The American Journal of Pathology, Vol. 192, No. 5, May 2022

The American Journal of PATHOLOGY
ajp.amjpathol.org

TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Inhibition of Cyclin-Dependent Kinase 8/Cyclin-Dependent Kinase 19 Suppresses Its Pro-Oncogenic Effects in Prostate Cancer

Anne Offermann,[*] Vincent Joerg,[*] Finn Becker,[*] Marie C. Roesch,[†] Duan Kang,[*] Anna-Lena Lemster,[*] Lars Tharun,[*] Jochen Behrends,[‡] Axel S. Merseburger,[†] Zoran Culig,[§] Verena Sailer,[*] Johannes Brägelmann,[¶‖**] Jutta Kirfel,[*] and Sven Perner[*††]

From the Institute of Pathology and the Department of Urology,[†] University Hospital Schleswig-Holstein, Campus Luebeck, Luebeck, Germany; the Core Facility Fluorescence Cytometry[‡] and the Institute of Pathology,[††] Research Center Borstel, Leibniz Lung Center, Borstel, Germany; Experimental Urology,[§] Department of Urology, Medical University of Innsbruck, Innsbruck, Austria; Molecular Pathology,[¶] Institute of Pathology, University Hospital of Cologne, Cologne, Germany; the Department of Translational Genomics,[‖] Center of Integrated Oncology Cologne-Bonn, Medical Faculty, University of Cologne, Cologne, Germany; and the Mildred Scheel School of Oncology, Cologne,** University Hospital Cologne, Medical Faculty, Cologne, Germany*

Accepted for publication January 14, 2022.

Address correspondence to Sven Perner, M.D., Ph.D., FRCPath, Institute of Pathology, University Hospital of Luebeck, Ratzeburger Allee 160 (Bldg. 50), 23538 Luebeck, Germany. E-mail: sven.perner@uksh.de.

Progression of prostate cancer (PCa) is characterized by metastasis and castration resistance after response to androgen deprivation. Therapeutic options are limited, causing high morbidity and lethality. Recent work reported pro-oncogenic implications of the Mediator subunits cyclin-dependent kinase (CDK) 8 and 19 for the progression of PCa. The current study explored the underlying molecular mechanisms of CDK8/CDK19 and tested effects of novel CDK8/CDK19 inhibitors. PC3, DU145, LNCaP, and androgen-independent LNCaP Abl were used for *in vitro* experiments. Two inhibitors and CDK19 overexpression were used to modify CDK8/CDK19 activity. MTT assay, propidium iodide staining, wound healing assay, Boyden chamber assay, and adhesion assay were used to investigate cell viability, cell cycle, migration, and adhesion, respectively. Peptide-kinase screen using the PamGene platform was conducted to identify phosphorylated targets. Combining CDK8/CDK19 inhibitors with anti-androgens led to synergistic antiproliferative effects and sensitized androgen-independent cells to bicalutamide. CDK8/CDK19 inhibition resulted in reduced migration and increased collagen I—dependent adhesion. Phosphorylation of multiple peptides linked to cancer progression was identified to be dependent on CDK8/CDK19. In summary, this study substantially supports recent findings on CDK8/CDK19 in PCa progression. These findings contribute to a better understanding of underlying pro-oncogenic effects, which is needed to develop CDK8/CDK19 as a therapeutic target in PCa. *(Am J Pathol 2022, 192: 813—823; https://doi.org/10.1016/j.ajpath.2022.01.010)*

Because of the significant role of deregulated gene expression in malignancies, there is a high demand to elaborate molecular mechanisms involved in transcriptional processes.[1] The Mediator complex constitutes an integral part of human gene transcription by interacting with RNA polymerase II and multiple transcription factors, thereby serving as a hub within the transcription machinery.[2,3] The Mediator is a multiprotein complex containing variably associated subunits that are structurally and functionally assigned to four different domains: the head, middle, tail, and kinase module.[4] As an evolutionarily highly conserved molecule,[4] it integrates cellular signaling and thereby modifies the expression of a variety of genes, including

Supported by the Rudolf Becker-Foundation grant T0321/36080/2020/kg (S.P. and J.K.); the University of Luebeck fellowship (A.O. and M.C.H.); a University of Luebeck medical doctoral fellowship (V.J.); the German Cancer Aid (Deutsche Krebshilfe) through a Mildred Scheel Nachwuchszentrum grant (grant number 70113307) and the Else Kröner-Fresenius Stiftung Memorial grant 2018_EKMS.35 (J.B.); and the German Research Foundation (Deutsche Forschungsgesellschaft) grant (J.K.).

A.O. and V.J. contributed equally to this work.

Disclosures: None declared.

https://doi.org/10.1016/j.ajpath.2022.01.010

oncogenes and tumor suppressor genes. On the basis of its crucial regulatory function in cells, parts of the Mediator complex have been identified to be involved in diverse pathomechanisms of human diseases, including cancer.

Recently, the Mediator subunit cyclin-dependent kinase 19 (CDK19) was found to be specifically up-regulated in primary prostate cancer (PCa) and was implicated in PCa progression.[5] Comprehensive tissue analyses showed that CDK19 is highly overexpressed in PCa metastases and castration-resistant PCa (CRPC), and that CDK19 expression in primary tumors predicts patient outcome after surgery.[6] Furthermore, *in vitro* studies revealed that CDK19 inhibition reduces the invasive potential of PCa cells, and that CDK19 associates with a prometastatic gene expression pattern.[5,7]

CDK19 belongs to the kinase module that also includes the subunits Mediator (MED) 12, MED13, cyclin C, and CDK8. The latter is the gene paralog of *CDK19*; thus, CDK8 and CDK19 harbor a high degree of structural and functional similarities.[8] Although CDK8 has been well investigated in numerous cancer entities including colon,[9] breast,[10] and melanoma,[11] and linked to many cancer-related pathways, such as WNT/ß-catenin-[9] and transforming growth factor-β/SMAD signaling,[12] less is known about the role of CDK19 in carcinogenesis. As part of the kinase module, CDK8 and CDK19 reversibly associate with the Mediator complex as part of the transcription machinery.[13] Besides its function as transcriptional regulator within the Mediator, CDK8 and CDK19 influence cellular signaling by phosphorylation of numerous downstream substrates. Recently, Poss et al[14] showed that blocking CDK8 and CDK19 by the selective inhibitor cortistatin A in colon cancer cells affects the phosphorylation of multiple proteins involved in inflammation, growth, and metabolic regulation. Of note, as seen after *CDK8/CDK19* gene knockdown, cellular changes occurred independent of these effects, highlighting the ability of *CDK8/CDK19* to modify cancer-related signaling by diverse mechanisms.

Because of its substantial effects in cancer cells, numerous selective CDK8/CDK19 inhibitors have been generated, aiming to develop CDK8/CDK19 as potential therapeutic target for cancer therapy. These inhibitors have shown tumor-suppressive effects in different cancer cell lines and *in vivo* models by affecting properties such as proliferation, cell cycle, migration, and invasion. CCT251545 has been identified as a selective CDK8/CDK19 inhibitor blocking the Wnt signaling pathway *in vivo*.[15,16] Subsequently, Koehler et al[17] developed additional compounds with high selectivity to CDK8/CDK19, which are able to phenocopy *CDK8/CDK19* knockout.

Findings so far suggest CDK19 to be pro-oncogenic molecule in PCa, which is supported by tissue analyses and *in vitro* experiments,[5] the aim of which was to further explore anti-tumor effects of CDK8/CDK19 inhibition in PCa. Herein, the focus was as follows: i) the establishment of recently published small-molecule inhibitors against CDK8/CDK19, ii) the investigation of cellular features that contribute specifically to PCa progression, and iii) the identification of molecular changes in PCa cells in response to CDK8/CDK19 inhibition.

Materials and Methods

Cell Lines

Prostate cancer cell lines LNCaP, DU145, and PC3 cells were purchased from ATCC (Manassas, VA) and maintained according to provider's instructions. The cell line LNCaP Abl[18] was a kind gift of Dr. Zoran Culig (University of Innsbruck, Innsbruck, Austria) and was grown in full growth medium without steroids (charcoal-stripped fetal calf serum; ThermoFisher Scientific, Waltham, MA).

