

Antitumor Effects of a Novel Small Molecule Targeting PCNA Chromatin Association in Prostate Cancer

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Abstract

Proliferating cell nuclear antigen (PCNA) plays an essential role in DNA replication and repair. Tumor cells express high levels of PCNA, identifying it as a potentially ideal target for cancer therapy. Previously, we identified nine compounds termed PCNA inhibitors (PCNA-Is) that bind directly to PCNA, stabilize PCNA trimer structure, reduce chromatin-associated PCNA, and selectively inhibit tumor cell growth. Of these compounds, PCNA-I1 is most potent. The purposes of this study were to further investigate the effects of targeting PCNA chromatin association on DNA damage and cytotoxicity and to evaluate the therapeutic potential of PCNA-I1 against tumors in mice. Given the important roles of tumor suppressor p53 in regulating sensitivity of tumor cells to chemotherapeutics, we performed studies in two human prostate cancer cell lines differing in p53 expression: LNCaP cells (wild-type p53) and PC-3 cells (p53-null). PCNA-I1 induced DNA damage and apoptosis in both LNCaP and PC-3 cells and enhanced DNA damage and apoptosis triggered by cisplatin. PCNA-I1 also induced autophagy in PC-3 cells. A short-term pretreatment with PCNA-I1 reduced colony formation by 50% in both cell lines. These data suggest that, unlike many other cytotoxic drugs, the effects of PCNA-I1 on tumor cells do not depend on expression of p53. Intravenous administrations of PCNA-I1 significantly retarded growth of LNCaP tumors of in nude mice without causing detectable effects on mouse body weight and hematology profiles. These data provide proof of concept that targeting PCNA chromatin association could be a novel and effective therapeutic approach for treatment of cancer. *Mol Cancer Ther*; 13(12); 2817–26. ©2014 AACR.

Introduction

Prostate cancer continues to be the most frequently occurring cancer in men in the United States and the second leading cause of cancer-related deaths in men (1). Although the 5-year survival rate of patients with localized prostate cancer is 100%, the prognosis for the 10% to 20% of patients who develop castration-resistant prostate cancer (CRPC) within that 5-year follow-up window is poor (1, 2). Currently, there is no cure for CRPC with treatment options limited to palliative care. Thus, indicating an urgent need for new and improved treatment modalities.

Proliferating cell nuclear antigen (PCNA) is a ubiquitous nuclear protein that plays an essential role in DNA replication and repair by providing replicative DNA polymerases and other partner proteins with the high processivity required to duplicate the entire genome (3–7). As a member of the DNA sliding clamp

family, functional PCNA is a ring-shaped homotrimer that is loaded onto chromatin by replication factor C (RFC; refs. 8–10). This allows PCNA to encircle and slide along DNA, increasing the efficiency of replicative DNA polymerases δ and ϵ during leading and lagging strand synthesis (7, 11). In addition, PCNA functions as a scaffold protein, binding a multitude of protein partners involved in many vital cellular processes such as DNA repair and cell-cycle control (7, 12). Collectively, these many functions of PCNA and its localization at the replisome put PCNA in a central position for determining the fate of the replication fork and numerous other cell signaling pathways.

Functional human PCNA are homotrimers joined in a head-to-tail arrangement (13, 14). Each PCNA monomer is composed of 261 amino acids and contains two globular domains, providing the trimeric ring 6-fold symmetry (15). The toroid structure of PCNA is evolutionarily well conserved; implicating the essential role for PCNA in basic cellular metabolism, which is underscored by the fact that homozygous deletion of PCNA results in embryonic lethality in mice (15–17). Trimerization of PCNA is crucial for carrying out its physiologic functions, as demonstrated in studies in which formation of the PCNA trimer was disrupted via a single mutation of tyrosine 114 (Y114A; ref. 18). This suggests that alterations to PCNA trimer structure and/or stability will affect PCNA function.

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PCNA is synthesized during all stages of the cell cycle; however, the rate of PCNA synthesis is increased 2 to 3-fold during early S phase (4, 5, 19). Furthermore, PCNA is present in two distinct populations, free PCNA and chromatin-associated PCNA; the latter is the functional form of PCNA (20). Gene deregulation and post-translational modifications of PCNA are hallmarks of malignant cells. Tumor cells, regardless of their origin, express high levels of PCNA, presumably to accommodate their high degree of uncontrolled replication (21). For these reasons, PCNA is a reliable diagnostic and prognostic biomarker (21–28).

Given that PCNA is a non-oncogenic mediator of DNA replication and is an essential component of the final common pathway that is shared by all mitogenic signals, we hypothesized that PCNA may be a valuable target for the development of novel cancer therapeutics. Previously, we performed an *in silico* screen of a compound library against a crystal structure of human PCNA and functional assays, these studies led to identification of nine compounds named as PCNA-inhibitors (PCNA-Is). These PCNA-Is bind directly to PCNA trimers, stabilize PCNA homotrimers structure, reduce PCNA association with chromatin, and attenuate DNA replication, and selectively inhibit growth of tumor cells of various tissue origins with IC_{50} values in the nanomolar range (29). Of those nine compounds, PCNA-II was the most potent. In this study, we show that treatment with PCNA-II induces DNA damage and programmed cell death and reduces clonogenicity of human prostate tumor cells. Furthermore, treatment with PCNA-II inhibited growth of LNCaP tumors in a xenograft model, providing proof of concept that targeting PCNA association with chromatin could be a novel and effective therapeutic approach for the treatment of cancer.

Materials and Methods

Mice

Specific pathogen-free male athymic nude mice were purchased from Jackson Laboratory and used in the study when they were 8 to 10 weeks of age. The mice were maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) and executed according to IACUC guidelines.

Reagents

Crystal violet, protease inhibitor cocktail, propidium iodide, and cisplatin were purchased from Sigma-Aldrich. Antibodies against pChk2 (T68), p53, phospho-p53 (S15), PCNA, cleaved PARP, and LC3B were purchased from Cell Signaling Technologies. Antibody to H2AX (S139) was purchased from Epitomics. Bcl-2 anti-

body was purchased from Santa Cruz Biotechnology. Alexa Fluor secondary antibodies were purchased from Invitrogen.

Cells and culture

LNCaP and PC-3 cells were obtained from ATCC in 2009 and 2011 and maintained at 37°C in 5% CO₂. LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% FBS. PC-3 cells were cultured in MEM/EBSS medium supplemented with 5% FBS, nonessential amino acids, sodium pyruvate, vitamin A, and glutamine. On the basis of the morphology, growth behaviors, and expression of androgen receptor and prostate specific antigen, we are certain they are LNCaP and PC-3 cells. However, no further authentication was performed. Cells in exponential growth phase were harvested by a 1- to 3-minute treatment with a 0.25% trypsin–0.02% EDTA solution and resuspended in the specified medium. Only suspensions of single cell with viability exceeding 95% (ascertained by Trypan blue exclusion) were used.

