Molecular and Cellular Endocrinology 333 (2011) 37–46

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

The antiprogestin Lonaprisan inhibits breast cancer cell proliferation by inducing p21 expression

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ARTICLE INFO

Article history:
Received 10 August 2010
Received in revised form
29 November 2010
Accepted 30 November 2010

Keywords:
Progesterone receptor
Lonaprisan
p21
Cell cycle arrest
Sp1

ABSTRACT

The ovarian steroid hormone progesterone is essential for normal mammary gland physiology but may also play a role in breast cancer. Highly potent and selective antiprogestins may therefore represent a new treatment option for this disease. Here we studied the effects of the new antiprogestin Lonaprisan on the T47D breast cancer cell line. Strong inhibition of cell proliferation and arrest in the G0/G1 phase were observed, as well as induction of a senescence-like phenotype. This was accompanied by p21 induction through direct binding of Lonaprisan-bound progesterone receptor (PR) to the promoter. Reduction of p21 levels blunted the antiproliferative effects of Lonaprisan. Mutation analysis showed that intact PR DNA-binding properties were needed for p21 induction. Phosphorylation of PR Ser345 was stimulated by Lonaprisan, but this post-translational modification was not required for p21 promoter activation, nor was the interaction with c-Src needed. These results support the rationale for using antiprogestins in breast cancer treatment and warrant further studies to better understand their mode of action.

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1. Introduction

Initial development of the mammary gland takes place during embryogenesis. Further important modifications occur at puberty but full development only takes place during pregnancy, in preparation for lactation (Conneely et al., 2007). These changes require progesterone, an ovarian steroid hormone with essential roles in the proliferation and differentiation of the mammary epithelium (Lange et al., 2008). A role of progesterone in mammary cancer has been debated for many years and is still controversial (Lange and Yee, 2008). Since the publication of the WHI and Million Women reports which show that some estrogen/progestagen combinations used in hormone replacement therapy are linked to increased breast cancer risk (Rossouw et al., 2002; Beral, 2003), many studies have been performed to clarify the role of the progesterone receptor (PR) in mammary cell carcinoma (Lange and Yee, 2008; Gadducci et al., 2009). One reason for the controversy is that progestins used in hormone replacement therapy have different mixed agonist/antagonist profiles and are not necessarily entirely selective for the PR (Spitz, 2006). Also, progesterone has a biphasic effect on cell proliferation and is difficult to study in isolation from other hormones that also contribute to breast cancer biology. One example is estrogen which directly stimulates PR expression upon binding to its cognate receptor, another one is prolactin, which is essential for epithelial mammary cell proliferation during lactation (Lange, 2008).

Studies with a mouse model deficient in PR show a lower incidence of carcinogen-induced mammary tumor (Lydon et al., 1999; Ismail et al., 2003). Mice lacking BRCA-1 and p53 expression in their mammary gland overexpress PR and develop aggressive tumors which are highly responsive to antiprogestins (Poole et al., 2006). A transgenic mouse model overexpressing the PR-A isoform shows abnormal mammary development and alterations in growth potential (Chou et al., 2003). This is not observed in mice overexpressing PR-B. *In vitro*, numerous experiments performed with breast cancer cell lines document the importance of PR in proliferation and colony formation (Musgrove et al., 1993; Faivre and Lange, 2007; Afhüppe et al., 2010), and crosstalk with growth factor signaling pathways have been evidenced (Skildum et al., 2005; Daniel et al., 2007; Faivre and Lange, 2007). In addition, protective effects of progestins against apoptosis have been reported (Moore et al., 2000).

The PR belongs to the steroid receptor family of ligand-activated transcription factors (Scarpin et al., 2009). Different PR isoforms encoded by the same gene have been described. The main isoforms, PR-A and PR-B, are transcribed from two different promoters and differ only at their N-terminal end which is shortened by 164 amino acids in the PR-A form (Kastner et al., 1990). Despite similar hormone- and DNA-binding activities, the two isoforms differ with regard to their transcriptional activities (Richer et al., 2002; Giangrande and McDonnell, 1999). PR-B is considered a stronger activator than PR-A, possibly because of its third transactivation

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doi:10.1016/j.mce.2010.11.034

domain (AF-3) present in the N-terminal region (Sartorius et al., 1994). Additional truncated forms have been described but their natural occurrence is a matter of debate (Samalecos and Gellersen, 2008). Following activation, the PR undergoes a conformational change leading to dissociation from chaperone proteins and translocates into the nucleus where it will bind to progesterone response elements (PREs) in the regulatory regions of target genes (Scarpin et al., 2009).

Post-translational modifications have been described for all steroid receptors, including the PR (Faus and Haendler, 2006). Several of these modifications have been linked to rapid, non-genomic effects. Concerning the PR, phosphorylation and sumoylation have been best studied (Daniel and Lange, 2009). Sumoylation at Lys388 leading to repression of PR transcriptional activity has been evidenced (Daniel et al., 2007). This is prevented by phosphorylation of Ser294 which takes place downstream of the MAPK pathway. Ser345 is also phosphorylated by MAPK, following activation of the pathway by c-Src, which is directly triggered by liganded PR (Boonyaratanakornkit et al., 2001; Faivre et al., 2008). Ser345 phosphorylation leads to binding of the PR to the Sp1 transcription factor and activation of Sp1-controlled target genes. These post-translational modifications play decisive roles in the fine expression control of specific gene subsets (Daniel and Lange, 2009).