CDK8/CDK19 Inhibitors

CDK8/CDK19 inhibitor CCT251545 was a kind gift of Julian Blagg (Cancer Research UK Cancer Therapeutics Unit, Division of Cancer Therapeutics, The Institute of Cancer Research, Sutton, UK).[15] CDK8/CDK19 inhibitor G02788177.93-1 was a kind gift of Michael F.T. Koehler (Discovery Chemistry, Genentech, South San Francisco, CA).[17] Sensitivity of PCa cell lines toward CDK8/CDK19 inhibitors was assessed by dilution series in 96-well plates compared with dimethyl sulfoxide (DMSO) control. Viability was measured by MTT assay at different time points and was dose dependent.

CDK19 Overexpression

CDK19 plasmids (human-tagged true open reading frame, pCMV6-Entry vector) and human cytomegalovirus control plasmids were purchased from OriGene (OriGene Technologies, Rockville, MD) and used to perform CDK19 overexpression experiments. Transfection was performed using Screenfect A (Genaxxon Bioscience GmbH, Ulm, Germany). Cells were seeded in transfection medium containing the plasmid. Forty-eight hours later, medium was exchanged. CDK19 overexpression was determined by Western blot analysis.

Cell Viability Assay

Cell viability following various inhibitor treatment conditions or plasmid transfection was assessed using thiazolyl blue tetrazolium bromide (MTT). A total of 7000 LnCaP, 7000 LNCaP Abl, 5000 PC3, or 5000 DU145 cells per well were plated in 96-well plates (Corning, Glendale, AZ), and 500 μg/mL MTT (Sigma-Aldrich, Steinheim, Germany) dissolved in phosphate-buffered saline (PBS; Gibco Life Technologies, Waltham, MA) was added. Four hours later, 100 μL solubilization buffer (40% v/v dimethylformamide [Alfa Aesar, Thermo Fisher (Kandel) GmbH, Kandel,

Germany], 2% glacial acetic acid [Merck, Darmstadt, Germany], 16% SDS [Applichem, Darmstadt, Germany], pH 4.7) was added; and absorbance was measured at 595 nm. For LNCaP Abl cells, the experiments were performed in medium containing charcoal-stripped fetal calf serum (ThermoFisher Scientific, Schwerte, Germany) without supplementation of dihydrotestosterone.

Cell Cycle Analysis

For cell cycle analysis, propidium iodide DNA staining followed by flow cytometric analysis was performed. In more detail, after 72 hours of specific cell treatments, cells were harvested and used for flow cytometric analysis. Cells were fixed in ice-cold 70% ethanol for 30 minutes at 4°C, washed three times with ice-cold PBS, and resuspended in 200 μL DNA staining solution [PBS containing 50 μg/mL propidium iodide (Sigma Aldrich, Steinheim, Germany) and 100 μg/mL Ribonuclease A (Sigma Aldrich, Steinheim, Germany)]. After 15 minutes of incubation in the dark, cell cycle was analyzed using an LSR II Cell Analyzer (BD Bioscience, Heidelberg, Germany). FCS Express 5 software (DeNovo Software, Glendale, CA) was used for analyzing flow cytometric data.

Wound Healing Assays

Wound healing assays were performed using IBIDI wound healing assays (ibidi GmbH, Graefelfing, Germany). Transfected or inhibitor treated cells were seeded in a concentration of 3×10^5 cells/mL in IBIDI chambers containing a 500-μm thick wall. After reaching a confluent cell layer, cells were treated with mitomycin to avoid overgrowth by proliferation. After 12 hours, cells were stained using crystal violet and migration was visually documented.

Cell Migration Assay

The 12-well cell inserts (ThermoFisher Scientific, Waltham, MA) were used to perform Boyden chamber migration assays. A total of 100 μL of serum-free medium was added to the upper chamber. Next, 200 μL serum-free medium containing 2.5×10^5 cells/mL was added to same chamber before adding 750 μL of 20% fetal calf serum−containing medium to the lower chamber. Cells were treated with DMSO or CDK8/CDK19 inhibitor at the time of plating. After 12 hours, the experiment was terminated by washing the inserts in Dulbecco's PBS twice before using cotton swabs to remove noninvasive cells on the insert surface. Remaining cells were stained using crystal violet. Next, inserts were washed using Dulbecco's PBS, and images were taken to document the amount of invasive cells.

Cell Adhesion Assay

A total of 25,000 cells were plated in 96-well plates, which were either nontreated or coated with 8 $\mu g/cm^2$ collagen type I (Sigma Aldrich, St. Louis, MO). Cells were treated with 5 or 10 μmol/L CDK8/CDK19 inhibitor either at the beginning of the assay or 24 hours in advance. After various measuring time points, the medium was aspirated and wells were washed twice with Dulbecco's PBS. Next, cells were stained using crystal violet for 20 minutes. After staining, remaining cells were washed four times with Dulbecco's PBS to remove remaining dye. The amount of adherent cells was documented by taking images. Afterwards, methanol was added to dissolve crystal violet taken up by the adherent cells. Absorbance was measured at 595 nm.

Kinase Profiling Assay

Cells used for analysis were treated with DMSO or CDK8/CDK19 inhibitors for 4 hours before being harvested and lysed. Protein concentration was measured using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Schwerte, Germany). Protein tyrosine kinase (PTK) activity profiling was performed using a PamStation12 (PamGene International B.V., Hertogenbosch, the Netherlands) and PTK PamChip4 with four identical arrays. On each array, 142 tyrosine-containing peptides (13 amino acids long), derived from known human phosphorylation sites, are immobilized. An assay incubation mixture, containing 2 to 4 μg of protein (depending on total protein available), 100 μmol/L ATP, and fluorescein isothiocyanate−labeled antiphosphotyrosine antibody (PamGene International B.V.), was added in each array. The mixture was pumped up and down through the porous membrane. Peptide phosphorylation was monitored during the incubation by taking images with a charge-coupled device camera in combination with Evolve software version 1.2 (PamGene International B.V.), allowing real-time recording of the reaction kinetics. After washing of the arrays, fluorescence was detected at different exposure times (20, 50, 100, and 200 milliseconds). Analysis was performed using the Bionavigator software version 2.2 (PamGene International B.V.).

Results

CDK8/CDK19 Blockade Shows Synergistic Effects with Androgen Blockade in Androgen-Sensitive LNCaP Cells without Affecting the Cell Cycle

Androgen receptor (AR) blockade is known to reduce cell viability of androgen-responsive PCa cells. Previous studies show CDK19 to be significantly overexpressed in CRPC. To find out whether CDK8/CDK19-related effects are connected to the AR-dependent cell growth, cell viability after dual inhibition of CDK8/CDK19 by two different CDK8/CDK19 inhibitors (CCT251545 and G02788177.93-1) and the AR pathway was analyzed. Bicalutamide (AR antagonist) was used to block AR activity. Treating cells only with 5 μmol/L G02788177.93-1 or with 20 μmol/L bicalutamide, cell viability was reduced by 15% to 25% compared with control cells at

different time points (Figure 1A). Combined treatment with 5 μmol/L G02788177.93-1 and 20 μmol/L bicalutamide had antiproliferative effects, which significantly increased after long-term treatment and reached almost 50% inhibition after 120 hours ($P < 0.05$) (Figure 1A), whereas other treatment conditions did not reach statistical significance.

