins. The lacking efficacy of cofactor recruitment by mifepristone and onapristone in the GST-pulldown experiment could result from a cofactor recruitment to the PR below detection limits, underlining the high affinity of ZK 230211-occupied PR.

4.2. Non-genomic signaling

Non-genomic activation of c-Src/p21ras/Erk and PI3K/Akt-dependent pathways has been implicated in the stimulation of human breast cancer cell cycle progression and proliferation evoked by the progesterone receptor [22–26]. An activation of c-Src kinase downstream MAPK signaling and cyclin D1 expression has already been demonstrated for the PR antagonist mifepristone [23]. Although the biological relevance in the absence of progesterone remains to be assessed we analyzed the differences of the three PR antagonists with regard to this PR stimulation. The mammalian two-hybrid model system for PR-B/c-Src interaction demonstrated that all PR modulators induce an association of PR-B with c-Src kinase in the absence of progesterone. The requirement of c-Src SH3 domain for receptor interaction (Fig. 4B) confirms previous reports on activation of non-genomic signaling by PR-SH3 domain interaction [44]. ZK 230211-occupied PR reveals the lowest efficacy for interaction with c-Src kinase in HeLa cells and subsequently, the lowest downstream cyclin D1 promoter activation in an optimized reporter gene assay. In these assay systems, mifepristone-occupied PR is fully active and similar to R5020 in efficacy and potency (Fig. 4A and C). Interestingly, onapristone-bound PR-B also displays a strong association with c-Src kinase and enhanced cyclin D1 promoter activity. The markedly weaker potency in the non-genomic signaling activities of onapristone (Fig. 4A and C) might be associated with lower receptor binding activity for onapristone. This is concordant with the reduced antagonistic potency in transactivation assays (Table 1). The reduced affinity of the ZK 230211-bound PR to c-Src kinase could be a direct consequence of the receptor conformation or might be affected by the increased nuclear accumulation of ZK 230211-occupied PR which was observed for endogenous receptor levels in T47D cells (Figs. 2 and 3). As a consequence, ZK 230211-binding would result in a reduced availability for PR rapid signaling in the cytoplasm. The biological consequence of residual stimulation of non-genomic signaling is unknown. However, it is not sufficient to stimulate cell cycle progression per se since ZK 230211 inhibits S phase entry when applied alone in T47D cells (Fig. 5B). Furthermore, gene expression profiling studies in T47D cells indicate an inhibition of transcription of cell cycle progressors after ZK 230211 treatment, cyclin D1 expression was not induced, while cyclin-dependent kinase inhibitors like p21 are increased (unpublished data). It is therefore reasonable to assume that the overall effect of PR-antagonist action is an integral of its non-genomic and genomic activities in the intact PR signaling system (like in T47D cells) [22] and that genomic signaling-induced inhibitory effects on cell cycle progression dominate.

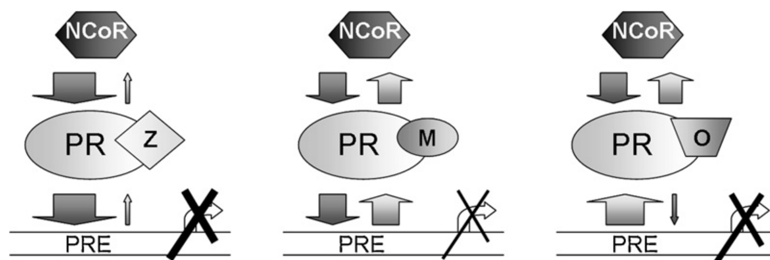

Fig. 6. The inhibition of PR-mediated genomic signaling by PR modulators is influenced by different determinants. Two of these which displayed differences between PR antagonists are depicted here: Firstly the DNA binding of the PR (strong for ZK 230211, left panel; weak or absent for onapristone, right panel) and secondly, the recruitment of corepressors, e.g. NCoR (strong for ZK 230211, left panel). PR = progesterone receptor, NCoR = nuclear receptor corepressor, PRE = progesterone responsive element, Z = ZK 230211, M = mifepristone, O = onapristone; size of the arrows define shift of the binding equilibrium.

4.3. Cell cycle regulation in the T47D breast cancer cell line

The inhibition of estradiol-induced S phase and G2/M phase entry provides evidence for a modulation of ERα signaling by ZK 230211-occupied PR. This modulation could be of direct or indirect manner. Interestingly, ZK 230211 antagonizes estradiol-induced distribution of cell cycle stages whereas onapristone does not (Fig. 5E and F). Mifepristone reverses enhanced G2/M phase, but is unable to affect estradiol-induced S phase entry, suggesting distinct mechanisms for the expression of S phase and G2/M phase regulator proteins that involve PR and ERα signaling. The inability of onapristone to antagonize estradiol actions on cell cycle progression under these experimental conditions suggests that nuclear localization and binding to the PREs might be prerequisite for the inhibition of ERα mediated T47D cell cycle progression.

5. Conclusion

Overall, the described characteristics of ZK 230211 are consistent with previous reports. In addition to the results described in the presented study, it does not display agonistic activities in the presence of the protein kinase A activator cAMP in a transactivation assay in T47D cells: ZK 230211 is similar to onapristone with regard to its pure antagonistic behavior in transcriptional activation while mifepristone becomes an agonist under identical experimental conditions [17,45]. Furthermore, in a recently reported gene expression analysis in T47D cells, a strong PR antagonistic activity of ZK 230211 was confirmed: ZK 230211 predominantly led to a down-regulation of PR target genes. The gene expression profile induced by ZK 230211-occupied PR clearly distinguishes from the gene expression profile induced by the antagonists onapristone and mifepristone in the absence of progesterone [46].

In summary, the data demonstrate ZK 230211 to be a PR antagonist with in vitro characteristics distinct from those demonstrated by the classical antagonists mifepristone and onapristone (Table 2). ZK 230211 displays higher PR antagonistic potency and higher selectivity at steroid hormone receptors. In combination with the weak activation of non-genomic c-Src kinase mediated signaling and the inhibition of estrogen-induced breast cancer cell cycle progression, ZK 230211 exhibits a spectrum of favorable in vitro properties which have not been identified in this extent for the classical antagonists. Our observations suggest ZK 230211 to be a more efficient type of antagonist, whose underlying antagonistic potency might be based on two direct molecular determinants which act additively or even synergistically: Firstly, the strong association of the ZK 230211-bound PR to PREs that prevents binding of agonist-bound PR or non-liganded PR with residual activity, and secondly, the intense recruitment of NCoR that leads to a transcriptionally inhibitory PR (Fig. 6). This type of antagonist, as represented by ZK 230211, might have favorable potential in clinical applications.

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