Clonogenic assay

Colony formation was assessed following a previously published protocol (30). Briefly, single cell suspensions of LNCaP and PC-3 cells were seeded into 6-well plates at 1×10^3 cells per well and allowed to adhere overnight. The cells were treated with 1 μ mol/L PCNA-II for 8 hours, washed with PBS, and cultured for 10 days. The colonies formed by the surviving cells were fixed with 10% formalin and stained with 0.5% crystal violet. Colonies containing more than 50 cells were viewed and counted under a stereomicroscope. The plating efficiency (PE) and surviving fraction (SF) were calculated (30).

Western blot analysis

LNCaP and PC-3 cells were seeded into 6-well plates at 5×10^5 cells per well and treated as described in the results. The cells were lysed using a lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor). Fifty mg of protein lysate was resolved by SDS-PAGE and analyzed immunoblotting with the specified antibodies. The immunoreactive signals were revealed using the enhanced chemiluminescence method (Millipore) and visualized using the Kodak IS4000MM Digital Imaging System (Carestream Health).

Immunofluorescence

Cells were seeded into a chamber slide at 2×10^4 cells per well. After an overnight incubation, the cells were treated with 1 μ mol/L PCNA-II for the times indicated, fixed with 2% paraformaldehyde, washed with PBS-0.1% Tween-20, permeabilized with methanol, and blocked using 5% normal goat serum. Primary antibodies were diluted per the manufacturer's recommendation and incubated overnight at 4°C. After washing, the cells were incubated with a fluorochrome-conjugated secondary

antibody, counterstained with DAPI, and mounted for analysis under a fluorescent microscopy. The images were captured with a cooled CCD camera using Spot Advanced software (Spot Imaging Solutions). The number of foci/cell was determined using ImageJ (NIH, Bethesda, MD).

Annexin V staining

Cells were seeded into 10 cm plates at 5×10^5 cells/plate. After an overnight incubation, the cells were treated for 48 hours with 1 $\mu\text{mol/L}$ PCNA-I1, tyrosinized, and collected in their respective media, stained using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen), and analyzed by flow cytometry for FITC Annexin V and propidium iodide (PI) using the Epics-XL-MCL system (Beckman Coulter). Data were analyzed using FCS Express (De Novo Software).

LNCaP tumor xenograft model

The xenograft LNCaP tumor model was detailed in our previous study (31). Briefly, mice were anesthetized and a small incision was created longitudinally on the dorsal lateral chest wall. LNCaP cells (2×10^6) soaked in a piece of gelfoam ("Vetspon", Novartis Animal Health) were placed under the skin. The wound was closed with a metal clip (Autoclip, Clay Adama) which was removed in 2 weeks after the surgery. Tumor size was measured twice a week using calipers. Tumor volume (mm^3) was calculated according to the formula: $(\text{width}^2 \times \text{Length})/2$.

Therapy procedure

Tumor-bearing mice were randomized into two groups and intravenously injected with vehicle (10% DMSO-10% Cremophor EL in PBS) or PCNA-I1. Control and treated tumor-bearing mice were monitored daily. Twice a week, mouse body weight was recorded for toxicity evaluation. Three days post therapy intervention, blood samples from control and treated mice were collected for evaluation of hematology profile (32). Experiments were terminated 6 weeks after the therapy intervention. Tumors were weighed and sampled for histology examination.

IHC analysis

Formalin-fixed tumor tissue was embedded in paraffin and cut into 4 μm sections and immunohistochemically stained as detailed previously (33). Briefly, tissue sections were deparaffinized in xylene followed by rehydration. Antigen was retrieved in Target Retrieval Solution (Dako). After treatment with 3% hydrogen peroxide, the sections were blocked with 5% goat serum and incubated with a primary antibody overnight at 4°C. The sections were rinsed and incubated with peroxidase-conjugated secondary antibodies. A positive reaction was visualized by incubating the slides with stable 3,3'-diaminobenzidine and with Liquid DAB-Plus Kit (Invitrogen) and counterstaining with Mayer's hematoxylin. Apoptosis in the tissue sections was analyzed using the terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay with a DeadEnd Fluorometric TUNEL

System (Promega) following the manufacturer's instructions. Images were examined under a microscope (a fluorescent microscope for the TUNEL staining) and captured using Spot camera (Spot Imaging Solutions).

Hematology profile analysis

The whole blood was collected from the submandibular vein of mice for hematology profile analysis. Briefly, animals were held by the scruff and a needle was used to puncture the vein. Blood (4 mice per group) was collected in microtainer tubes with EDTA. Samples were analyzed the same day using a Hemavet 950FS (Drew Scientific). Data are represented as mean \pm SD.

Statistical analysis

Data from each assay were expressed as means \pm SD. Statistical differences between two groups were determined by the Student *t* test. $P < 0.05$ was considered significantly different.

Results

Treatment with PCNA-I1 activates the DNA damage response in prostate cancer

Previously, we showed that treatment with PCNA-I1 reduced PCNA association with chromatin, inhibited cell growth and bromodeoxyuridine incorporation in cells, and induced S and G₂-M arrest (29). Because PCNA is required for DNA synthesis and repair, the attenuation of PCNA association to chromatin by PCNA-I1 may result in prolonged stalling of replication forks and cause collapse of the replication machinery, potentially leading to DNA damage and programmed cell death (7). As shown in Fig. 1A, treatment of both LNCaP and PC-3 cells with PCNA-I1 enhanced phosphorylation of the DNA damage response proteins Chk2. Total p53 and the DNA damage effector phosphor-p53 were increased in LNCaP cells but not in PC-3 cells, which are p53 null (Fig. 1A). Immunofluorescence staining showed that expression of γH2AX , the DNA double-strand break marker, was significantly enhanced in cells treated for 24 hours with PCNA-I1 (Fig. 1B). The numbers of γH2AX foci were elevated by 2.4- and 4.5-fold in LNCaP cells and PC-3 cells (Fig. 1B and C), respectively. The PCNA-I1-triggered expression of γH2AX was further elevated at 48 and 72 hours, revealed by immunoblotting (Fig. 1D).