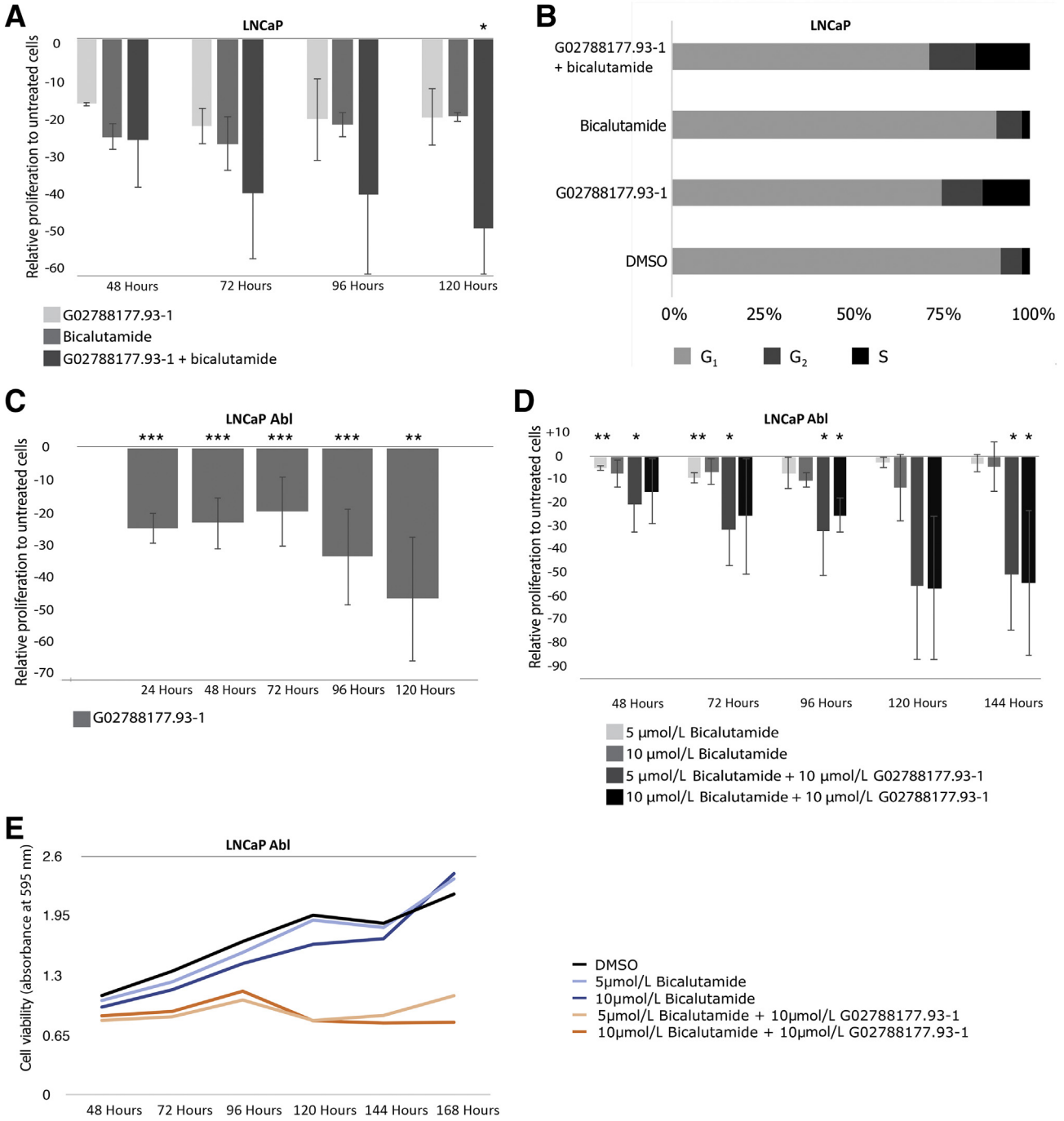

Figure 1 Effects of cyclin-dependent kinase (CDK) 8/CDK19 inhibitor treatment in combination with androgen blockade on the viability of prostate cancer cells. **A:** Cell viability measured by MTT (absorbances at 595 nm; *y* axis) of LNCaP cells treated with 5 μmol/L G02788177.93-1 or 20 μmol/L bicalutamide alone or in combination for 48, 72, 96, and 120 hours. Bar graphs show relative cell viability [normalized to dimethyl sulfoxide (DMSO)—treated cells]. **B:** Percentage distribution of LNCaP cells in G_1, G_2, and S phase of the cell cycle, assessed by flow cytometric analysis after treatment with 5 μmol/L G02788177.93-1 or 20 μmol/L bicalutamide alone or in combination compared with DMSO-treated cells. **C:** Cell viability measured by MTT of LNCaP Abl cells treated with 10 μmol/L G02788177.93-1 for 24, 48, 72, 96, 120, and 144 hours. Bar graphs show relative cell viability (normalized to DMSO-treated cells). **D** and **E:** Cell viability measured by MTT of LNCaP Abl cells treated with 5 or 10 μmol/L bicalutamide with or without 10 μmol/L G02788177.93-1 for 48, 72, 96, and 120 hours. Bar graphs show relative cell viability (normalized to DMSO-treated cells). Time course indicates absorbance at 595 nm after 48, 72, 96, 120, 144, and 168 hours of treatment. In all cell viability assays, the mean of each experimental triplicate and the SD are indicated. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Recent studies suggest that CDK8/CDK19 inhibition through small-molecule inhibitors exerts antiproliferative activity on VCaP cell lines by inducing a premature G_1/S-phase transition, leading to DNA damage and consequently cell death.[7] A structurally different small-molecule inhibitor compared with the above-mentioned study was used to confirm this observation in LNCaP cells. A significant reduction of cells in G_1 phase (92% in control and 75% after CDK8/CDK19 inhibition) and consequently an increased proportion of cells in the S phase (3% in control and 14% after CDK8/CDK19 inhibition) was observed (Figure 1B). Because CDK8/CDK19 inhibition exerts stronger antiproliferative effects in combination with androgen blockade, whether combined cell treatment with 5 μmol/L CDK8/CDK19 inhibitors and AR antagonist, 20 μmol/L bicalutamide, further affected the G_1/S phase ratio was examined. After treatment, there was no difference in the distribution of cells in G_1, G_2, and S phases compared with untreated cells or G02788177.93-1 treatment alone (Figure 1B).

CDK8/CDK19 Blockade Impacts Cell Viability of Androgen-Independent LNCaP Abl Cells

To connect CDK8/CDK19 inhibition to castration resistance, LNCaP Abl cells, which are long-term androgen deprived and characterized by an androgen-independent growth, were used. Inhibition of CDK8/CDK19 activity by G02788177.93-1 impaired cell viability of LNCaP Abl cells in a time-dependent manner. Cell viability was reduced for 24.3% ($P < 0.001$), 22.8% ($P < 0.001$), 19.4% ($P < 0.001$), 33.2% ($P < 0.001$), 37.2% ($P < 0.001$), and 45.8% ($P < 0.01$) after 24, 48, 72, 96, 120, and 144 hours, respectively, in response to 10 μmol/L G02788177.93-1 compared with DMSO (Figure 1C).

In contrast, cell treatment with CCT251545 and G02788177.93-1 did not show a significant antiproliferative effect on basal cell viability in PC3, DU145, and LNCaP cells after various time points (Supplemental Figure S1).

CDK8/CDK19 Blockade Shows Synergistic Effects with Androgen Blockade in Androgen-Independent LNCaP Abl Cells

Treatment of LNCaP Abl cells with 5 or 10 μmol/L bicalutamide induced a slight decrease in cell viability (2% to 10% compared with control cells) and had no time-dependent effect (Figure 1D). In contrast, combined treatment with CDK8/CDK19 inhibitor G02788177.93-1 and bicalutamide showed a drastic decrease in cell viability of LNCaP Abl cells in comparison to control cells. The antiproliferative effect increased in a time-dependent manner and reached a cell viability reduction of >50% after 120 hours (no statistical significance) and 144 hours ($P < 0.05$) independently of the bicalutamide dose (Figure 1D).

Overall, a steady increase of cell viability over the observed time period for all treatment conditions (DMSO controls and single 20 μmol/L bicalutamide- and 20 μmol/L flutamide-treated cells), except for the dual application of androgen inhibitor and CDK8/CDK19 inhibitor, could be observed. This indicates a synergistic effect of androgen receptor and CDK8/CDK19 inhibition rather than merely toxic effects of the experimental conditions (Figure 1E).

