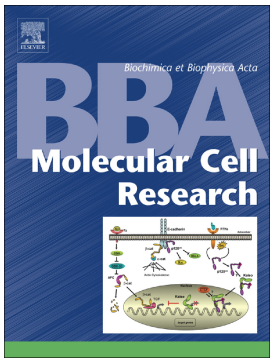

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Selective inhibition reveals cyclin-dependent kinase 2 as another kinase that phosphorylates the androgen receptor at serine 81

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Abstract

Several studies have revealed that cyclin-dependent kinases (CDK) can mediate phosphorylation of steroid receptors at multiple sites, including serine 81 of the androgen receptor (AR). Phosphorylation of S81 is required for AR nuclear translocation in association with chromatin and also regulates endogenous AR-regulated transcription in response to hormones. Up to date, S81-phosphorylation has been studied using different CDK inhibitors. Nevertheless, most inhibitors are non-selective or have unknown selectivity. We investigated the selectivity of commercially available CDK inhibitors and identified compounds that will be suitable for further studies to identify the CDKs responsible for S81-AR phosphorylation. We confirmed the positive impact of CDK1 and CDK9 on phosphorylation of S81 of AR and its transcriptional activity. Although CDK1-mediated phosphorylation was previously shown to occur physiologically during mitosis, our experiments did not confirm this hypothesis. By using chemical and genetic inhibition techniques, we identified that CDK2 contributes to S81-AR phosphorylation and transactivation while CDK4 was not shown to be involved in this process.

Highlights

- Cyclin-dependent kinases 1 and 9 have the positive impact on S81-phosphorylation of androgen receptor
- G2/M arrest does not mediate S81 phosphorylation and transcription of androgen receptor
- Chemical and molecular inhibition of CDK2, but not CDK4 contributes to S81-phosphorylation of androgen receptor

Keywords

Androgen receptor, cyclin-dependent kinase, phosphorylation, serine 81, inhibitor

Abbreviations

CDK - cyclin-dependent kinases; PTMs - post-translational modifications; AR - androgen receptor, PCa - prostate cancer; PSA - prostate specific antigen; ENZ - enzalutamide; CSS - charcoal stripped serum; FBS - fetal bovine serum; ER - estrogen receptor; PR - progesterone receptor; DRB - 5,6-dichloro-1-*D*-ribofuranosylbenzimidazole

1. Introduction

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that belong to the CMGC group of kinome and their activities are regulated by cyclin-binding and inhibitors from the CIP/KIP and INK4 families in response to several extra- and intracellular signals [1, 2]. Deregulation of CDKs is frequently associated with human cancers [3, 4], and therefore, the development of synthetic inhibitors is a therapeutic strategy that has recently resulted in the approval of palbociclib (PD0332991, Ibrance®) and ribociclib (LEE011, Kisqali®) for the treatment of advanced breast cancer [5-7]. The CDK family comprises 20 members that are responsible for the phosphorylation of substrates that are important for cell cycle progression and transcription. Several studies have revealed that CDKs can also mediate steroid receptor phosphorylation at multiple sites, including the androgen receptor [8-10].

The androgen receptor (AR) is a steroid hormone receptor that plays a crucial role in the normal development of male reproductive tissues, and its high expression and/or relaxation of its regulation are strongly implicated in prostate cancer (PCa) [11]. Androgen binding induces conformational changes of AR that influence its interactions with other proteins and DNA as well as its subcellular localization and transcriptional activity. AR is further regulated by numerous post-translational modifications (PTMs) that affect its physiological role, especially its transcriptional program. Most phosphorylation events are mediated by different kinases, including phosphorylation of serine, threonine or tyrosine residues, which are distributed along the whole AR sequence, unlike other types of PTMs [8, 10]. While the proximal kinases for some phosphosites have been identified, the function of several phospho-residues and the kinases responsible for their phosphorylation are still unknown. One of the most frequently studied phosphosites, serine 81, has been shown to be phosphorylated by the CDK1, CDK5 and CDK9 kinases [12-16]. Notably, overexpression of CDK1 and cyclin B have been suggested to contribute to increased AR activity in prostate cancer and its resistance to AR antagonists [16]. Several studies have suggested that CDK1 can also cooperate with CDK7 and CDK11 to phosphorylate the other two residues of the androgen receptor, serine 515 [17-19] and serine 308 [20, 21], respectively.

Our study focuses on the phosphorylation of S81, the function of which was previously investigated using various approaches, including S81-phospho-site mutants, AR overexpression, inhibition, siRNA, chromatin-immunoprecipitation and cell reporters [12-16]. Previous studies have demonstrated that S81 phosphorylation is required for AR nuclear translocation and association with chromatin and also regulates endogenous AR-regulated transcription in response to hormones, resulting in positive effects on cell growth.

Previous reports were mostly supported by chemical inhibition of CDKs, but in our opinion, the observed effects are usually pharmacologically corroborated using non-selective CDK inhibitors or CDK inhibitors with unknown selectivity. The use of inhibitors with unclear selectivity might lead to misleading conclusions, especially when these inhibitors are used as a tool for evaluating certain biological processes that are linked with the function of a particular CDK. Roscovitine is an example, and it was initially believed to be a relatively specific inhibitor of CDK1, 2 and 5. However, subsequent studies have demonstrated that it also blocks transcription through inhibition of CDK7 and 9 [22, 23]. Importantly, at least two groups used roscovitine as a tool in studies related to the function of AR and showed its ability to block androgen-stimulated phosphorylation of S81 of AR in LNCaP cells [12, 15], but each group pointed to a different CDK as the responsible enzyme. Independently, CGP74514A was shown to explain the role of CDK1 in the phosphorylation

of S81-AR [13]. Nevertheless, the available selectivity profile and our own data suggest that this compound is less selective and not suitable as a tool.

We therefore decided to verify the potential role of different CDKs in the phosphorylation of S81-AR using a combination of chemical inhibition and siRNA silencing. We systematically characterized the selectivity of different CDK inhibitors on a panel of recombinant CDK/cyclin complexes to complete or verify information that was already published. To improve our understanding of the selectivity of inhibitors, we evaluated their cellular activities in PCa cells by monitoring their substrates using immunoblotting analysis. A key objective of this part of the study was to identify the optimal concentrations of inhibitors and treatment durations for the inhibition of particular CDKs without side effects (e.g., off-target effects or massive apoptosis). Most selective compounds were then used for analyses of the phosphorylation of androgen receptors at S81. A comparison of transient silencing of individual CDKs in PCa cells correlated well with the effects of pharmacological inhibition of CDKs as well as with the AR transcriptional activity in a cellular reporter.

We confirmed the positive impact of CDK1 and CDK9 on the phosphorylation of S81 of AR and its transcriptional activity. Although CDK1-mediated phosphorylation has been shown to occur physiologically during mitosis, our experiments did not confirm this hypothesis. Using chemical and genetic inhibition techniques, we showed that CDK2 can contribute to S81-AR phosphorylation and transactivation, while CDK4 was shown to not be involved in this process. Taken together, our observations contribute to the further understanding of the relationship between S81-AR and CDKs.