PCNA-I1 treatment induces programmed cell death in prostate tumor cells

Given the DNA damage inflicted by treatment with PCNA-I1, we analyzed the effects of PCNA-I1 on apoptosis by using Annexin V staining and flow cytometry (Fig. 2A). Treatment with PCNA-I1 for 48 hours reduced the percentages of viable cells in both LNCaP (Fig. 2A, top and B) and PC-3 (Fig. 2A, bottom and C) cells (Annexin V⁻/PI⁻). PCNA-I1 treatment increased the percentages of dead cells (Annexin⁺/PI⁺) and apoptotic cells (Annexin V⁺/PI⁻; Fig. 2A-C). There was no significant increase in

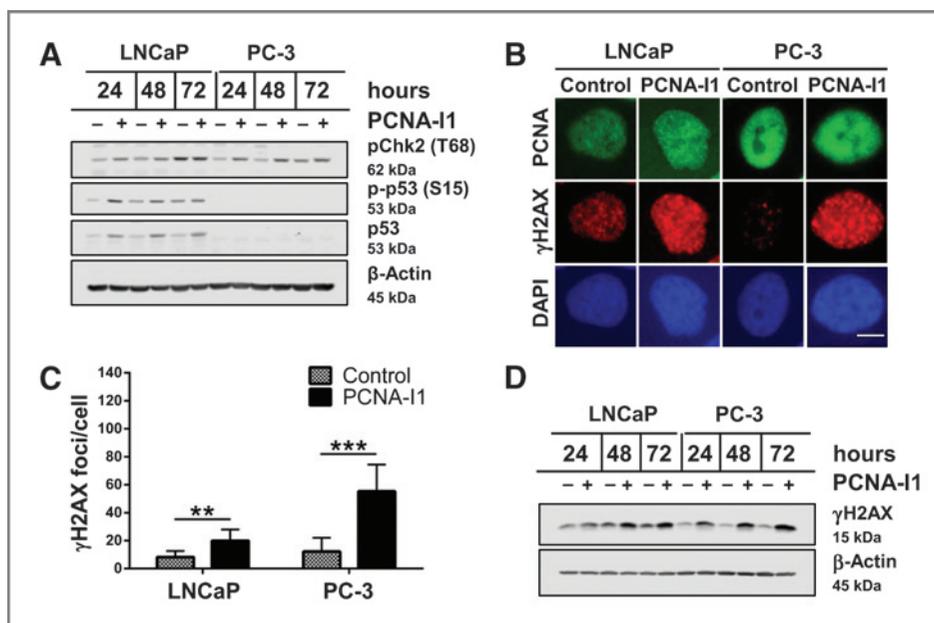


Figure 1. PCNA-I1 treatment activates the DNA damage response and induces DNA double-strand breaks in prostate cancer cells. **A**, expression of DNA damage response proteins of LNCaP and PC-3 cells treated with 1 μ mol/L PCNA-I1 for 24, 48, and 72 hours was analyzed by Western blot analysis. β -actin was used as a loading control. **B**, LNCaP and PC-3 cells were plated in chamber slides and treated with 1 μ mol/L PCNA-I1 for 24 hours. PCNA and γ H2AX expressions were visualized using fluorescently labeled secondary antibodies. Nuclei were stained with DAPI and visualized using $\times 40$ magnification. **C**, the number of γ H2AX foci in LNCaP and PC-3 cells treated with 1 μ mol/L PCNA-I1 were counted using ImageJ. **D**, γ H2AX expression was determined in LNCaP and PC-3 treated with 1 μ mol/L PCNA-I1 at 24, 48, and 72 hours by Western blot analysis. β -Actin was used as a loading control. **, $P < 0.01$, ***, $P < 0.001$.

necrotic cells (Annexin V⁻/PI⁺; Fig. 2A–C). We next examined the effects of PCNA-I1 on expression of the antiapoptotic protein Bcl2 in over a 72-hour period. Basal Bcl-2 expression was higher in PC-3 cells than LNCaP cells. Treatment with PCNA-I1 reduced Bcl-2 expression in both LNCaP and PC-3 cells at 48 and 72 hours (Fig. 2D), potentially causing the cells to be more susceptible to the induction of apoptosis. Therefore, we determined whether treatment of LNCaP cells with PCNA-I1 and cisplatin would produce additive or synergistic effects on DNA damage and apoptosis. LNCaP cells were treated for 12, 18, and 24 hours with PCNA-I1 and cisplatin alone or in combination. The combination treatment significantly increased expression of phosphorylated p53 and γ H2AX. Moreover, expression of cleaved apoptotic protein PARP was also significantly elevated (Fig. 2E). Furthermore, the combination treatment increased the percentage of necrotic cells (Annexin V⁻/PI⁺) and dead cells (Annexin V⁺/PI⁺) compared with cisplatin treatment alone (Fig. 2F), confirming the recent findings that inhibiting PCNA function sensitizes cells to DNA damage and cell death induced by cisplatin (34, 35).

PCNA-I1 treatment induces autophagy in PC-3 cells

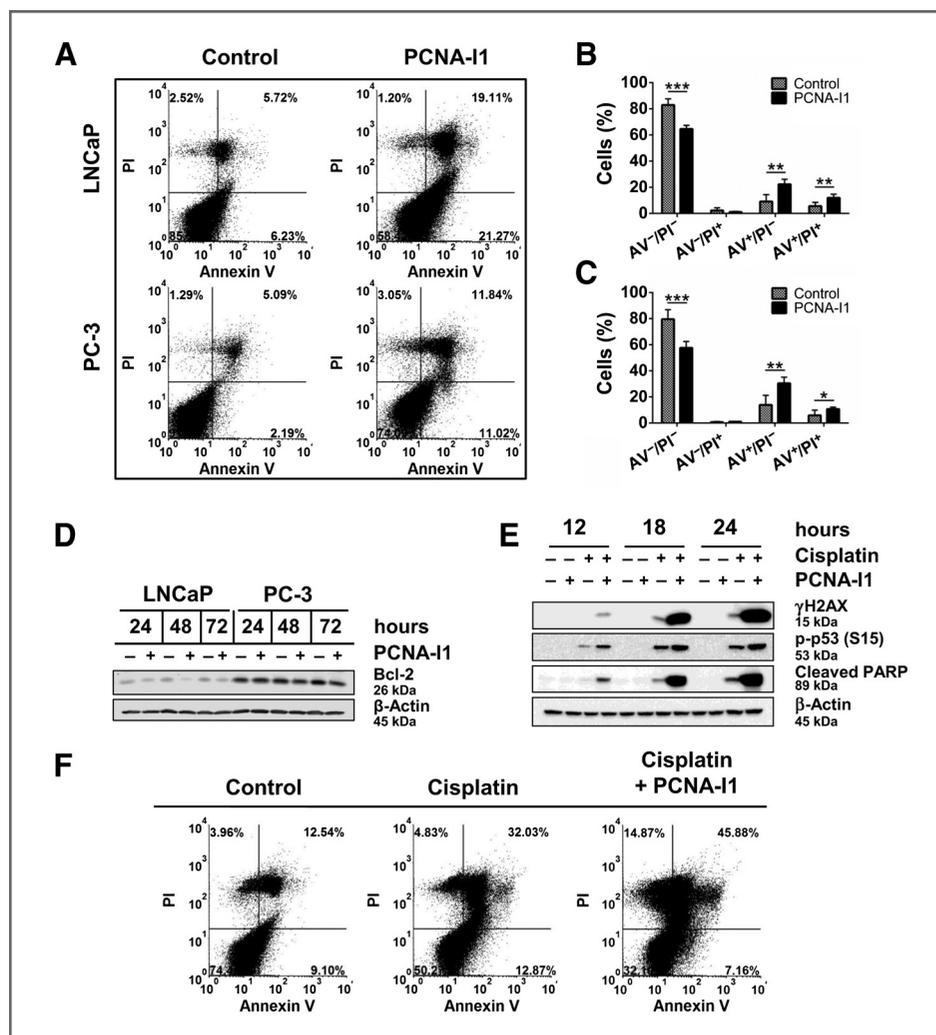
We next determined whether treatment with PCNA-I1 induced autophagy, the type-II programmed cell death. The phosphatidylethanolamine conjugated form of LC3B-I, known as LC3B-II, is commonly used as an autophagosomal marker. Immunofluorescent staining was used to