CDK8/CDK19 Inhibition Influences Cell Migration and Invasion *in Vitro*

Recent studies indicate that CDK8/CDK19 inhibition by senexin A impacts migration and invasion of several PCa cell lines. To test the novel inhibitor G02788177.93-1 and to specify these observations, a wound healing assay with DU145 and PC3 control cells, G02788177.93-1 treated cells, and cells with CDK19 overexpression was performed. In concordance with the recent findings, G02788177.93-1–treated cells showed a reduced extent of motility, whereas CDK19 overexpressing cells migrated significantly more efficiently compared with control cells (Figure 2, A and B). Next, Boyden chamber migration assays with the same treatment and cell conditions were performed. Both DU145 and PC3 cells showed less migratory potential after CDK8/CDK19 inhibition by G02788177.93-1, whereas CDK19 overexpression led to an increased extent of migrated cells in both cell lines (Figure 2, C and D).

CDK8/CDK19 Inhibition Leads to Increased Affinity to Collagen I

The underlying mechanisms of impaired migration and motility after CDK8/CDK19 inhibition were investigated further. Prior transcriptome analyses showed that senexin A significantly affects genes involved in cytoskeleton organization, cell motion, and adhesion.[5] Because adhesion of epithelial cells is essentially mediated by integrin–extracellular matrix interaction, the effects of CDK8/CDK19 on PCa cell adhesion and its relationship to collagen I, which constitutes an integral part of the extracellular matrix, were performed. Herein, CDK8/CDK19 inhibition by G02788177.93-1 significantly enhanced cell adhesion of PC3 cells in the presence of collagen I (Figure 3A). In absence of collagen I, there was no significant difference in cell adhesion (Figure 3A). Absorbance at 595 nm, reflecting cell density in the adhesion assay, quantified and confirmed the visual results and was significantly higher after CDK8/CDK19 inhibition normalized to DMSO (Figure 3B).

To further specify and verify these results, adhesion assays using DU145 cells under different experimental conditions were performed. More specifically, DU145 cells were treated with G02788177.93-1 24 hours in advance or at the beginning of the experiment in the absence or presence of collagen I. Over time, adhesion of DU145 cells increased and was significantly reduced in

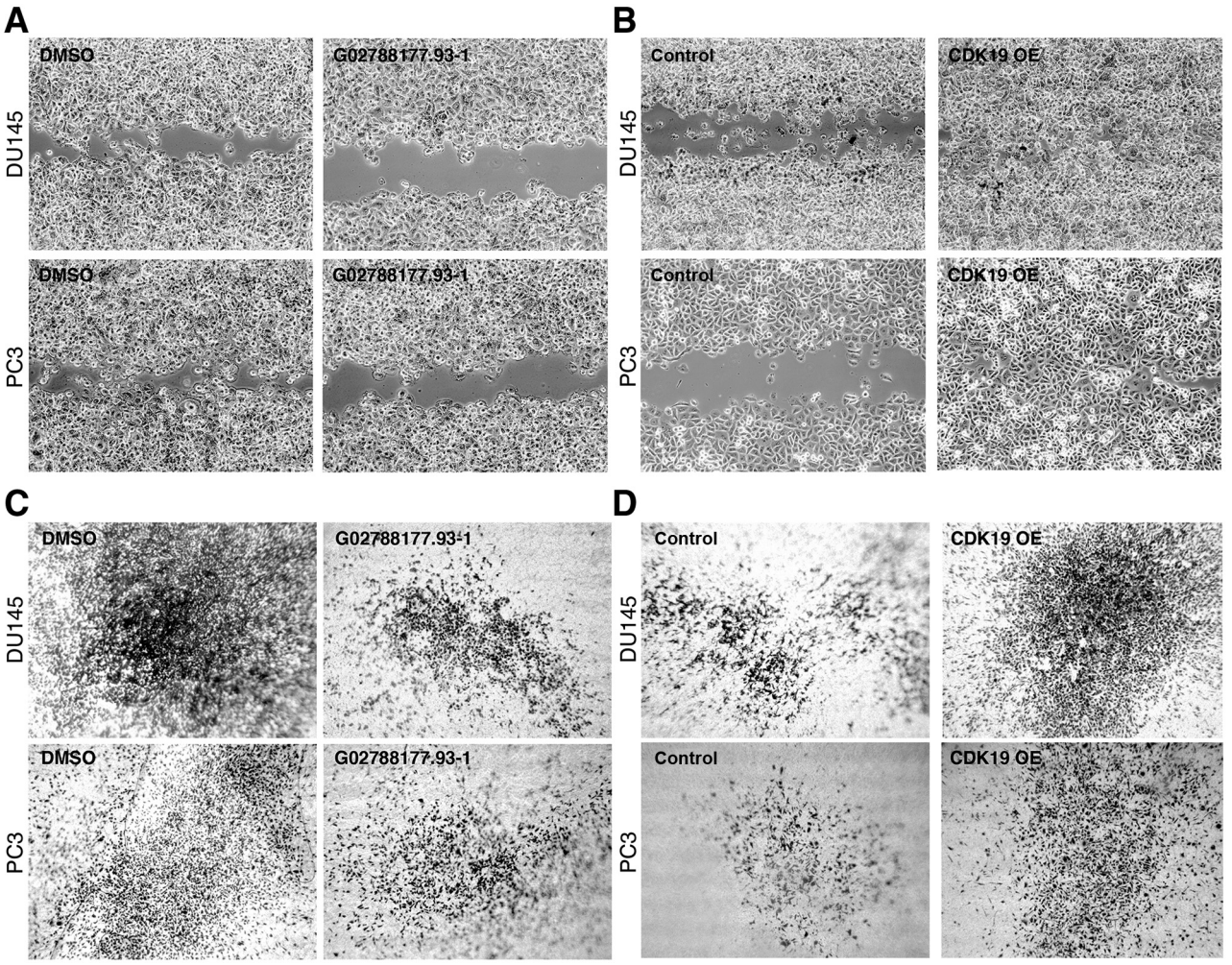

Figure 2 Effects of cyclin-dependent kinase (CDK) 8/CDK19 inhibitor treatment and CDK19 overexpression (OE) on migration and cell motility of prostate cancer cells. **A** and **B:** Migration of DU145 and PC3 cells assessed by wound healing assay after treatment with G02788177.93-1 compared with dimethyl sulfoxide (DMSO)—treated cells (**A**) and after CDK19 overexpression compared with control cells (**B**). **C** and **D:** Migration of DU145 and PC3 cells assessed by Boyden chamber assay under similar treatment conditions. Representative images are used for illustration. Original magnification, ×40 (**A**—**D**).

G02788177.93-1—treated cells (Figure 3C). Of note, this effect was only measurable when cells were pretreated with G02788177.93-1 for 24 hours in advance (Figure 3C) and subsequently absent when the inhibitor was applied at the beginning of the adhesion assay (Figure 3D). Likewise, in the absence of collagen I, there was no detectable difference in cell adhesion after G02788177.93-1 treatment or DMSO control (Figure 3E).

CDK8/CDK19 Inhibition Leads to Decreased Phosphorylation of Various Pathway Molecules

To identify downstream pathways affected by the kinase activity of CDK8/CDK19, the PamGene peptide microarray for detailed, high-throughput substrate identification was used. Figure 4 shows substrates whose phosphorylations are significantly reduced after G02788177.93-1 treatment in PC3 (Figure 4A), LNCaP (Figure 4B), and LNCaP Abl (Figure 4C) cells. There was a significant difference in the mean pattern of listed substrate phosphorylation comparing cells treated with G02788177.93-1 or DMSO (control cells). Substrates are listed according to their level of significance in experiments performed in triplicates each (Figure 4, A—C). Overall target analysis revealed a high level of substrate similarity in all cell lines. Substrates that are recurrently affected among PCa cell lines were further assigned to cancer-related features. Most affected features were cell survival and cell viability (43% of selected substrates), adhesion, migration, and invasion (15%), as well as angiogenesis (20%) and immune cell signaling (22%) (Figure 4D).