2. Materials and Methods

2.1 Reagents

Specific reagents were purchased from PerkinElmer (R1881), Sigma Aldrich (nocodazole, RO3306, PHA767491), MedChemExpress (abemaciclib, palbociclib, LDC000067), Santa Cruz Biotechnology (NU6102, GW8510), Enzo Life Sciences (CGP74514A) and Calbiochem (SU9516). Enzalutamide was kindly provided by Dr. Frederic R. Santer from Innsbruck Medical University. Specific siRNAs were purchased from Dharmacon (CDK9, L-003243-00-0020; CDK1, L-003224-00-0020; CDK2, L-003236-00-0020; CDK4, L-003238-00-0010) or Santa Cruz Biotechnology (control siRNA, sc-37007).

2.2 Cell lines and transfection

Prostate cancer cell lines (C4-2, LAPC-4 and LNCaP) were authenticated using the AmpFLSTR® Identifiler® PCR Amplification Kit (Applied Biosystems). LNCaP cells were purchased from ECACC. LAPC-4 and C4-2 cells were kindly provided by Dr. Frederic R. Santer from Innsbruck Medical University and Dr. Marian Hajduch from Palacky University in Olomouc, respectively. The 22Rv1-ARE14 reporter cell line (Bartoňková et al., 2016) was a generous gift from Prof. Zdeněk Dvořák (Department of Cell Biology and Genetics, Palacký University). The C4-2 and LNCaP cell lines were grown in 25 mM HEPES-modified RPMI-1640 medium, The LAPC-4 cell line was grown in Iscove´s Modified Dulbecco´s medium, and the 22Rv1-ARE14 cell line was grown in RPMI-1640 medium. All media were supplemented with 10 % normal or charcoal-stripped fetal bovine serum, 4 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate. Cells were maintained in a humidified CO_2 incubator at 37 °C. The cells were transfected by siRNAs specific to certain CDKs using Lipofectamine® RNAiMAX (ThermoFisher Scientific) according to the manufacturer´s protocol.

2.3 Immunoblotting and antibodies

Briefly, cell lysates were prepared by harvesting cells in Laemmli sample buffer. Proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. After blocking the membranes, they were incubated with specific primary antibodies overnight, washed and then incubated with peroxidase-conjugated secondary antibodies. Finally, peroxidase activity was detected using Pierce™ ECL Western blotting substrates and a CCD camera LAS-4000 (Fujifilm). The specific primary and secondary antibodies are listed in **Supplementary Table S2.**

2.4 Kinase inhibition assay

CDK/cyclin complexes were assayed with appropriate substrates in the presence of ATP (**see Supplementary Table S3**), 0.05 µCi [γ-^{33}P]ATP and the test compound in in a reaction buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 2.5 µg / 50 µl $PEG_{20.000}$) to a final total volume of 10 µL. The reactions were stopped by adding 5 µL of 3 % aq. H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3× with 0.5 % aq. H_3PO_4 and air-dried. Kinase inhibition was quantified using an FLA-7000 digital image analyser (Fujifilm). The concentration of the test compounds required to decrease CDK activity by 50 % was determined from the dose-response curves and designated as IC_{50}.

2.5 Cell cycle analysis

PCa cells were treated with tested compounds at different concentrations for 24 h and were then trypsinized, washed with PBS, fixed with ice-cold 70 % ethanol and incubated on ice overnight. Following hydration, the cells were stained with propidium iodide (0.01 mg/mL, Sigma Aldrich) for 1 h at room temperature in the dark and finally analysed by flow cytometry using a 488-nm laser (BD FACS Verse with software BD FACSuite™, version 1.0.6.).

2.6 AR-transcriptional reporter assay

22Rv1-ARE14 cells were seeded into a 96-well plate. The next day, the cultivation medium was discarded and the cells were incubated in the absence or presence of tested compounds dissolved in RPMI-1640 medium supplemented with charcoal-stripped serum and 1 nM R1881 for 7 hours. After an incubation period, the cells were washed with PBS and lysed for 10 min in a lysis buffer (10 mM TRIS pH = 7.4, 2 mM DCTA, 1 % nonidet P40, 2 mM DTT). After lysing, a flash mix solution (20 mM tricin pH = 7.8, 1.07 mM $MgSO_4$. $7H_20$, 5 mM ATP, 9.4 µM luciferin) was added to the wells and the luminescence of the samples was measured using a Tecan M200Pro microplate reader.

3. Results

3.1 Activation of S81-AR phosphorylation in PCa cells after androgen stimuli

Phosphorylation of AR at S81 in response to androgen treatment in some PCa cells has been previously documented [12, 13, 24, 25]. Nevertheless, we decided to verify S81-phosphorylation upon androgen (R1881) treatment in two prostate cancer cell lines (PCa), C4-2 and LAPC-4, both showing androgen-independent growth, but an androgen-responsive phenotype.

In both cell lines, we observed a dose-dependent increase of S81-AR phosphorylation upon R1881 stimulation up to a concentration of 100 nM (**Fig. 1**). The relationship between S81-AR phosphorylation and the transcriptional activity of AR was confirmed by monitoring the expression of the known AR transcriptional targets PSA and NKX3.1. Only C4-2 cells showed a relatively high basal expression of PSA in the absence of R1881. Nevertheless, this trend refers to a high level of constitutive activity of AR [24, 26].

Time-dependent experiments showed that phosphorylation at S81 in C4-2 cells was maximally induced between 4 - 24 hours of androgen treatment, as previously documented [12, 13], but the increase in S81-phosphorylation in LAPC-4 cells in response to androgen was relatively slow (**Fig. 1**). Phosphorylation and expression of PSA and NKX3.1 slightly increased after 16 hours, and the effects of androgen treatment were observed until 48 hours.

Based on the different responses to androgen stimulation in these experiments, C4-2 cells were treated simultaneously with sub-lethal concentrations of an inhibitor (**Supplementary Fig. 1**) and R1881 for 8 hours, whereas LAPC-4 cells were treated for 24 hours.

3.2 Selectivity of commercially available CDK inhibitors

To identify the CDKs that are responsible for S81-AR phosphorylation, we first investigated the selectivity of 31 commercially available CDK inhibitors (**Table 1 and S1**). We performed profiling under the same experimental conditions and then selected the compounds that displayed the highest selectivity for further studies. The role of CDK1 in the process of AR phosphorylation has been previously studied using inhibitors with varying degrees of potency towards CDKs, including roscovitine, CGP74514A and RO3306 [12, 13, 16]. Whereas roscovitine is known to inhibit most CDKs and its suitability as a tool is therefore limited, CGP74514A [27] and RO3306 [28] are sold by many commercial vendors as being specific for CDK1. The CDK selectivity profile of RO3306 has not been published (with the exception of CDK2 and CDK9, which were reported to be poorly sensitive) [29], but targeting CDK1 in cells was observed independently in many studies with different cell types and usually resulted in the blockade of mitosis [30-33] or cyclin B accumulation [28, 34]. Our biochemical profiling confirmed its stated preference for CDK1 (IC_{50} = 40 nM) over other members of the CDK family (see **Table 1**). We, as well other authors, noted that CGP74514A displays different cellular effects than RO3306, which suggests that they have different selectivities [30, 32, 35]. We therefore examined the selectivity of CGP74514A and found that it primarily targets CDK2 and CDK5 (**Table 1**). These results agree with the broad kinase selectivity profiling according to a previous radiometric filtration binding assay [36, 37]. Based on these results, RO3306 was selected for pharmacological inhibition of CDK1 in this study.