visualize the LC3B puncta, an indicator of autophagosome formation, in LNCaP and PC-3 cells. LC3B puncta were present in both control and PCNA-I1-treated LNCaP cells; however, there was no statistical difference in the number of puncta per cell (Fig. 3A and B). In contrast, there was a significant increase in the number of LC3B puncta present in PC-3 cells treated with PCNA-I1 (Fig. 3A and B). The differential expression of LC3B in LNCaP and PC-3 cells was further determined using immunoblotting. Although an increase in LC3B-I was observed in LNCaP cells treated with PCNA-I1, there was no expression of LC3B-II (Fig. 3C). In contrast, treatment with PCNA-I1 increased the expression of LC3B-II at all time points in PC-3 cells (Fig. 3C). Together, these data indicate that treatment with PCNA-I1 induced autophagy in PC-3 but not LNCaP cells.

Treatment with PCNA-I1 decreases clonogenicity of prostate tumor cells

Given that PCNA-I1 induced DNA damage and apoptosis in both LNCaP and PC-3 cells, and autophagy in PC-3 cells, we assessed the cytotoxic effects of a short-term (8 hours) PCNA-I1 exposure in a colony formation assay (30). The untreated PC-3 cells formed 247 ± 28 colonies, which is approximately two times more than those formed by LNCaP cells (109 ± 25 ; Fig. 4A and B). The colonies formed by PC-3 cells were also significantly larger than those formed by LNCaP cells (Fig. 4A). Despite differences in colony formation efficiencies between the

Figure 2. Treatment with PCNA-I1 induces apoptosis in LNCaP and PC-3 cells and combination treatment with cisplatin has synergistic effects in LNCaP cells. A, Annexin V staining was determined by flow cytometry in LNCaP and PC-3 cells treated with 1 $\mu\text{mol/L}$ PCNA-I1 for 48 hours. The percentage of normal, necrotic, apoptotic, and dead cells were plotted for LNCaP (B) and PC-3 cells (C). D, the expression of Bcl-2 in LNCaP and PC-3 cells treated with 1 $\mu\text{mol/L}$ PCNA-I1 for 24, 48, and 72 hours were analyzed by Western blot analysis. β -actin was used as a loading control. E, expression of DNA damage and apoptotic proteins in LNCaP cells treated with 1 $\mu\text{mol/L}$ PCNA-I1 and 5 $\mu\text{mol/L}$ cisplatin either alone or in combination for 12, 18, and 24 hours was determined by Western blot analysis. β -actin was used as a loading control. F, Annexin V staining was determined by flow cytometry in LNCaP cells treated with 5 $\mu\text{mol/L}$ cisplatin alone or in combination with 1 $\mu\text{mol/L}$ PCNA-I1 for 48 hours. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



two cell lines, the short-term treatment with PCNA-I1 resulted in approximately a 50% reduction in the colony formation by both LNCaP and PC-3 cells (Fig. 4C). The treatment with PCNA-I1 also significantly reduced the sizes of the colonies (Fig. 4A). These data indicated that a short-term pretreatment of PCNA-I1 was sufficient to produce the cytotoxic effects on LNCaP and PC-3 cells.

Intravenous injection of PCNA-I1 inhibits prostate tumor growth *in vivo*

One week after inoculation of LNCaP cells, tumor-bearing mice were intravenously injected either with vehicle or 10 mg/kg body weight of PCNA-I1, 5 days a week for 2 consecutive weeks. As shown in Fig. 5A, the treatment with PCNA-I1 significantly retarded growth of LNCaP tumors ($P < 0.01$). At the end of the therapy study, tumor weight in PCNA-I1-treated mice was approximately 28% of the weight of tumors in vehicle-treated mice ($P < 0.01$; Fig. 5B). The body weights were not significantly different between vehicle- and PCNA-I1-treated mice (Fig. 5C). To further evaluate potential acute (2 weeks after

therapy intervention) systemic toxic effects of PCNA-I1, we examined the hematology profiles and found that the treatment of PCNA-I1 did not cause significant alterations to the profiles of leukocytes, erythrocytes, and thrombocytes (Table 1). These data indicate that the therapy with PCNA-I1 was effective against the growth of LNCaP tumors and did not cause significant toxicity to the host.

IHC analysis of tumor lesions showed that treatment with PCNA-I1 reduced expression of PCNA by approximately 26% ($P < 0.01$, Fig. 5D and E) and increased the number of apoptotic cells (TUNEL staining) by approximately 5-fold ($P < 0.01$, Fig. 5D and F), respectively.