Discussion

Previous studies showed the Mediator complex subunit CDK19 to be specifically up-regulated in PCa and to be

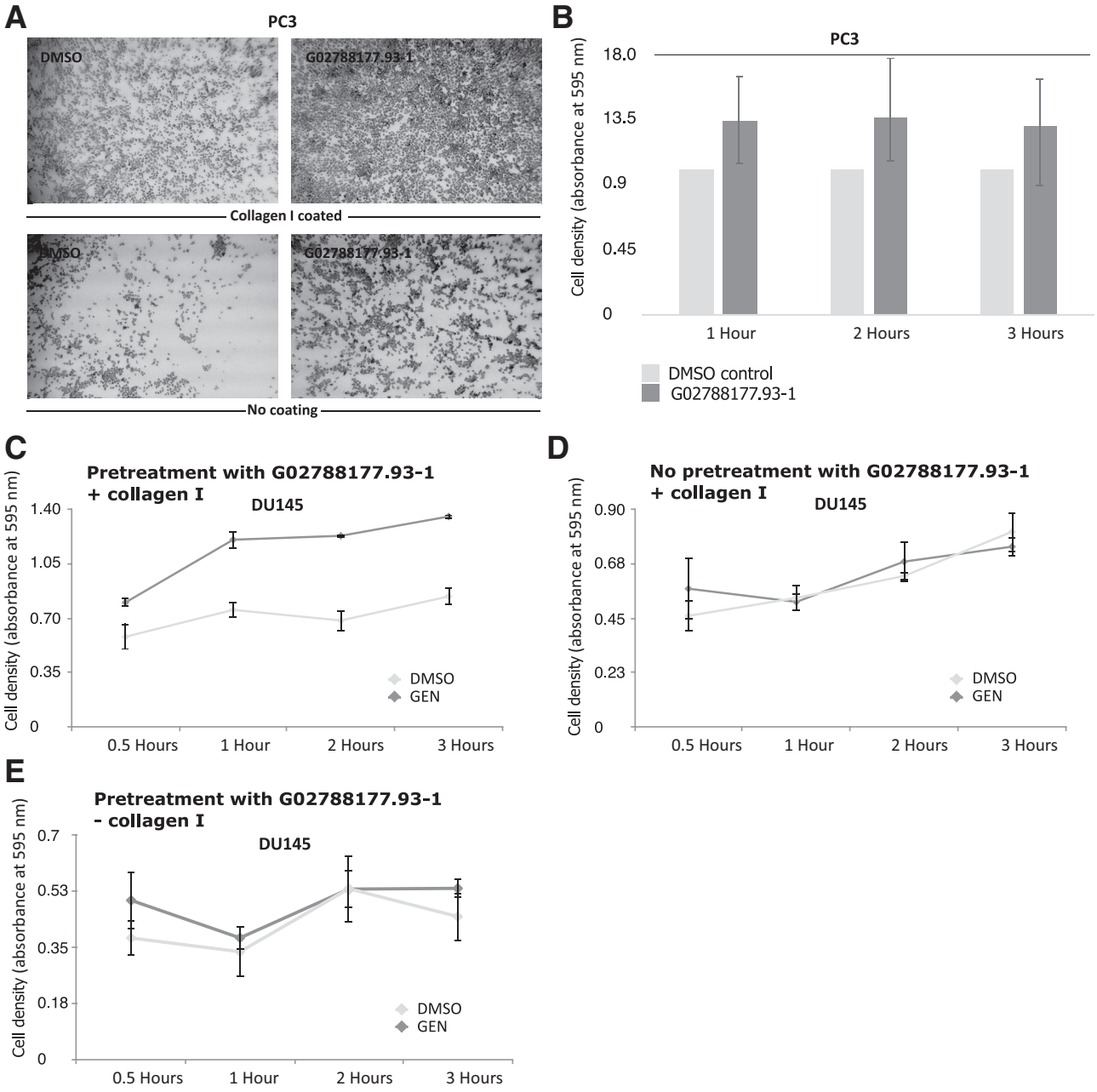

Figure 3 Effects of cyclin-dependent kinase (CDK) 8/CDK19 inhibitor treatment on cell adhesion of prostate cancer cells. **A:** Representative images showing adherent PC3 cells treated with G02788177.93-1 (GEN) compared with dimethyl sulfoxide (DMSO)—treated cells after performing adhesion assay. The experiment was conducted in the presence or absence of collagen I coating of wells. **B:** Density of PC3 cells following adhesion assay reflected by absorbance at 595 nm after crystal violet staining of adherent cells. Bar graphs show the mean absorbance of G02788177.93-1 normalized to DMSO-treated cells. **C:** Density of DU145 cells, which were treated with G02788177.93-1 or DMSO for 1 hour following adhesion assay. Cell density is reflected by absorbance at 595 nm after crystal violet staining of adherent cells. Absorbance was measured 0.5, 1, 2, and 3 hours after cell plating on collagen I—coated wells. Bar graphs show the mean of each experimental triplicate and the SD. **D** and **E:** The same experiment was performed with G02788177.93-1 or DMSO treatment at beginning of the adhesion assay (**D**) or in the absence of collagen I (**E**). Original magnification, ×40 (**A**).

implicated in the pathogenesis of PCa progression.[5,6] Both findings, based on tissue-related data and the results of functional *in vitro* studies, give strong evidence for an involvement of CDK19 in the growth, progression, and metastasis of PCa as well as the development of castration resistance. In this study, the aim was to explore underlying molecular mechanisms of pro-oncogenic effects of CDK19, and to further characterize recently published CDK8/CDK19 inhibitors in the context of PCa.

Senexin A and senexin B are widely used inhibitors to investigate the effects of CDK8/CDK19 inhibition.[19,20] Subsequently, structurally different and potent CDK8/CDK19 inhibitors have been developed, aiming to optimize the potency and specificity of inhibitors.[15,17] In this study,