Studies investigating the impact of the pharmacological inhibition of CDK9 on the phosphorylation of AR at S81 were performed using flavopiridol, 5,6-dichloro-1-*D*-ribofuranosylbenzimidazole (DRB) and CDK9 inhibitor II (CAN508) [13, 14, 16], which display relatively low selectivity or potency for CDK9 [38, 39]. Recently, two novel inhibitors, PHA767491 [40] and LDC000067 [38], with high selectivity for CDK9 were identified. Our results confirmed that both of these compounds prefer CDK9, and the IC_{50} values for other CDKs were at least ten-fold higher (**Table 1**).

A link between AR phosphorylation and CDK2 or CDK4 has not been mentioned in the literature to date. For pharmacological inhibition of CDK2, we selected NU6102 [41-43], which is a compound that displays higher potency and better selectivity over tested CDK2 inhibitors and seems to be the most selective CDK2

inhibitor available (**Table 1**). For chemical inhibition of CDK4, we picked two structurally unrelated compounds, palbociclib (PD0332991) [44] and abemaciclib (LY2835619) [45], which are well-characterized tools that have been used in several studies. Our assays confirmed that they had nanomolar potency towards CDK4, whereas other CDKs were inhibited with concentrations up to the micromolar level (**Table 1**).

3.3 RO3306-mediated CDK1 inhibition suppresses the phosphorylation of AR at S81

Our data show that the PCa cell lines C4-2 and LAPC-4 cannot activate S81-phosphorylation in the presence of RO3306 after androgen stimulation. As shown in **Fig. 2**, a 10 µM concentration of RO3306 is sufficient to clearly suppress S81-phosphorylation compared to control cells after androgen activation and also diminished the transcriptional activity of AR, as shown through monitoring NKX3.1 and PSA expression. Inhibition of CDK1 by RO3306 in PCa cells after 24 h of treatment was clearly documented by increasing the number of cells in a G2/M population, especially in LAPC-4 cells, which reached up to ~ 80% (**Fig. 2c**). During the treatment of both PCa cell lines, no inhibition of transcriptional CDK7 and 9 was observed, which was evident from the constant levels of phosphorylated S2 and S5 of the carboxy-terminal domain of RNA polymerase II. In addition, we did not observe significant changes in phosphorylation of the retinoblastoma protein at the S807/811 and S780 phosphosites, which are the major targets of CDK2 and CDK4 (**Fig. 2a, b**). In a control experiment, siRNA silencing of CDK1 clearly blocked R1881-stimulated AR phosphorylation at S81 (**Fig. 2d**). These results, along with the results from flow cytometry, indicate that the CDK1 inhibitor RO3306 can decrease androgen-mediated phosphorylation of AR at S81 due to selective inhibition of CDK1 in PCa cells.

3.4 G2/M phase arrest does not lead to S81-phosphorylation and transcriptional activity of AR

Recent observations have suggested that phosphorylation of S81 is increased even in the absence of androgen in nocodazole-synchronized LNCaP cells [13, 16], which was explained by the physiologically high activity of CDK1 in mitosis. We were interested in whether transactivation of AR would be observed despite the fact that transcription is largely inhibited in mitosis [46].

For this experiment, we used an AR-dependent reporter cell line, 22Rv1-ARE14 (expressing endogenous AR), that was transfected with a reporter plasmid containing the sequence of an androgen response element from the promoter of human PSA [47]. Treatment of 22Rv1-ARE14 cells with different concentrations of nocodazole did not increase luciferase activity, in contrast to cells stimulated by R1881 (**Fig. 3a**). We therefore performed an immunoblotting analysis of S81-phosphorylation, and surprisingly, we did not observe increased S81-phosphorylation in nocodazole-treated 22Rv1-ARE14 cells (**Fig. 3b**). Consistent with the accumulation of cells in the G2/M phase of the cell cycle, we observed increased expression of cyclin B and phosphorylation of another mitotic marker, protein phosphatase 1 alpha (PP1α), at T320 (**Fig. 3c**). We first speculated whether the reporter cell line may have a mechanism to avoid S81 phosphorylation in the presence of nocodazole. Nevertheless, we did not observe increased phosphorylation of S81 in C4-2, LAPC-4 and LNCaP cells (**Supplementary Fig. 2**). Our data demonstrate that G2/M arrest does not mediate S81 phosphorylation and transcription of AR in different PCa cells.

3.5 Pharmacological inhibition of CDK9 diminishes androgen-mediated phosphorylation of AR at S81

To investigate the role of CDK9 in the phosphorylation of AR at S81, sub-lethal doses of LDC000067 were applied to cells. S81-AR phosphorylation was repressed in C4-2 cells after an 8-hour treatment with LDC000067 in a concentration-dependent manner (**Fig. 4a**). In a control experiment, R1881-stimulated phosphorylation at S81 was suppressed by CDK9 siRNA (**Fig. 4d**). Cellular inhibition of CDK9 was obvious due to the decreased phosphorylation of S2 and S5 of RNA polymerase II. Additionally, this decline correlated well with a dose-dependent decrease of Mcl-1, which belongs to antiapoptotic proteins that have a short half-life (**Fig. 4a**). Stronger effects were observed in LAPC-4 after 24 hours of treatment (**Fig. 4b**). All of these results were observed for the CDK9 inhibitor PHA767491 as well (see **Supplementary Fig. 3**) and corresponded with previous findings [13, 14, 16] that CDK9 can participate in the phosphorylation of AR at S81.

Interesting changes were observed in NKX3.1 expression: 10 μM LDC000067 decreased NKX3.1 and PSA to basal levels as expected, whereas higher doses completely diminished NKX3.1 expression. This trend was observed for PHA767491 as well (**Supplementary Fig. 3**), which clearly confirmed that the decline of NKX3.1 expression after treatment with higher doses of CDK9 inhibitors occurred due to general inhibition of transcription, which is not related to an AR-mediated process.

3.6 Pharmacological inhibition of CDK4 does not inhibit androgen-mediated phosphorylation of AR at S81

Many CDKs have been investigated in relation to AR phosphorylation, but no study has examined the role of CDK4 in this process to date. Pharmacological inhibition was examined with palbociclib (PD0332991), which is a selective nanomolar CDK4/6 inhibitor with a high selectivity index over 274 tested human kinases [48]. Palbociclib treatment did not change S81-AR phosphorylation in any cell line (**Fig. 5a, b**). The AR-transcriptional targets NKX3.1 and PSA were also not affected. Cell cycle analysis revealed that a 250 nM concentration of palbociclib caused accumulation of cells in the G1 phase of the cell cycle (**Fig. 5c**). In addition, dephosphorylation of Rb at S780 confirmed that palbociclib selectively targeted CDK4/6 in both PCa cell lines (**Fig. 5a, b**). All of these results correlate with experiments using abemaciclib (see **Supplementary Fig. 4**), which is another well-profiled and selective CDK4 inhibitor [45, 48]. We also silenced CDK4 expression with siRNA, which led to no changes in S81-AR phosphorylation after androgen activation in C4-2 and LAPC-4 cells (**Fig. 5d, e**). Based on these findings, we conclude that CDK4 does not phosphorylate AR at S81-phosphosite in PCa cell lines.

3.7 CDK2 contributes to the phosphorylation of the androgen receptor at S81

We finally asked whether CDK2 is involved in S81-AR phosphorylation. As shown in **Fig. 6d**, siRNA-mediated inhibition of CDK2 expression suppressed androgen-stimulated S81-AR phosphorylation in C4-2 cells.