Discussion

Previously we reported a series of novel small-molecule compounds which bind directly to PCNA trimers, stabilize the trimer structure, reduce PCNA association with chromatin, inhibit DNA replication, and selectively inhibit tumor cell growth (29). In the present study, PCNA-I1, which is most potent among the nine PCNA-I1s, was chosen for further investigation to determine the effects of

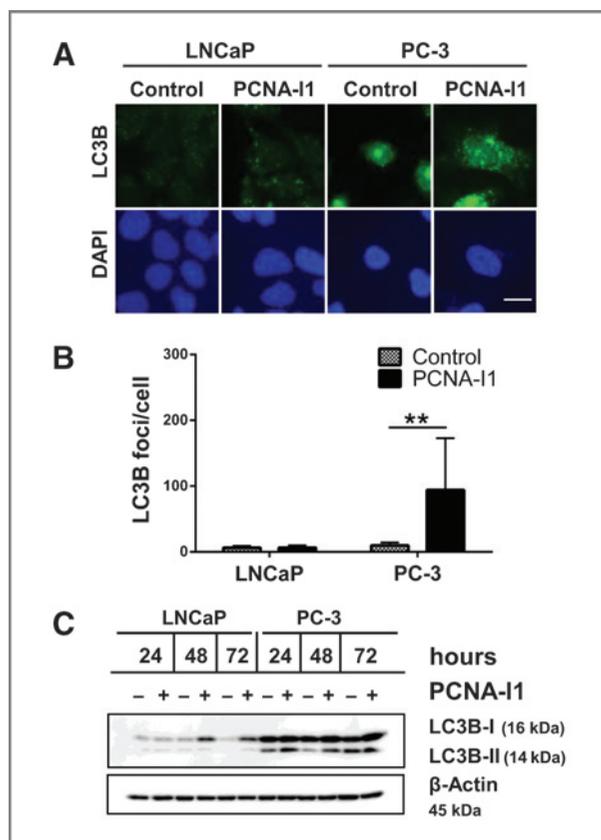


Figure 3. PCNA-I1 treatment induces autophagy in PC-3 cells. A, LNCaP and PC-3 cells were seeded into chamber slides and treated with 1 mol/L PCNA-I1 for 24 hours. LC3B puncta were visualized using a fluorescently labeled secondary antibody. Nuclei were stained with DAPI and visualized using $\times 40$ magnification. B, the number of LC3B puncta in LNCaP and PC-3 cells were quantified using ImageJ. C, expression of autophagy proteins was analyzed in LNCaP and PC-3 cells treated with 1 M PCNA-I1 for 24, 48, and 72 hours by Western blot analysis. β -actin was used as a loading control. **, $P < 0.01$.

targeting PCNA chromatin association on DNA damage and cytotoxicity and to evaluate therapeutic potential in a xenograft model of human prostate cancer in nude mice.

Replication stress and stalling of replication forks have been shown to increase susceptibility to DNA damage, resulting in the formation of double-strand breaks, the activation of ATM (36), and potentially cell death. The inhibitory effects of PCNA-I1 on DNA replication and the observed S-G₂-M phase arrest (31) implicate replication stress and fork stalling. Consistent with these findings, treatment with PCNA-I1 resulted in activation of Chk2, leading to an increase expression of p53 as well as an increased phosphorylation of p53 in LNCaP cells. Moreover, we found that the DNA double-strand break marker γ H2AX was increased in both LNCaP and PC-3 cells treated with PCNA-I1. These findings indicate that replication stress induced by PCNA-I1 causes the accumulation of DNA damage in prostate tumor cells.

The accumulation of DNA damage beyond the repair capability of cells will eventually result in cell death.

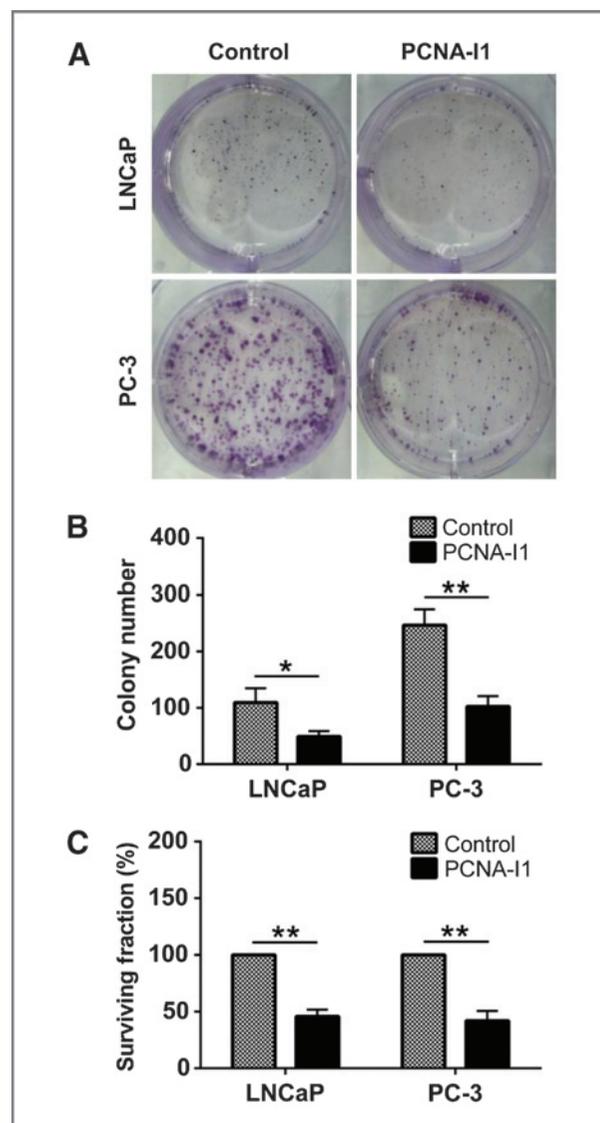
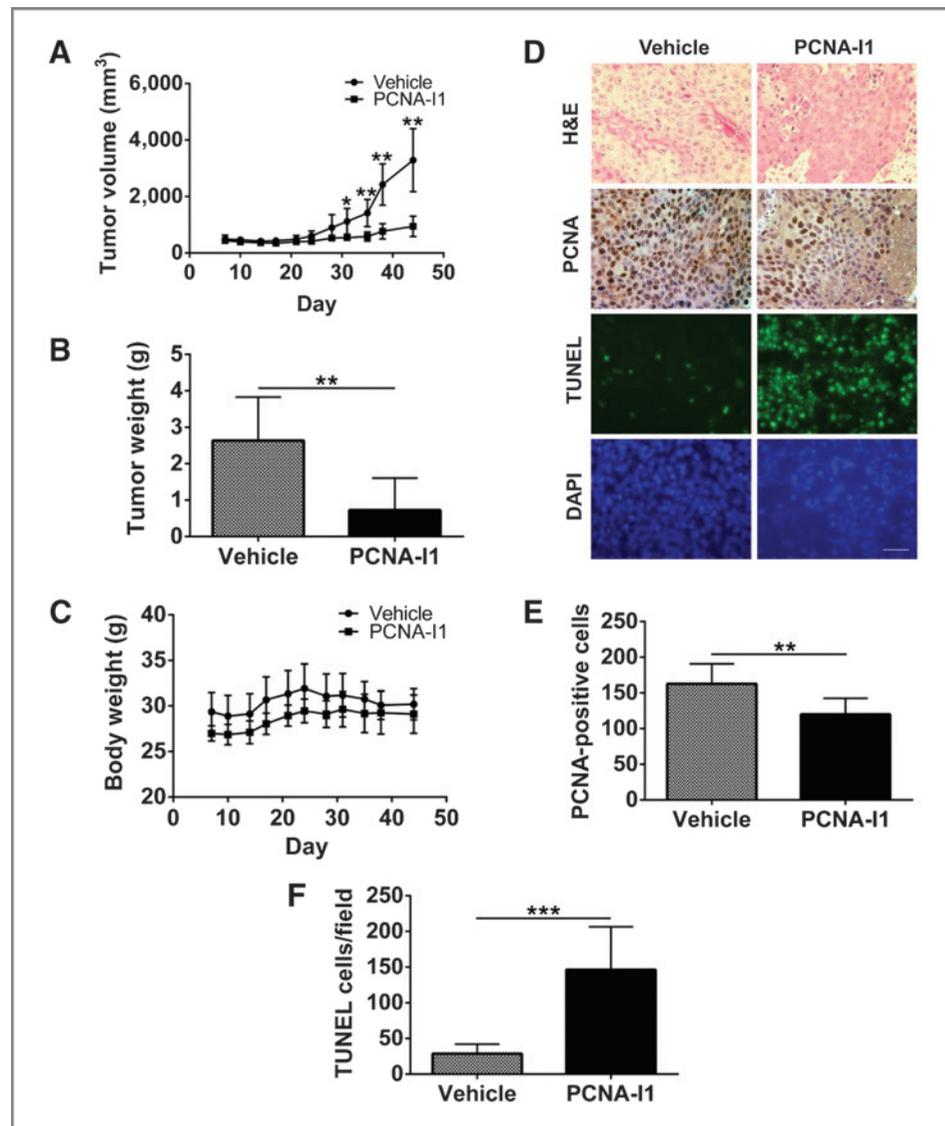