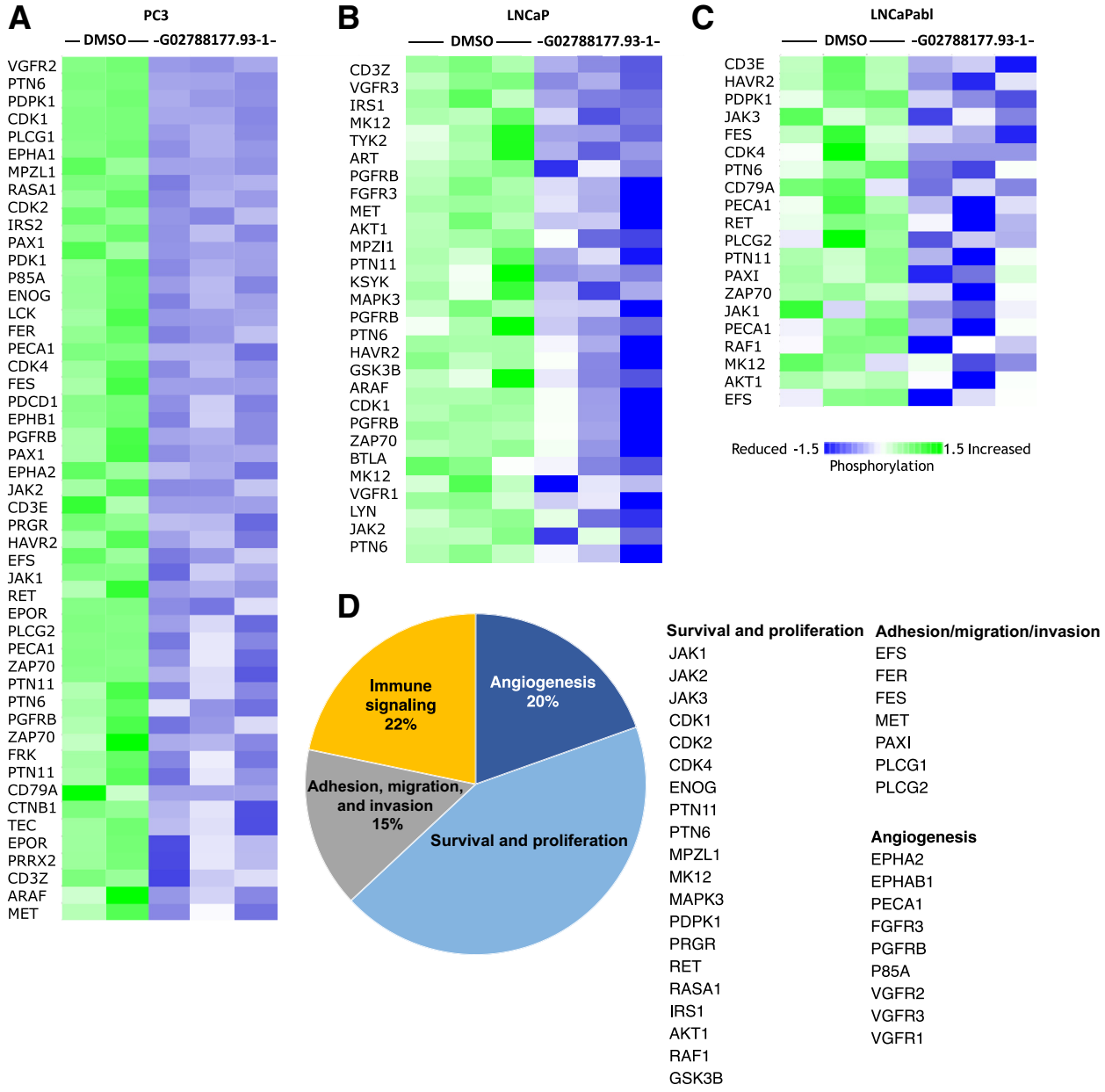

Figure 4 High-throughput peptide phosphorylation assay for the identification of cyclin-dependent kinase (CDK) 8/CDK19 substrates in prostate cancer cells. **A-C:** Heat map indicating peptide substrates that show a significant differentially phosphorylation level after CDK8/CDK19 inhibition by G02788177.93-1, according to significance after quality control in PC (**A**), LNCaP (**B**), and LNCaP Abl (**C**) cells. Rows show treatment conditions [triplicate for each, except for PC3 dimethyl sulfoxide (DMSO) control, due to technical issues]. **D:** Recurrently altered substrates in two or three cell lines assigned to biological processes.

the response of different PCa cell lines to CCT251545[15] and G02788177.93-1, and report their effects on cell proliferation, androgen-independent cell growth, migration, and the phosphorylation of CDK8/CDK19 substrates, was characterized. The results of this study support the efficiency of these inhibitors because doses of 5 to 10 μmol/L lead to CDK8/CDK19 inhibition confirmed by reduction of STAT1-S727 phosphorylation (data not shown), a substrate commonly used to measure CDK8/CDK19 kinase activity.[21]

In concordance with the recent observation that CDK/CDK19 inhibition does not significantly affect proliferation at doses that are sufficient to reduce migration and invasion of PCa cells,[5] CCT251545 and G02788177.93-1 influenced the cell viability of PC3, DU145, and LNCaP cells at 10 μmol/L, whereas lower doses showed no significant effect (Supplemental Figure S1). Interestingly, similar results have been reported in colon cancer cells (HCT116). Bergeron et al[22] demonstrated a dramatically decreased proliferation at doses around 10 μmol/L, which was phenocopied by *CDK8* knockout. Of note, the effects observed herein were most pronounced after 24 hours of treatment, which most probably might be explained by pharmacokinetics, which was not a further focus of this study.

The progression of PCa is characterized by the development of castration resistance after initial response to androgen deprivation. Progressing or recurring disease in a hormone-naive status is commonly treated with luteinizing hormone-releasing hormone agonists, which remains the standard of care for androgen deprivation.[23] For metastatic PCa, androgen deprivation is combined with novel antiandrogens, such as apalutamide, enzalutamide, and abiraterone, or with conventional chemotherapy (docetaxel).[23] In the setting of metastatic CRPC, the use of enzalutamide rather than bicalutamide is recommended on the basis of clinical trials showing that enzalutamide has significantly improved progression-free survival in patients.[24] Bicalutamide is currently recommended in combination with radiotherapy for patients with prostate-specific antigen relapse and high risk for cancer progression.[23]

Bicalutamide is a nonsteroidal AR antagonist that competitively inhibits the action of androgens by binding to the AR. Thereby, the androgen-induced expression of AR target genes and consequently subsequent oncogenic effects on AR-sensitive PCa cells are attenuated. Mechanistically, bicalutamide stimulates AR nuclear translocation and its binding to AR responsive genes, and ultimately induces a transcriptionally inactive state of the AR.[25] As a result, bicalutamide inhibits proliferation and the progression to S phase of the cell cycle in a dose-dependent manner, which is abrogated by adding dihydrotestosterone.[25] Interestingly, bicalutamide acquires agonistic properties during long-term androgen ablation of LNCaP cells,[18] whereas the new generation of antiandrogens, such as enzalutamide, prevent AR nuclear translocation and do not exhibit any agonistic properties.

Prior work showed CDK19 to be significantly upregulated in CRPC[6] and the expression of CDK8 and CDK19 to be increased in androgen-sensitive LNCaP cells[5] under androgen deprivation (versus androgen stimulation). On the basis of these results, we hypothesized that CDK8/CDK19 influences PCa growth in an androgen-dependent manner and therefore contributes to castration resistance. To verify this hypothesis, cell viability of androgen-sensitive LNCaP cells in response to dual blockade of CDK8/CDK19 and the AR was measured. Hereby, a synergistic effect of combined G02788177.93-1 and bicalutamide treatment was observed, which reduced LNCaP cell viability to 50% in a time-dependent manner (Figure 1A). In contrast, G02788177.93-1 or bicalutamide treatment alone had only modest antiproliferative effects, which were stable over time. Therefore, these results might indicate that CDK8/CDK19 at least partially mediates its pro-oncogenic effects via the AR axis and that dual therapy combining AR and CDK8/CDK19 blockade might be a strategy to increase treatment efficiency.

On the basis of a recently published study suggesting that CDK8/CDK19 mediates antiproliferative activity by inducing a premature G_1/S-phase transition leading to DNA damage and subsequent cell death,[7] whether the synergistic effect of dual G02788177.93-1 and bicalutamide treatment can be explained by an altered cell cycle was explored further. Comparing the distribution of cell cycle phases in response to G02788177.93-1 alone or in combination with bicalutamide, there was no significant difference (Figure 1B), suggesting a different mechanism conveying the synergistic effects on cell viability. However, previous results[7] in LNCaP cells, showing a significant reduction of cells in G_1 phase and an increased proportion of cells in the S phase in response to CDK8/CDK19 blockade, were confirmed (Figure 1B). Strikingly, there was no effect of bicalutamide alone on the distribution of cell cycle phases (Figure 1B). In previous studies, bicalutamide was shown to block LNCaP cells from entering S phase by regulating the expression of specific cell-cycle regulatory genes.[26] Of note, in the current experiments, cell cycle phases were measured 72 hours after treatment with bicalutamide, whereas previous studies used an observation period of 24 hours after treatment, which might explain this disconcordance. But because there was an antiproliferative effect of bicalutamide 72 hours after treatment (Figure 1A), it can be assumed that bicalutamide still has an inhibitory effect on LNCaP cells in this experimental setting.

To further explore the effects of CDK8/CDK19 on castration resistance, the effects of G02788177.93-1 alone or in combination with bicalutamide on LNCaP Abl cells were investigated. LNCaP Abl is an LNCaP subline that is long-term androgen deprived and characterized by androgen-independent growth. Interestingly, CDK8/CDK19 inhibition alone had moderate effects on cell viability in a time-dependent manner, highlighting that androgen-independent growth might be partially mediated by CDK8/CDK19 (Figure 1C). AR blockade alone by bicalutamide had no antiproliferative effects in LNCaP Abl (Figure 1D), as expected and described before. Intriguingly, combining bicalutamide with G02788177.93-1 dramatically reduced cell viability of LNCaP Abl in particular after long-term treatment (Figure 1D), providing evidence that CDK8/CDK19 might support androgen-independent growth and its blockade rescues androgen dependency. The general course of cell viability over time indicates that the dual treatment leads to synergistic antiproliferative effects rather than conveying toxic effects (Figure 1E). In conclusion, in the light of previous findings, these results provide evidence that CDK8/CDK19 inhibition might be a promising therapeutic approach to overcome resistance against antiandrogenic therapy, the main reason for PCa-specific death.