To confirm our hypothesis, we also carried out a chemical approach to promote CDK2 inhibition. Short-time treatment of C4-2 cells with NU6102 (**Fig. 6a**) abrogated androgen-induced S81-AR phosphorylation. Subsequently, the expression level of NKX3.1 did not increase as it did in the androgen-stimulated control cells. Cellular inhibition of CDK2 by NU6102 was confirmed by dephosphorylation of the retinoblastoma protein at the S807/811 phosphosite. Importantly, unchanged phosphorylation of RNA polymerase II confirmed that CDK9 was not inhibited. Similar results were also observed after prolonged treatment of LAPC-4 cells (**Fig. 6b**). Surprisingly, whereas expression of the AR-transcription target NKX3.1 was suppressed, expression of PSA

remained unchanged under the same conditions. Exposure of C4-2 and LAPC-4 cells to NU6102 did not cause substantial changes in the cell cycle, which confirmed that there was no cellular interaction with CDK4 and CDK1 (**Fig. 6c**).

3.8 The impact of the pharmacological inhibition of different CDKs on the transcriptional activity of AR

Previous studies demonstrated that S81-phosphorylation is required for activation of AR and transcription of androgen-regulated genes [13, 14].

To verify and compare the effects of CDK inhibitors with different selectivity, we used 22Rv1-ARE14 that received short treatments (to minimize the cytotoxicity) with increasing concentrations of compounds in the presence of R1881 and then assayed luciferase activity and immunoblotted some AR-regulated genes. As shown in **Fig. 7a**, R1881 markedly induced luciferase activity in 22Rv1-ARE14 cells (~ 4-fold increase), whereas the activity was completely blocked in the presence of antiandrogen enzalutamide. As expected, luciferase activity was not diminished by the CDK4 inhibitor palbociclib up to a 1 μM concentration (**Fig. 7a**). On the other hand, inhibitors of CDK2 (NU6102), CDK1 (RO3306) and CDK9 (LCD000067) reduced luciferase activity in a dose-dependent manner (**Fig. 7a**). Immunoblot analysis revealed that levels of NKX3.1, PSA and S81-AR phosphorylation decreased, which correlated with the luciferase activity in these cells (**Supplementary Fig. 5**).

Finally, we assayed other commercially available CDK inhibitors (SU9516, GW8510 and CGP74514A) that also had preferences for CDK2/cyclin complexes and induced the same cell cycle arrest in cells [30, 49, 50] but displayed lower selectivity (see **Table 1** for CDK inhibition data). All of these inhibitors suppressed

4. Discussion

CDK1 is responsible for the phosphorylation of several cellular substrates that are predominantly important in the progression of mitosis and cytokinesis. Recently, it was suggested that CDK1 may be able to provide a pool of activated AR during mitosis [13]. Protein expression of AR during the cell cycle remains unchanged, but transcription of most AR-responsive genes is suppressed in mitosis [20]. Surprisingly, one study indicated that an increased level of S81-phosphorylation can be observed in nocodazole-G2/M-arrested LNCaP cells independently on androgen addition [13]. Because S81-phosphorylation is connected with transactivation of AR, we investigated whether increased transcription activity of AR would be observed in synchronized G2/M cells after nocodazole treatment. In the experiments reported here, we observed neither S81-phosphorylation nor transcriptional activation of AR in synchronized cells, which contrasted with previously published data [13, 16]. Therefore, phosphorylation of S81-AR during mitosis requires further investigation. Possible differences of the abovementioned results may be explained by the different experimental conditions used during LNCaP cultivation, which can clearly affect AR signalling. For example, a recent study revealed that a high cell density, time of cultivation and presence of androgens during cultivation can deregulate expression of several cell cycle regulators and influence the proliferation status of PCa cells [32]. Other reports also showed that the same type of PCa cells can display different patterns of AR expression and that its target PSA is probably influenced by the concentration of androgens in the media or by the duration of cultivation in charcoal-stripped serum at the beginning of the experiment [24, 51].

Published results have revealed relationships between serine 81 in AR and some CDKs, especially between S81 and CDKs 1, 5 and 9 [12-16]. These results are often supported by pharmacological inhibition of a

certain CDK. An effect of CDK1 inhibition on suppression of S81-phosphorylation was previously confirmed by many CDK inhibitors, including roscovitine and CGP74514A [12, 13, 16]. Nevertheless, the low selectivity of these inhibitors can influence the proper interpretation of the results. CGP74514A is a typical example of an incorrectly used CDK1 probe because it has other targets (**Table 1**) [36, 37]. RO3306 is widely considered to be a selective CDK1 inhibitor, but its kinase profile has not yet been published. We confirmed here for the first time that RO3306 selectively inhibits CDK1 over other CDKs and conclude that its effect on the phosphorylation of serine 81 is indeed caused by this kinase. Similarly, our experiments with two novel selective CDK9 inhibitors, LDC000067 and PHA767491, supported previous findings about the role of CDK9 in S81-phosphorylation of AR [13, 14, 16].

Refinement of the selectivity of CDK inhibitors led us to speculate on whether other interphase CDKs could be involved in this process. We focused on CDK2 and CDK4, which are known to interact with steroid receptors either directly or indirectly, but to the best of our knowledge, no previous reports have indicated a possible role of any G1-regulating CDK in S81-AR phosphorylation. The CDK2/A complex causes transcriptional activation of the estrogen receptor through its phosphorylation of serines 104 and 106 [52, 53]. Interaction of cyclin A with CDK2 also contributes to transactivation of the progesterone receptor via phosphorylation of multiple phosphosites [54, 55]. For the first time, we demonstrate a relationship between phosphorylation of S81-AR and CDK2 through siRNA-based silencing and pharmacological inhibition in PCa cells. The compound NU6102 has been shown to have a high preference for CDK2 and inhibit it at the lowest concentrations (see **Table 1** and references [41-43]), and therefore, it is a suitable tool for cellular studies. In C4-2 and LAPC-4 cells, NU6102 selectively inhibited CDK2 in subtoxic doses, diminished androgen-stimulated S81-phosphorylation and also supressed androgen-mediated transcription of AR-regulated genes in the reporter cell line 22Rv1-ARE14 in a concentration-dependent manner. Finally, similar results were observed with other CDK inhibitors with lower selectivity for CDK2 as well (**Table 1**, **Fig. 8**). Taken together, these results from chemical and molecular inhibition indicated that CDK2 contributes to S81-phosphorylation and transcriptional activity of AR.

Interestingly, cyclin D was reported to bind to AR and inhibit ligand-dependent AR activity [56-58]. Another study suggested that CDK6 can bind to AR in the presence of androgen and markedly enhance its transcriptional activity, but this process was documented as being independent of cyclin D1 and CDK6 kinase activity [59]. The stimulation impact on AR may be linked to frequent overexpression of CDK6 in human PCa, while cyclin D amplification is observed only rarely [60, 61]. Our results provide no evidence that CDK4 is involved in phosphorylation of AR at S81 and its transactivation. Silencing of CDK4 (as well as palbociclib and abemaciclib application) did not cause significant changes in the expression and phosphorylation of AR as well as of NKX3.1 and PSA expression. In addition, there was no effect on R1881-stimulated transcriptional activity in a reporter cell line, which also suggests that CDK4 has no effect on the transcriptional activity of AR in PCa cells.