Figure 4. PCNA-I1 treatment reduces clonogenicity of prostate tumor cells. A, LNCaP and PC-3 cells were seeded into a 6-well plate and allowed to adhere overnight before treatment with 1 μ mol/L PCNA-I1 for 8 hours. Cells were allowed to grow into colonies for 10 days before being fixed and stained with crystal violet. B, the number of colonies containing ≥ 50 cells was counted using a stereomicroscope. C, the surviving fraction was calculated using the formula: surviving fraction = (plating efficiency of treated/plating efficiency of control) \times 100. *, $P < 0.05$; **, $P < 0.01$.

Analysis of programmed cell death demonstrated that the PCNA-I1-mediated inhibition of DNA replication (29) and DNA damage were sufficient for inducing apoptosis in LNCaP and PC-3 cells. Consistent with the effects on apoptosis, treatment with PCNA-I1 reduced the expression of the antiapoptotic protein Bcl-2 in both cell lines. Bcl-2 protein, not detectable in normal human prostatic tissue, is expressed in primary prostatic adenocarcinoma and is further elevated in CRPC (37). This expression of Bcl-2 has been shown to confer resistance

Figure 5. Administering PCNA-I1 intravenously inhibits prostate tumor growth *in vivo*. A total of 2×10^6 LNCaP cells were absorbed into a gelatin sponge and implanted subcutaneously in to the flanks of nude mice. One week later, tumor-bearing mice were treated with vehicle or 10 mg/kg PCNA-I1 by intravenous injection 5 days/week for 2 consecutive weeks. A, tumor volume was measured by calipers twice per week over a 6-week period. B, tumors were isolated from mice at the end of treatment and weighed. C, the body weight of mice harboring LNCaP tumors were monitored twice per week over a 6-week period. D, tumor tissues were fixed in formaldehyde and embedded in paraffin. Tissue sections were then stained with H&E, PCNA, and TUNEL with DAPI counterstain and visualized at $\times 40$ magnification. E, the number of PCNA-positive cells were quantified in vehicle and PCNA-I1-treated tissue sections. F, the number of TUNEL-positive cells were quantified in vehicle and PCNA-I1-treated tissue sections. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



to apoptotic stimuli both *in vitro* and *in vivo* and allow the normally androgen-sensitive LNCaP cells to form tumors in an androgen-depleted host, thus promoting progression of prostate cancer to CRPC (37, 38). Therefore, the observed decrease in Bcl-2 expression upon treatment with PCNA-I1 suggests that these cells may be sensitized to apoptosis. This finding is further confirmed by the fact that PCNA-I1 treatment sensitizes LNCaP cells to cisplatin treatment. Typically, prostate cancer is intrinsically resistant to cisplatin-based therapies (39). However, combination treatment of PCNA-I1 and cisplatin synergistically increased γ H2AX, phospho-p53, and cleaved PARP expression and the percentage of apoptotic cells compared with cisplatin treatment alone. Similar findings of improved sensitivity to cisplatin via inhibition of translesion synthesis (TLS) using a small-molecule inhibitor of PCNA that binds to the PIP-BOX have been reported

(34, 35). Whether PCNA-I1 improves sensitivity to cisplatin treatment through inhibition of TLS remains to be determined. The tumor suppressor protein p53, often mutated in human tumors, regulates apoptosis and cell survival upon DNA damage (39–42). Tumor cells with p53 mutations are often resistant to cytotoxic drugs, such as cisplatin (43–45). Given that PC-3 cells do not express tumor suppressor p53, our data indicate that the cytotoxic effects of PCNA-I1 were likely mediated by both p53-dependent and -independent pathways.

Autophagy, the type-II programmed cell death, has been described as having both cytoprotective and cytotoxic functions in tumor cells, both of which have implications for the treatment of cancer (46). Although autophagy is traditionally thought of as a cell-survival pathway, it has been demonstrated that excessive or prolonged autophagy results in "autophagic death" that

Table 1. PCNA-I1 did not affect hematology profiles

	Vehicle	PCNA-I1
Leukocytes		
WBC (K/ μ L)	8.57 \pm 5.14	7.00 \pm 2.23
NE (K/ μ L)	1.26 \pm 0.65	1.45 \pm 0.43
LY (K/ μ L)	6.64 \pm 4.58	4.97 \pm 2.08
MO (K/ μ L)	0.61 \pm 0.45	0.37 \pm 0.17
EO (K/ μ L)	0.04 \pm 0.03	0.15 \pm 0.12
BA (K/ μ L)	0.01 \pm 0.02	0.06 \pm 0.07
NE (%)	20.27 \pm 16.52	22.55 \pm 9.44
LY (%)	72.14 \pm 15.69	69.31 \pm 9.04
MO (%)	6.36 \pm 2.47	5.31 \pm 2.09
EO (%)	0.92 \pm 1.30	2.14 \pm 1.14
BA (%)	0.30 \pm 0.59	0.69 \pm 0.61
Erythrocytes		
RBC (M/ μ L)	8.96 \pm 1.92	9.55 \pm 1.08
Hb(g/dL)	12.5 \pm 2.05	13.27 \pm 1.16
HCT (%)	52.28 \pm 9.75	55.82 \pm 5.78
MCV (fL)	58.67 \pm 1.92	58.57 \pm 3.35
MCH (pg)	14.08 \pm 0.77	13.93 \pm 0.83
MCHC (g/dL)	23.98 \pm 0.58	23.82 \pm 0.63
RDW (%)	18.97 \pm 1.92	18.35 \pm 1.11
Thrombocytes		
PLT (K/ μ L)	704.67 \pm 220.30	701.83 \pm 201.87
MPV (fL)	4.6 \pm 0.46	4.95 \pm 0.30