Next, the aim was to verify previous results showing that CDK8/CDK19 inhibition reduces metastatic capacity of PCa cells.[5] Two different assays clearly confirmed that CDK8/CDK19 inhibition by the two novel inhibitors reduces migration of PCa cells. Opposite effects were seen in CDK19 overexpressing cells, specifying our observations (Figure 2). As described above, a previous study showed that CDK8/CDK19 inhibition has major impact on cellular features that mediate cell migration and invasion. In

subsequent experiments, CDK8/CDK19-mediated effects observed on migration and invasion can be at least partially explained by modulation of cell adhesion dependence in the presence of collagen I (Figure 3). Collagen I is part of the extracellular matrix of the bone, but also of prostatic periglandular stromal tissue, which was confirmed by immunohistochemistry (data not shown). On the basis of these results, we hypothesize that CDK8/CDK19 deregulation might reduce the capacity of PCa cells to bind collagen I and therefore affect cellular adhesion and specifically facilitate migration through collagen I–containing stromal tissue. Intracellular mediators of collagen I interaction include integrins, which were found to negatively correlate with high CDK19 levels using cBioportal *in silico* analysis (data not shown). The observation that 24 hours pretreatment with G02788177.93-1 is required for subsequent increase in adhesion might indicate that CDK8/CDK19 act through changes in transcriptional regulation.

CDK8 and CDK19 are part of the Mediator complex and therefore exert major impact on gene transcription.[13] Apart from that, recent studies identified multiple peptides to be phosphorylated by CDK8/CDK19 kinase activity.[14] Through phosphorylation of specific pathway molecules, CDK8/CDK19 is involved in immune-oncological processes[27,28] and cancer-related signaling, such as NF-κB activity.[10] To identify downstream substrates of CDK8/CDK19 kinase function, a large-scale phosphoarray screen (PamGene International B.V.) was performed with three different PCa cell lines after treatment with the CDK8/CDK19 inhibitor G02788177.93-1. The phosphorylation of numerous substrates was found to be recurrently altered in more than one cell line, which are listed in Figure 4D. These substrates were subsequently assigned to cell functions (Figure 4D), providing evidence that CDK8/CDK19 affects the metastatic potential of PCa cells through their kinase activity. Of note, further studies are needed to experimentally explore the biological significance of these findings and to differentiate direct from indirect effects of CDK8/CDK19. Regarding the latter, it seems possible that the changed phosphorylation of some substrates results from altered upstream signaling due to CDK8/CDK19 inhibition but not by direct kinase interaction between CDK8/CDK10 and substrates. This seems particularly likely because the signaling molecules listed in Figure 4 are closely connected. However, these results provide clues about the pathways affected by CDK8/CDK19 inhibition with regard to their kinase function and may serve as a result tool for further studies.

Additionally, these observations underline the importance of CDK8/CDK19 as kinases, apart from their function as transcriptional coregulators within the Mediator complex. The shift of focus to this specific property of CDK8/CDK19 unrelated to the Mediator complex has yielded promising results in the past because other groups were able to show direct CDK8/CDK19 kinase impact on essential oncogenic signaling pathways, such as the β-catenin/Wnt pathway.[29] The overall high similarity of affected substrates between different cell lines indicates relevance of CDK8/CDK19 kinase function in all stages of the disease, including hormone-sensitive PCa and CRPC phenotypes.

Collectively, the results of these functional experiments strengthen recent findings identifying CDK8/CDK19 as contributors to the progression of PCa. This study, for the first time, showed i) that CDK8/CDK19 inhibition sensitizes PCa cells to androgen blockade, ii) that reduced migratory capacity of PCa cells after CDK8/CDK19 inhibition is linked to increased adhesion in a collagen I–dependent manner, possibly via altered integrin signaling, and iii) that multiple phosphorylated substrates of CDK8/CDK19 highlight the significance of the CDK8/CDK19 kinase function.

Acknowledgments

We thank Eva Dreyer and Wenzel Vogel for excellent technical support; Zoran Culig for providing LNCaP Abl cells; Julian Blagg (Cancer Research UK Cancer Therapeutics Unit, Division of Cancer Therapeutics, The Institute of Cancer Research, Sutton, UK) for providing the cyclin-dependent kinase (CDK) 8/CDK19 inhibitor CCT251545; and Michael F.T. Koehler (Discovery Chemistry, Genentech) for providing G02788177.93-1.

Author Contributions

S.P., A.O., M.C.R., J.B., and V.J. conceptualized and designed the study; A.O., V.J., F.B., D.K., A.-L.L., and J.B. acquired data and performed experiments; A.O., V.J., M.C.R., F.B., S.P., J.B., A.S.M., V.S., L.T., J.K., and A.S.M. analyzed and interpretated data; Z.C. provided resources; S.P., A.O., V.J., J.B., M.C.R., V.S., L.T., and J.K. participated in data discussion; A.O. and M.C.R. performed statistical analysis; S.P., A.O., and V.J. drafted the manuscript; all coauthors revised the manuscript.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2022.01.010*.

References

1. Bradner JE, Hnisz D, Young RA: Transcriptional addiction in cancer. Cell 2017, 168:629–643
2. Malik S, Roeder RG: The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat Rev Genet 2010, 11:761–772
3. Soutourina J: Transcription regulation by the Mediator complex. Nat Rev Mol Cell Biol 2018, 19:262–274
4. Bourbon H-M: Comparative genomics supports a deep evolutionary origin for the large, four-module transcriptional mediator complex. Nucleic Acids Res 2008, 36:3993–4008
5. Brägelmann J, Klümper N, Offermann A, von Mässenhausen A, Böhm D, Deng M, Queisser A, Sanders C, Syring I, Merseburger AS,

Vogel W, Sievers E, Vlasic I, Carlsson J, Andrén O, Brossart P, Duensing S, Svensson MA, Shaikhibrahim Z, Kirfel J, Perner S: Pan-cancer analysis of the Mediator complex transcriptome identifies CDK19 and CDK8 as therapeutic targets in advanced prostate cancer. Clin Cancer Res 2017, 23:1829–1840