Based on published reports and our own findings, we propose to combine genetic inactivation with pharmacological inhibition in mechanistic studies because the selectivity of many commercially available inhibitors is often limited. Experiments with non-selective inhibitors easily lead to misleading conclusions, as discussed for SB203508 or dasatinib [62]. Fortunately, selectivity profiles of many CDK inhibitors are increasingly found in publications [36, 37, 48, 63-66], which allow researchers to pick the most suitable

chemical tools for their studies. We also suppose that the role of CDKs in the phosphorylation of AR cannot be integrated into a definite model yet mainly because several studies have shown that cyclins or CDKs can behave as coactivators of steroid receptors independently of their kinase activities. Furthermore, CDK/cyclin complexes have been shown to be able to substitute for each other in a compensatory mechanism in cells [67]. In this study, we confirmed the importance of CDK1 and CDK9 in AR activation and identified CDK2 as another kinase that phosphorylates S81 in AR.

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Figure captions

Fig. 1 Activation of S81-AR phosphorylation in PCa cells after androgen stimulation. C4-2 (**a**) and LAPC-4 (**b**) were cultivated in charcoal-stripped serum medium (CSS) for 24 hours and then treated with different concentrations of R1881 (left panels) or with 1 nM R1881 for the indicated times (right panels). Lysates were blotted for detection of total AR, pS81-(AR) and AR transcriptional targets PSA or NKX3.1. Hsp70 and tubulin were included as controls for protein loading.

Fig. 2 The impact of CDK1 inhibition on phosphorylation of the androgen receptor at Ser81. C4-2 (**a**) or LAPC-4 (**b**) cells were grown in charcoal-stripped serum medium (CSS) for 24 hours and then stimulated by R1881 alone or with different doses of RO3306 (RO), as indicated. RO3306 was added 30 min before androgen treatment. Cellular expression of all proteins was detected by immunoblotting analysis. β-actin and Hsp70 were included as controls for protein loading. Representative western blots are shown; see the Supplementary data for the biological replicates. (**c**) Effects of RO3306 on the distribution of the cell cycle of C4-2 and LAPC-4 cells treated for 24 hours in FBS medium. Flow cytometric analysis (10 000 counts) of DNA stained by propidium iodide. (**d**) C4-2 cells were transfected with siRNA against CDK1 and cultivated in CSS medium for an additional 48 hours. Induction of phosphorylation of S81-(AR) was performed by adding R1881 8 hours before the harvest. Cellular expression of all proteins was detected through immunoblotting analysis. β-actin was included as a control for protein loading.

Fig. 3 Phosphorylation and transcriptional activity in a nocodazole-treated PCa cell line. (**a**) 22Rv1-ARE14 cells were grown in charcoal-stripped serum medium (CSS) for 24 hours in the presence of different doses of nocodazole for 24 hours, and the luciferase activity was measured. Cells stimulated by 1 nM R1881 (R) for 7 hours served as an internal control. (**b**) 22Rv1-ARE14 cells were cultivated in CSS medium for 24 hours and then treated with 40 ng/ml of nocodazole for the indicated times. Cells stimulated by 1 nM R1881 (R) for 24 hours served as an internal control. Lysates were blotted for detection of appropriate proteins and Hsp70 was included as a control for equal protein loading.

Fig. 4 The impact of pharmacological inhibition of CDK9 on the phosphorylation of the androgen receptor at Ser81. C4-2 (**a**) or LAPC-4 (**b**) cells were grown in charcoal-stripped serum medium (CSS) for 24 hours and then stimulated by R1881 alone or with different doses of LDC000067 as indicated. LDC000067 was added 30 min before androgen treatment. Cellular expression of all proteins was detected by immunoblotting analysis. β-actin was included as a control for protein loading. (**c**) Effects of LDC000067 on the distribution of the cell cycle of C4-2 and LAPC-4 cells treated for 24 hours in FBS medium. Flow cytometric analysis (10 000 counts) of DNA stained by propidium iodide. (**d**) C4-2 cells were transfected with siRNA against CDK9 and cultivated in CSS medium for an additional 48 hours. Induction of phosphorylation of S81-(AR) was performed by adding R1881 8 hours before the harvest. Cellular expression of all proteins was detected by immunoblotting analysis. β-actin was included as a control for protein loading.

Fig. 5 The impact of inhibition of CDK4 on the phosphorylation of the androgen receptor at Ser81. C4-2 (**a**) or LAPC-4 (**b**) cells were grown in charcoal-stripped serum medium (CSS) for 24 hours and then stimulated by

R1881 alone or with different doses of palbociclib (PD), as indicated. Palbociclib was added 30 min before androgen treatment. Cellular expression of all proteins was detected by immunoblotting analysis. β-actin was included as a control for protein loading. Representative western blots are shown; see the Supplementary data for biological replicates. (**c**) Effects of palbociclib on the distribution of the cell cycle of C4-2 and LAPC-4 cells treated for 24 hours in FBS medium. Flow cytometric analysis (10 000 counts) of DNA stained by propidium iodide. C4-2 (**d**) and LAPC-4 (**e**) cells were transfected with siRNA against CDK4 and cultivated in CSS medium for an additional 48 hours. Induction of phosphorylation of S81-(AR) was performed by adding R1881 8 hours (24 hours for LAPC-4) before the harvest. Lysates were blotted for detection of total AR, pS81-(AR) and CDK1. β-actin was included as a control for equal protein loading.

Fig. 6 The impact of pharmacological inhibition of CDK2 on the phosphorylation of the androgen receptor at Ser81. C4-2 (**a**) or LAPC-4 (**b**) cells were grown in charcoal-stripped serum medium (CSS) for 24 hours and then stimulated by R1881 alone or with different doses of NU6102 (NU) as indicated. NU6102 was added 30 min before the androgen treatment. Cellular expression of all proteins was detected by immunoblotting analysis. Hsp70 was included as a control for equal protein loading. Representative western blots are shown; see the Supplementary data for the biological replicates. (**c**) Effects of NU6102 on the distribution of the cell cycle of C4-2 and LAPC-4 cells treated for 24 hours in FBS medium. Flow cytometric analysis (10 000 counts) of DNA stained by propidium iodide. (**d**) C4-2 cells were transfected with siRNA against CDK2 and cultivated in CSS medium for an additional 48 hours. Induction of phosphorylation of S81-(AR) was performed by adding R1881 8 hours before the harvest. Cellular expression of all proteins was detected by immunoblotting analysis. β-actin and Hsp70 were included controls for protein loading.

Fig. 7 The influence of different CDK inhibitors on the AR-mediated transcription in the 22Rv1-ARE14 reporter cell line. Cells were grown in charcoal-stripped serum medium (CSS) for 24 hours and stimulated with 1 nM R1881 (R) alone or with different doses of CDK inhibitors for 7 hours, and then, the luciferase activity in the cell lysate was measured. Enzalutamide (ENZ) served as a negative control.