NOTE: Blood was collected from 4 mice per group by submandibular puncture following the described treatment. Data shown are mean \pm SD from 4 mice.

occurs either independent of or in conjunction with apoptosis (47–49). We examined the effects of PCNA-I1 on autophagy in LNCaP and PC-3 cells. Treatment of LNCaP cells with PCNA-I1 did not induce autophagosome formation. However, it did significantly increase autophagosome formation in PC-3 cells. Given the observed increase in Annexin V staining and γ H2AX expression upon treatment with PCNA-I1 in PC-3 cells, it is possible that this treatment induces the cytotoxic form of autophagy. However, future studies using a pharmacologic inhibitor of autophagy such as bafilomycin, chloroquine, or 3-methyl adenine will be necessary to confirm PCNA-I1 induction of autophagic death. If in fact autophagy is playing a cytoprotective role in PC-3 cells, these inhibitors could be used to improve sensitivity to PCNA-I1 and promote apoptosis. Regardless of the mechanism of programmed cell death, the cytotoxic effects of PCNA-I1 on both LNCaP and PC-3 cells were further confirmed by data from the clonogenic assay.

The therapeutic effects of targeting PCNA chromatin association using PCNA-I1 were investigated in the xeno-

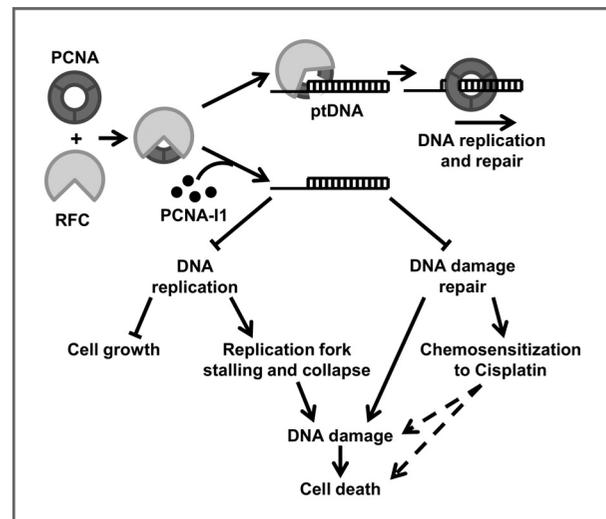


Figure 6. Summary of findings. Under normal conditions, PCNA is loaded onto chromatin by RFC at primer-template junctions (ptDNA) facilitating both DNA synthesis and repair. Treatment with PCNA-I1 stabilizes PCNA homotrimers inhibiting PCNA loading onto chromatin. This results in inhibition of DNA replication and DNA damage repair. Inhibition of DNA replication inhibits tumor cell growth and leads to stalling of replication forks. This prolonged stalling ultimately leads to replication fork collapse that induces DNA damage and cell death. Inhibition of DNA damage repair was also found to chemosensitize tumor cells to treatment with cisplatin, resulting in a synergistic effect on both DNA damage accumulation and cell death.

graft model of LNCaP tumors. Our data show that intravenous administrations of PCNA-I1 significantly retarded growth of LNCaP tumor in nude mice. The treatment induced massive apoptosis and growth inhibition, as evidenced by the TUNEL staining and IHC analysis of PCNA expression in tumors.

One of the important toxic side effects of many chemotherapeutic agents is depression of bone marrow, leading to leukopenia and thrombocytopenia, which may subsequently cause severe infection and septicemia. We found that the therapeutic dose of PCNA-I1 did not significantly change the body weights and hematology profiles of tumor-bearing mice, indicating that the treatment did not cause significant systemic toxicity. This is possibly due to the fact that normal cells, including the primary cultures of bone marrow mesenchymal stem cells, endothelial cells, lymphocytes, mammary epithelial cells, and prostate epithelial cells, are nine times less sensitive to PCNA-I1 than tumor cells of various tissue origins (29). This property of therapeutic dose of PCNA-I1 provides a strong rationale for future clinical applications of PCNA-I1 or its derivatives for cancer therapy.

In summary, our data show that treatment with PCNA-I1 induced DNA damage, apoptosis, and autophagic cell death in two lines of human prostate cancer. The potential pathways leading to cell death induced by PCNA-I1 are summarized in Fig. 6. Significant therapeutic effects of PCNA-I1 were also observed. Importantly, the beneficial therapeutic effects of PCNA-I1 are likely not limited to