Together with the preclinical data described above, the presence of PR in about one third of breast cancer cases (Sorlie et al., 2001) and the observed link between PR isoform dysbalance and tumor aggressiveness (Mote et al., 2007; Kariagina et al., 2008) suggest that potent and selective PR antagonists may help preventing tumor progression. Several antiprogestins have already been described, but only a few have reached the clinic for various indications including contraception, endometriosis and uterine leiomyoma (Spitz, 2006). These compounds are either pure antagonists or mixed agonists/antagonists. The first PR antagonist mifepristone (RU486) was described in the early eighties (Baulieu, 1987), followed a few years later by onapristone (Wiechert and Neef, 1987). Mixed agonists/antagonists such as asoprisnil have also been identified (DeManno et al., 2003). All these compounds are derived from steroids and more recently, non-steroidal PR antagonists have also been described, but none of them has reached the clinic (Spitz, 2006). Herein we analysed the effects of the steroidal PR antagonist Lonaprisan (ZK 230211) (Fuhrmann et al., 2000; Afhüppe et al., 2010) a type III PR antagonist, on the breast cancer cell line T47D by investigating the molecular mechanisms responsible for its antiproliferative activity. Exposure to Lonaprisan inhibited T47D cell growth and led to an arrest in the G0/G1 phase of the cell cycle, while inducing senescence. This was associated with induction of the cyclin-dependent kinase inhibitor p21 and involved the Sp1 transcription factor.

2. Materials and methods

2.1. Drugs

PR antagonists (Lonaprisan, onapristone, mifepristone (RU486), asoprisnil, the standard PR agonist (R5020), the ER agonist 17β-estradiol (E2), the ER antagonist tamoxifen, and Taxol were synthesized at Bayer Schering Pharma AG (Berlin, Germany). Doxorubicin and saponin were from Sigma–Aldrich (Steinheim, Germany).

2.2. Cell lines

The T47D and MCF-7 human breast cancer cell lines, and the HeLa cervical carcinoma cell line were grown at 37 °C in a 5% CO_2 humidified environment in T75 flasks. T47D cells were grown routinely in phenol red-free RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% charcoal-stripped hormone-free fetal bovine serum, 4 mmol/l L-glutamine (both from Biochrom AG), 200 mU/ml insulin (Sigma–Aldrich) and 0.1 nM estradiol. MCF-7 cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% fetal bovine serum (Biochrom AG) and 4 mmol/l L-glutamine. HeLa cells were grown in DMEM/Ham's F-12 medium (Biochrom AG) supplemented with 10% fetal bovine serum. For chromatin immunoprecipitation (ChIP) experiments, transcription assays and for the analysis of PR phosphorylation, cells were grown for 48 h with starvation media consisting of phenol red-free RPMI 1640 medium supplemented with 5% charcoal-stripped hormone-free fetal bovine serum.

2.3. Cell proliferation

Cells were seeded at 10,000 cells/well in 96-well plates. After 24 h, compounds were added in fresh medium. At the desired timepoint Alamar Blue (Invitrogen, Karlsruhe, Germany) was added to the plates at 10% of the culture medium. After 2 h of incubation, proliferation was monitored by measuring the fluorescence emitted by a REDOX indicator using a microtiter well plate reader.

2.4. Viability assay

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

2.5. Cell cycle analysis

To perform cell cycle analysis, 200,000 cells per well were seeded in six-well plates in their growth medium and allowed to attach overnight. 10 nM Lonaprisan or vehicle (EtOH) were then added. After the indicated timepoints, cells were harvested by trypsinisation, pelleted by centrifugation and washed once with PBS (Biochrom AG). Media, washes and the pelleted cells were kept together. The cells were resuspended by pipetting several times in 1 ml ethanol (70%) in order to get a homogenous single-cell suspension. They were stored at −20 °C until the day of analysis when they were centrifuged and washed once with PBS. Cell pellets were then resuspended in 0.2 ml PBS containing 1.25 mg/ml ribonuclease A and 50 μg/ml propidium iodide (both from Sigma–Aldrich). After careful resuspension, cells were transferred into a filter cap-fitted polystyrene tube (Falcon; BD Biosciences, Heidelberg, Germany) and incubated at 4 °C in the dark for 4 h. Cells were then analysed in a fluorescence-activated cell sorting Caliber flow cytometer (FACS Calibur; BD Biosciences). The results were processed using the ModFit LT software (Verity Software House, Topsham, ME).

2.6. Apoptosis assay

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

2.7. Senescence assay

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

2.8. Western blot analysis

Whole-cell lysates were collected on ice by scraping in M-PER Mammalian protein extraction reagent (Thermo Fisher Scientific, Dreieich, Germany), supplemented with complete protease cocktails inhibitor tablets (Roche Diagnostics, Mannheim, Germany), 0.5 mM DTT (Sigma–Aldrich) and benzonase (Merck KGaA, Darmstadt, Germany). They were then incubated for 30 min on ice with periodic vortexing. Lysates were clarified by centrifugation for 10 min at 14,000 rpm and at 4 °C. Soluble proteins were quantified by the Bradford method using the BCA protein assay (Thermo Fisher Scientific), and equal amounts of protein were resolved by SDS-PAGE. The proteins were electrotransferred to a polyvinylidene difluoride membrane (Invitrogen), immunoblotted with a specific antibody, and developed using AmershamTM ECLTM Western Blotting detection reagents according to the manufacturer's protocol (Amersham Biosciences, Freiburg, Germany).

2.9. Antibodies for Western blot analysis

Primary antibodies for the following proteins were used at the indicated dilutions: p21 (C-19; 1:200) and Sp1 (PEP-2; 1:200) antibodies were obtained from

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