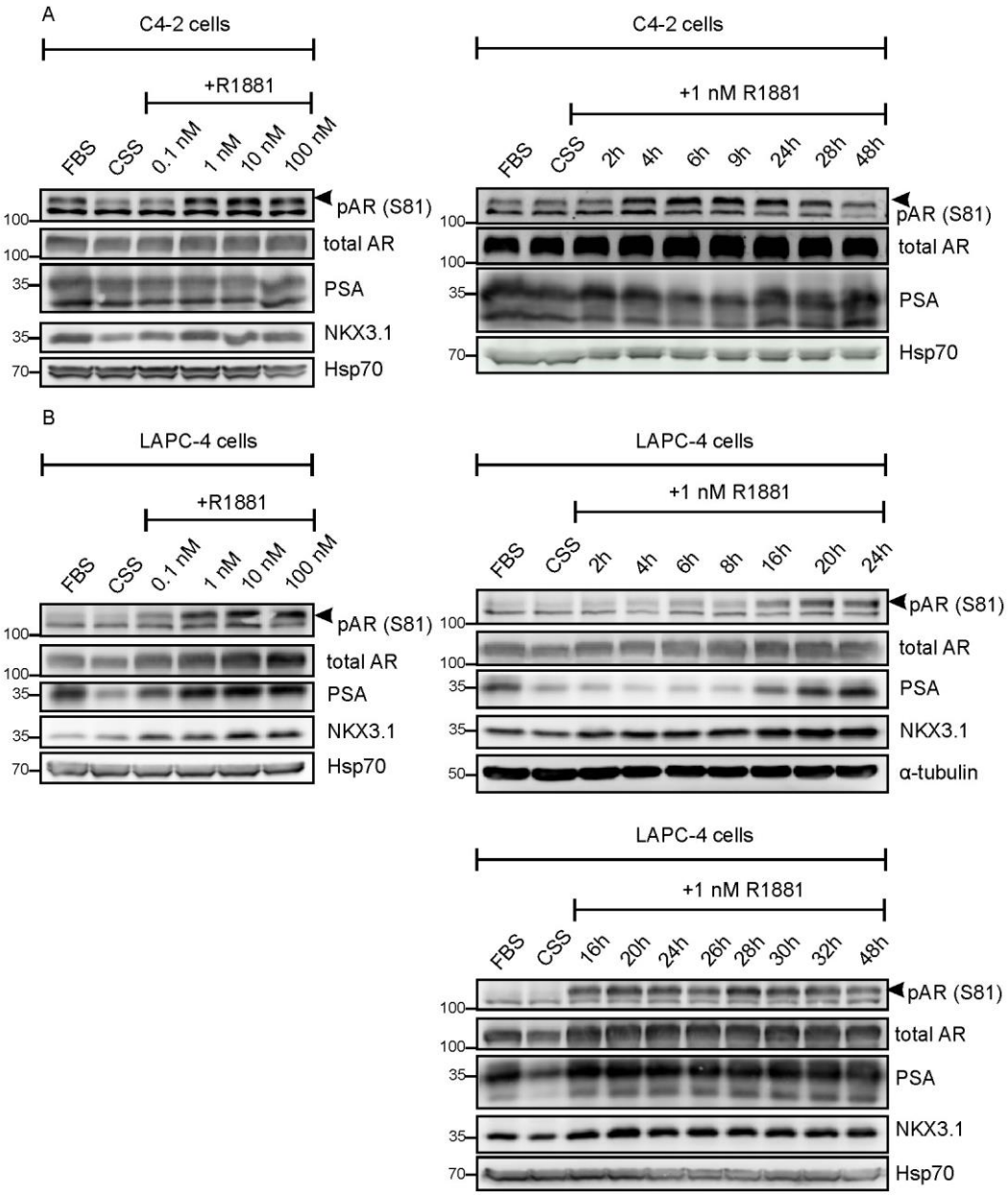

Fig. 1

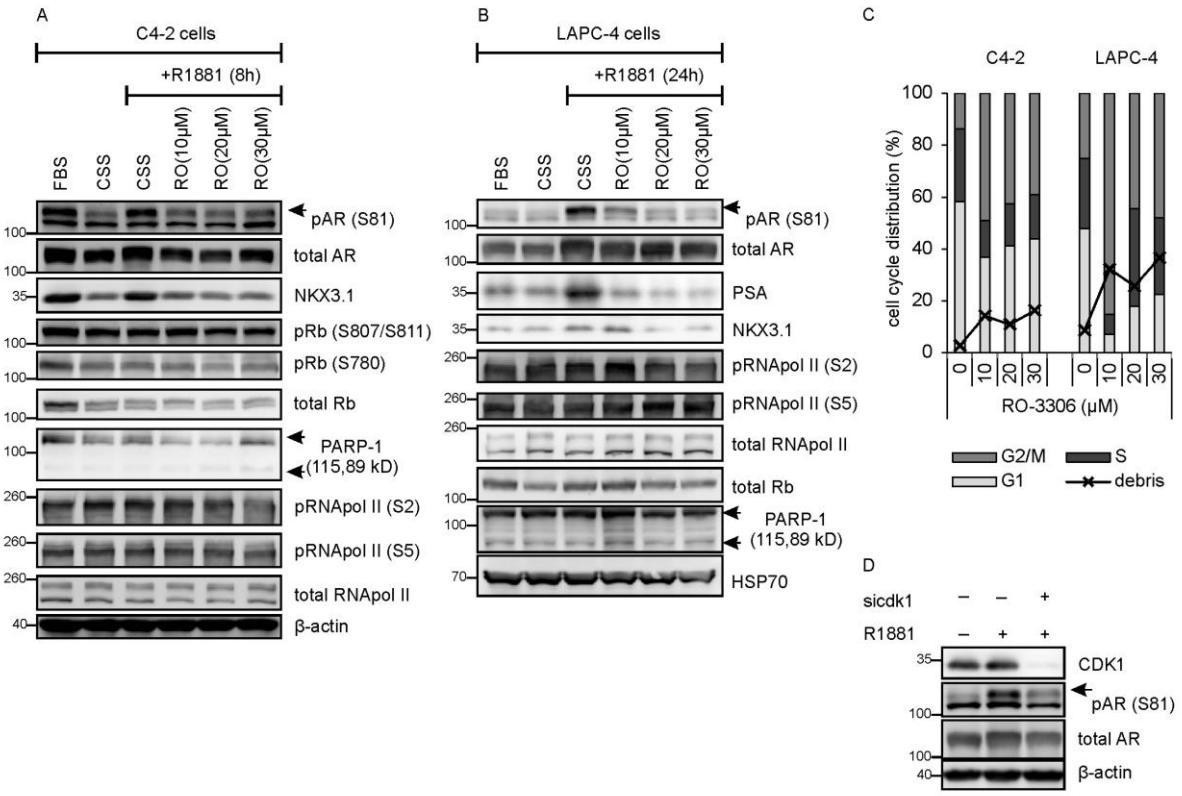

Fig. 2

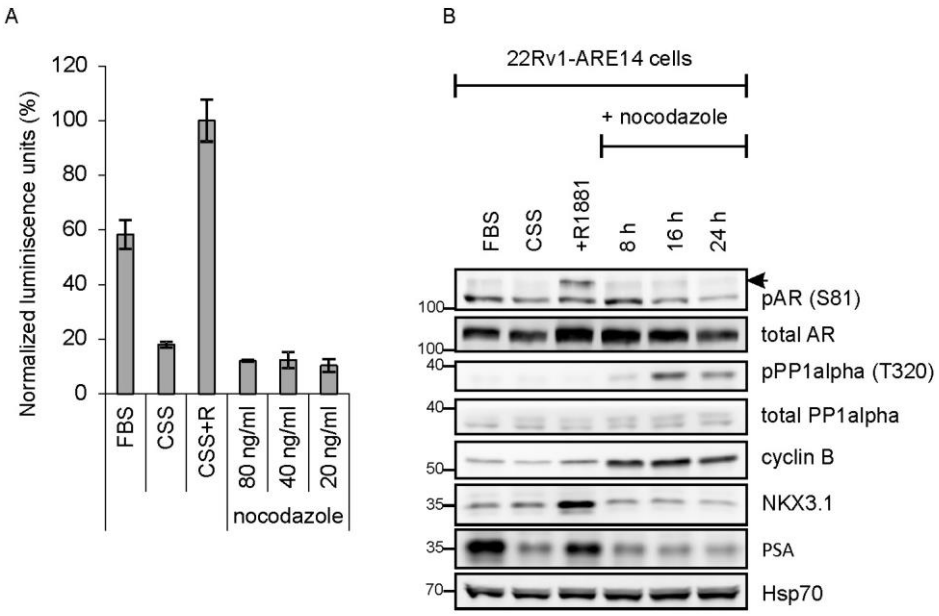

Fig. 3

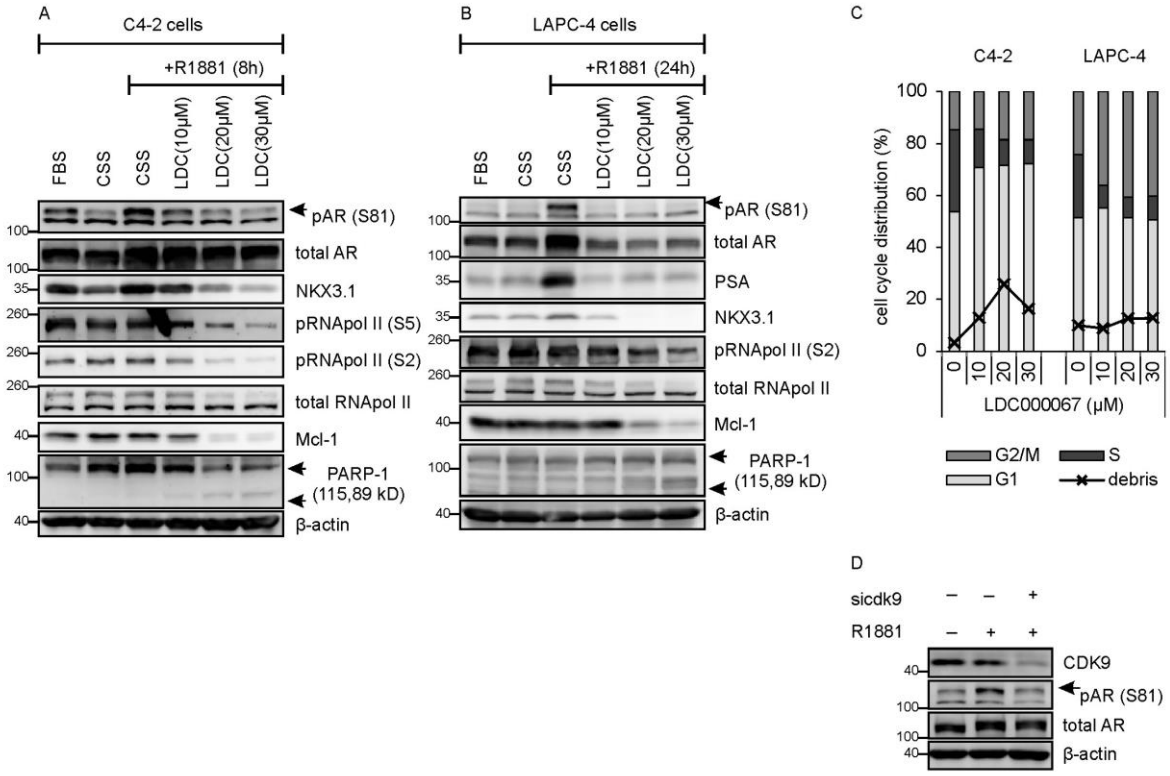

Fig. 4

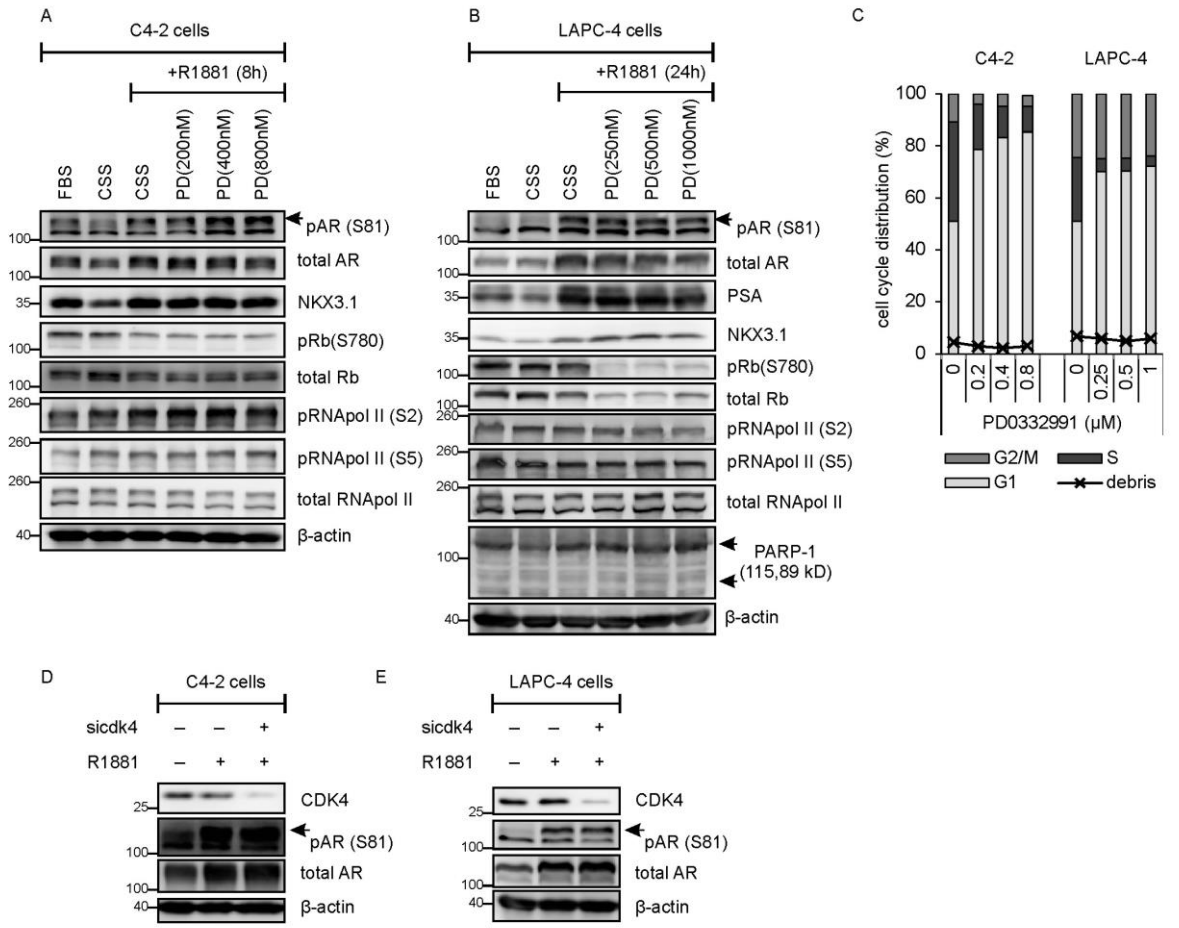

Fig. 5

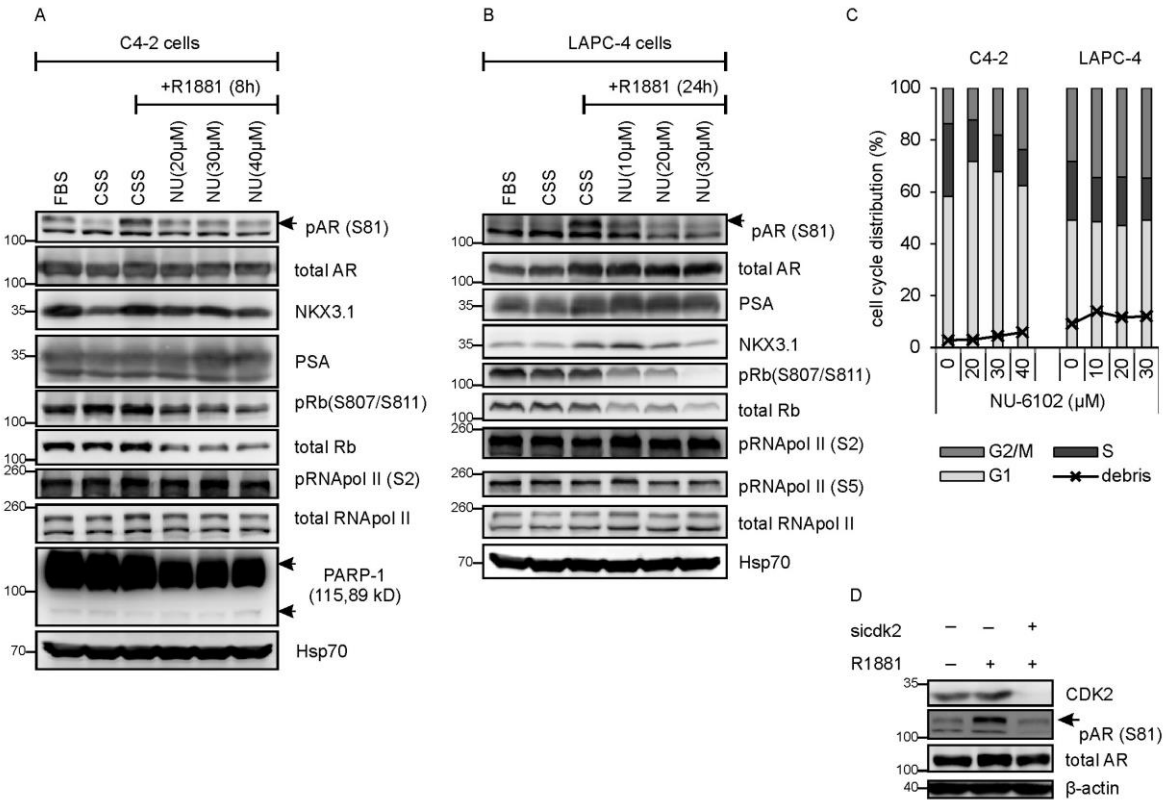

Fig. 6

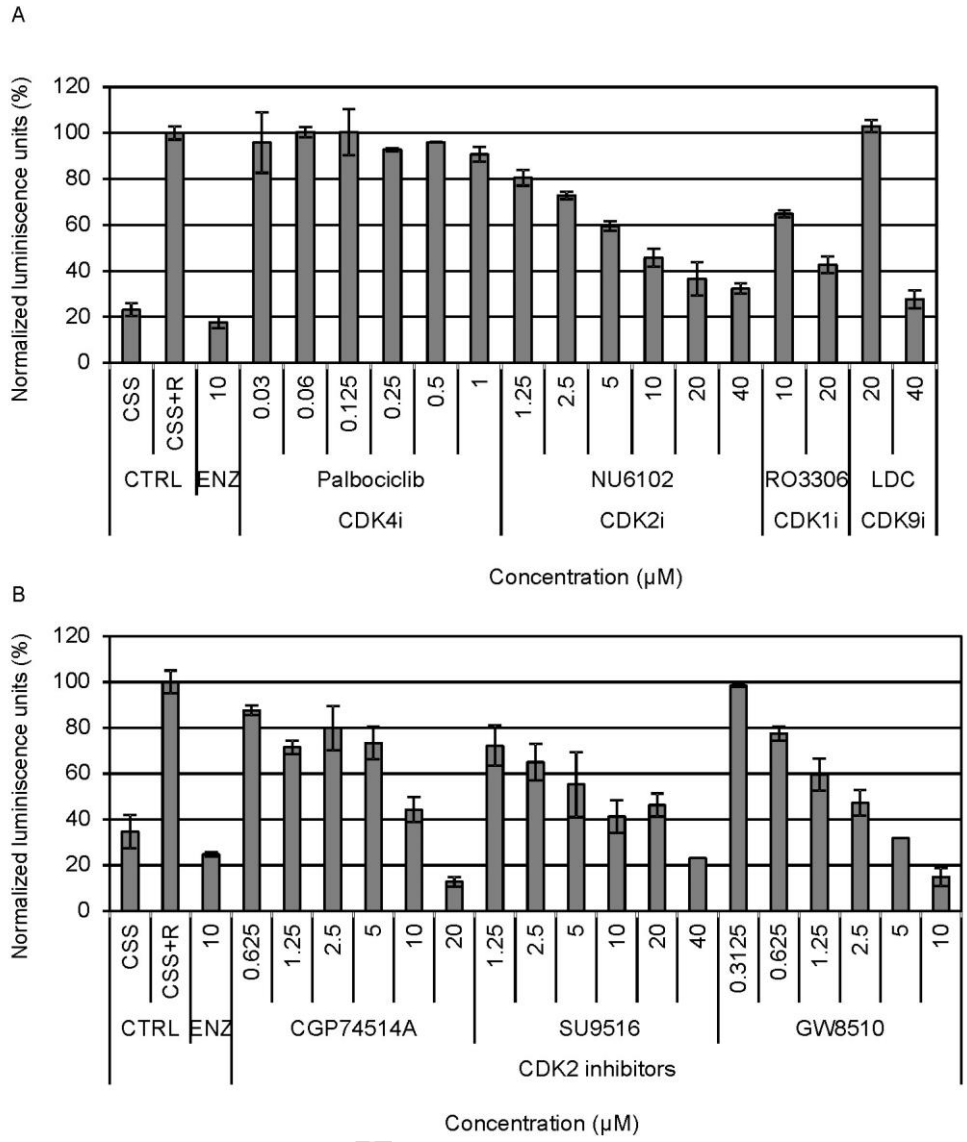

Fig. 7

Table 1.

Target	Inhibitor	CDK/cyclin complexes, IC_{50} (μM)*						
		CDK1/B1	CDK2/E1	CDK2/A2	CDK4/D1	CDK5/p25	CDK7/H/MAT1	CDK9/T1
CDK1	RO3306	0.040	0.146	0.253	40.985	1.153	15.990	14.290
CDK2	NU6102	0.146	0.006	0.014	0.184	0.122	2.530	4.120
	CGP74514A	0.147	0.018	0.037	2.498	0.058	0.279	1.431
	SU9516	0.168	0.013	0.026	1.730	0.022	0.899	1.513
	GW8510	0.049	0.002	0.002	0.139	0.007	0.317	0.543
CDK4	Palbociclib	9.785	8.920	1.84	0.009	11.250	>20	1.240
	Abemaciclib	0.371	0.347	0.013	0.005	0.405	3.112	0.101
CDK9	LDC000067	3.950	2.334	0.841	3.160	4.969	20.000	0.227
	PHA767491	0.459	1.539	0.353	10.045	1.321	36.667	0.103

*measured at least in triplicates.