6. Becker F, Joerg V, Hupe MC, Roth D, Krupar R, Lubczyk V, Kuefer R, Sailer V, Duensing S, Kirfel J, Merseburger AS, Brägelmann J, Perner S, Offermann A: Increased mediator complex subunit CDK19 expression associates with aggressive prostate cancer. Int J Cancer 2020, 146:577–588
7. Nakamura A, Nakata D, Kakoi Y, Kunitomo M, Murai S, Ebara S, Hata A, Hara T: CDK8/19 inhibition induces premature G1/S transition and ATR-dependent cell death in prostate cancer cells. Oncotarget 2018, 9:13474–13487
8. Fant CB, Taatjes DJ: Regulatory functions of the Mediator kinases CDK8 and CDK19. Transcription 2019, 10:76–90
9. Firestein R, Bass AJ, Kim SY, Dunn IF, Silver SJ, Guney I, Freed E, Ligon AH, Vena N, Ogino S, Chheda MG, Tamayo P, Finn S, Shrestha Y, Boehm JS, Jain S, Bojarski E, Mermel C, Barretina J, Chan JA, Baselga J, Tabernero J, Root DE, Fuchs CS, Loda M, Shivdasani RA, Meyerson M, Hahn WC: CDK8 is a colorectal cancer oncogene that regulates β-catenin activity. Nature 2008, 455:547–551
10. Xu D, Li C-F, Zhang X, Gong Z, Chan C-H, Lee S-W, Jin G, Rezaeian A-H, Han F, Wang J, Yang W-L, Feng Z-Z, Chen W, Wu C-Y, Wang Y-J, Chow L-P, Zhu X-F, Zeng Y-X, Lin H-K: Skp2–MacroH2A1–CDK8 axis orchestrates G2/M transition and tumorigenesis. Nat Commun 2015, 6:6641
11. Kapoor A, Goldberg MS, Cumberland LK, Ratnakumar K, Segura MF, Emanuel PO, Menendez S, Vardabasso C, LeRoy G, Vidal CI, Polsky D, Osman I, Garcia BA, Hernando E, Bernstein E: The histone variant macroH2A suppresses melanoma progression through regulation of CDK8. Nature 2010, 468:1105–1109
12. Alarcón C, Zaromytidou A-I, Xi Q, Gao S, Yu J, Fujisawa S, Barlas A, Miller AN, Manova-Todorova K, Macias MJ, Sapkota G, Pan D, Massagué J: Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-β pathways. Cell 2009, 139:757–769
13. Allen BL, Taatjes DJ: The Mediator complex: a central integrator of transcription. Nat Rev Mol Cell Biol 2015, 16:155–166
14. Poss ZC, Ebmeier CC, Odell AT, Tangpeerachaikul A, Lee T, Pelish HE, Shair MD, Dowell RD, Old WM, Taatjes DJ: Identification of Mediator kinase substrates in human cells using cortistatin A and quantitative phosphoproteomics. Cell Rep 2016, 15:436–450
15. Dale T, Clarke PA, Esdar C, Waalboer D, Adeniji-Popoola O, Ortiz-Ruiz M-J, Mallinger A, Samant RS, Czodrowski P, Musil D, Schwarz D, Schneider K, Stubbs M, Ewan K, Fraser E, TePoele R, Court W, Box G, Valenti M, de Haven Brandon A, Gowan S, Rohdich F, Raynaud F, Schneider R, Poeschke O, Blaukat A, Workman P, Schiemann K, Eccles SA, Wienke D, Blagg J: A selective chemical probe for exploring the role of CDK8 and CDK19 in human disease. Nat Chem Biol 2015, 11:973–980
16. Mallinger A, Schiemann K, Rink C, Stieber F, Calderini M, Crumpler S, Stubbs M, Adeniji-Popoola O, Poeschke O, Busch M, Czodrowski P, Musil D, Schwarz D, Ortiz-Ruiz M-J, Schneider R, Thai C, Valenti M, de Haven Brandon A, Burke R, Workman P, Dale T, Wienke D, Clarke PA, Esdar C, Raynaud FI, Eccles SA, Rohdich F, Blagg J: Discovery of potent, selective, and orally bioavailable small-molecule modulators of the Mediator complex-associated kinases CDK8 and CDK19. J Med Chem 2016, 59:1078–1101
17. Koehler MFT, Bergeron P, Blackwood EM, Bowman K, Clark KR, Firestein R, Kiefer JR, Maskos K, McCleland ML, Orren L, Salphati L, Schmidt S, Schneider Ev, Wu J, Beresini MH: Development of a potent, specific CDK8 kinase inhibitor which phenocopies CDK8/19 knockout cells. ACS Med Chem Lett 2016, 7:223–228
18. Culig Z, Hoffmann J, Erdel M, Eder IE, Hobisch A, Hittmair A, Bartsch G, Utermann G, Schneider MR, Parczyk K, Klocker H: Switch from antagonist to agonist of the androgen receptor blocker bicalutamide is associated with prostate tumour progression in a new model system. Br J Cancer 1999, 81:242–251
19. Chen M, Liang J, Ji H, Yang Z, Altilia S, Hu B, Schronce A, McDermott MSJ, Schools GP, Lim C, Oliver D, Shtutman MS, Lu T, Stark GR, Porter DC, Broude Ev, Roninson IB: CDK8/19 Mediator kinases potentiate induction of transcription by NFκB. Proc Natl Acad Sci U S A 2017, 114:10208–10213
20. Menzl I, Zhang T, Berger-Becvar A, Grausenburger R, Heller G, Prchal-Murphy M, Edlinger L, Knab VM, Uras IZ, Grundschober E, Bauer K, Roth M, Skucha A, Liu Y, Hatcher JM, Liang Y, Kwiatkowski NP, Fux D, Hoelbl-Kovacic A, Kubicek S, Melo Jv, Valent P, Weichhart T, Grebien F, Zuber J, Gray NS, Sexl V: A kinase-independent role for CDK8 in BCR-ABL1+ leukemia. Nat Commun 2019, 10:4741
21. Nitulescu II, Meyer SC, Wen QJ, Crispino JD, Lemieux ME, Levine RL, Pelish HE, Shair MD: Mediator kinase phosphorylation of STAT1 S727 promotes growth of neoplasms with JAK-STAT activation. EBioMedicine 2017, 26:112–125
22. Bergeron P, Koehler MFT, Blackwood EM, Bowman K, Clark K, Firestein R, Kiefer JR, Maskos K, McCleland ML, Orren L, Ramaswamy S, Salphati L, Schmidt S, Schneider Ev, Wu J, Beresini M: Design and development of a series of potent and selective type II inhibitors of CDK8. ACS Med Chem Lett 2016, 7:595–600
23. Cornford P, van den Bergh RCN, Briers E, Van den Broeck T, Cumberbatch MG, De Santis M, Fanti S, Fossati N, Gandaglia G, Gillessen S, Grivas N, Grummet J, Henry AM, der Kwast THV, Lam TB, Lardas M, Liew M, Mason MD, Moris L, Oprea-Lager DE, der Poel HGV, Rouvière O, Schoots IG, Tilki D, Wiegel T, Willemse PM, Mottet N: EAU-EANM-ESTRO-ESUR-SIOG guidelines on prostate cancer: part II-2020 update: treatment of relapsing and metastatic prostate cancer. Eur Urol 2021, 79:263–282
24. Shore ND, Chowdhury S, Villers A, Klotz L, Siemens DR, Phung D, van Os S, Hasabou N, Wang F, Bhattacharya S, Heidenreich A: Efficacy and safety of enzalutamide versus bicalutamide for patients with metastatic prostate cancer (TERRAIN): a randomised, double-blind, phase 2 study. Lancet Oncol 2016, 17:153–163
25. Masiello D, Cheng S, Bubley GJ, Lu ML, Balk SP: Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. J Biol Chem 2002, 277:26321–26326
26. Bai VU, Cifuentes E, Menon M, Barrack ER, Reddy GP-V: Androgen receptor regulates Cdc6 in synchronized LNCaP cells progressing from G1 to S phase. J Cell Physiol 2005, 204:381–387
27. Putz EM, Gotthardt D, Hoermann G, Csiszar A, Wirth S, Berger A, Straka E, Rigler D, Wallner B, Jamieson AM, Pickl WF, Zebedin-Brandl EM, Müller M, Decker T, Sexl V: CDK8-mediated STAT1-S727 phosphorylation restrains NK cell cytotoxicity and tumor surveillance. Cell Rep 2013, 4:437–444
28. Rzymski T, Mikula M, Żyłkiewicz E, Dreas A, Wiklik K, Gołas A, Wójcik K, Masiejczyk M, Wróbel A, Dolata I, Kitlińska A, Statkiewicz M, Kuklinska U, Goryca K, Sapała Ł, Grochowska A, Cabaj A, Szajewska-Skuta M, Gabor-Worwa E, Kucwaj K, Białas A, Radzimierski A, Combik M, Woyciechowski J, Mikulski M, Windak R, Ostrowski J, Brzózka K: SEL120-34A is a novel CDK8 inhibitor active in AML cells with high levels of serine phosphorylation of STAT1 and STAT5 transactivation domains. Oncotarget 2017, 8:33779–33795
29. Cai W, Shen F, Feng Z, Chen J, Liu Q, Li E, Xu B, Cao J: Down-regulation of CDK-8 inhibits colon cancer hepatic metastasis by regulating Wnt/β-catenin pathway. Biomed Pharmacother 2015, 74:153–157