prostate cancer because PCNA is required and is over-expressed in almost all cancer cells. The therapeutic implications for PCNA-II are vast in that regardless of factors driving the uncontrolled replication of tumor cells, PCNA is an essential component of DNA replication in the final common pathway shared by all mitogenic signals. This notion is supported by the fact that PCNA-II was shown to inhibit growth of all tumor cells examined in our previous study, including human breast cancer, prostate cancer, and melanoma and mouse prostate and colon cancer, melanoma, and fibrosarcoma, as well as tumor cells with multidrug resistance phenotype (29). Therefore, future studies will focus on further characterizing the effects of this class of compounds on the myriad of PCNA functions that could potentially be exploited for the treatment of a variety of cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64:9–29.
- Kirby M, Hirst C, Crawford ED. Characterising the castration-resistant prostate cancer population: a systematic review. *Int J Clin Pract* 2011;65:1180–92.
- Miyachi K, Fritzier MJ, Tan EM. Autoantibody to a nuclear antigen in proliferating cells. *J Immunol* 1978;121:2228–34.
- Bravo R, Celis JE. Up-dated catalogue of HeLa cell proteins: percentages and characteristics of the major cell polypeptides labeled with a mixture of 16 ¹⁴C-labeled amino acids. *Clin Chem* 1982;28:766–81.
- Morris GF, Mathews MB. Regulation of proliferating cell nuclear antigen during the cell cycle. *J Biol Chem* 1989;264:13856–64.
- Garg P, Burgers PM. DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit Rev Biochem Mol Biol* 2005;40:115–28.
- Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. *Cell* 2007;129:665–79.
- Gomes XV, Schmidt SL, Burgers PM. ATP utilization by yeast replication factor C. II. Multiple stepwise ATP binding events are required to load proliferating cell nuclear antigen onto primed DNA. *J Biol Chem* 2001;276:34776–83.
- Majka J, Burgers PM. The PCNA-RFC families of DNA clamps and clamp loaders. *Prog Nucleic Acid Res Mol Biol* 2004;78:227–60.
- Bowman GD, O'Donnell M, Kuriyan J. Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex. *Nature* 2004;429:724–30.
- Pursell ZF, Isoz I, Lundstrom EB, Johansson E, Kunkel TA. Yeast DNA polymerase epsilon participates in leading-strand DNA replication. *Science* 2007;317:127–30.
- Naryzhny SN. Proliferating cell nuclear antigen: a proteomics view. *Cell Mol Life Sci* 2008;65:3789–808.
- Gulbis JM, Kelman Z, Hurwitz J, O'Donnell M, Kuriyan J. Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA. *Cell* 1996;87:297–306.
- Schurtenberger P, Egelhaaf SU, Hindges R, Maga G, Jonsson ZO, May RP, et al. The solution structure of functionally active human proliferating cell nuclear antigen determined by small-angle neutron scattering. *J Mol Biol* 1998;275:123–32.
- Krishna TS, Kong XP, Gary S, Burgers PM, Kuriyan J. Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell* 1994;79:1233–43.
- Kelman Z, O'Donnell M. Structural and functional similarities of prokaryotic and eukaryotic DNA polymerase sliding clamps. *Nucleic Acids Res* 1995;23:3613–20.
- Roa S, Avdievich E, Peled JU, Maccarthy T, Werling U, Kuang FL, et al. Ubiquitylated PCNA plays a role in somatic hypermutation and class-switch recombination and is required for meiotic progression. *Proc Natl Acad Sci U S A* 2008;105:16248–53.
- Jonsson ZO, Podust VN, Podust LM, Hubscher U. Tyrosine 114 is essential for the trimeric structure and the functional activities of human proliferating cell nuclear antigen. *EMBO J* 1995;14:5745–51.
- Bravo R, Celis JE. A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. *J Cell Biol* 1980;84:795–802.
- Bravo R, Macdonald-Bravo H. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J Cell Biol* 1987;105:1549–54.
- Naryzhny SN, Lee H. Characterization of proliferating cell nuclear antigen (PCNA) isoforms in normal and cancer cells: there is no cancer-associated form of PCNA. *FEBS Lett* 2007;581:4917–20.
- Kimos MC, Wang S, Borkowski A, Yang GY, Yang CS, Perry K, et al. Esophagin and proliferating cell nuclear antigen (PCNA) are biomarkers of human esophageal neoplastic progression. *Int J Cancer* 2004;111:415–7.
- Stuart-Harris R, Caldas C, Pinder SE, Pharoah P. Proliferation markers and survival in early breast cancer: a systematic review and meta-analysis of 85 studies in 32,825 patients. *Breast* 2008;17:323–34.
- Gould Rothberg BE, Bracken MB, Rimm DL. Tissue biomarkers for prognosis in cutaneous melanoma: a systematic review and meta-analysis. *J Natl Cancer Inst* 2009;101:452–74.
- Kallakury BV, Sheehan CE, Rhee SJ, Fisher HA, Kaufman RP Jr, Rifkin MD, et al. The prognostic significance of proliferation-associated nucleolar protein p120 expression in prostate adenocarcinoma: a comparison with cyclins A and B1, Ki-67, proliferating cell nuclear antigen, and p34cdc2. *Cancer* 1999;85:1569–76.
- Malkas LH, Herbert BS, Abdel-Aziz W, Dobrolecki LE, Liu Y, Agarwal B, et al. A cancer-associated PCNA expressed in breast cancer has implications as a potential biomarker. *Proc Natl Acad Sci U S A* 2006;103:19472–7.
- Miyamoto S, Ito K, Kurokawa K, Suzuki K, Suzuki K, Yamanaka H. Clinical validity of proliferating cell nuclear antigen as an objective marker for evaluating biologic features in patients with untreated prostate cancer. *Int J Urol* 2006;13:767–72.
- Zhong W, Peng J, He H, Wu D, Han Z, Bi X, et al. Ki-67 and PCNA expression in prostate cancer and benign prostatic hyperplasia. *Clin Invest Med* 2008;31:E8–15.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Dillehay, Z. Dong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.L. Dillehay, S. Lu, Z. Dong
Writing, review, and/or revision of the manuscript: K.L. Dillehay, S. Lu, Z. Dong
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.L. Dillehay, Z. Dong
Study supervision: Z. Dong

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29. Tan Z, Wortman M, Dillehay KL, Seibel WL, Evelyn CR, Smith SJ, et al. Small-molecule targeting of proliferating cell nuclear antigen chromatin association inhibits tumor cell growth. *Mol Pharmacol* 2012;81:811–9.
30. Munshi A, Hobbs M, Meyn RE. Clonogenic cell survival assay. *Methods Mol Med* 2005;110:21–8.
31. Cui L, Chen P, Tan Z, Li W, Dong Z. Hemostatic gelatin sponge is a superior matrix to matrigel for establishment of LNCaP human prostate cancer in nude mice. *Prostate* 2012;72:1669–77.
32. Tan Z, Chen P, Schneider N, Glover S, Cui L, Torgue J, et al. Significant systemic therapeutic effects of high-LET immunoradiation by ²¹²Pb-trastuzumab against prostatic tumors of androgen-independent human prostate cancer in mice. *Int J Oncol* 2012;40:1881–8.
33. Zhang F, Lee J, Lu S, Pettaway CA, Dong Z. Blockade of transforming growth factor-beta signaling suppresses progression of androgen-independent human prostate cancer in nude mice. *Clin Cancer Res* 2005;11:4512–20.
34. PUNCHIHEWA C, INOUE A, HISHIKI A, FUJIKAWA Y, CONNELLY M, EVISON B, et al. Identification of small molecule proliferating cell nuclear antigen (PCNA) inhibitor that disrupts interactions with PIP-box proteins and inhibits DNA replication. *J Biol Chem* 2012;287:14289–300.
35. Inoue A, Kikuchi S, Hishiki A, Shao Y, Heath R, Evison BJ, et al. A small molecule inhibitor of monoubiquitinated proliferating cell nuclear antigen (PCNA) inhibits repair of interstrand DNA cross-link, enhances DNA double strand break, and sensitizes cancer cells to cisplatin. *J Biol Chem* 2014;289:7109–20.
36. Zeman MK, Cimprich KA. Causes and consequences of replication stress. *Nat Cell Biol* 2014;16:2–9.
37. Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS, Buttyan R. Overexpression of bcl-2 protects prostate cancer cells from apoptosis *in vitro* and confers resistance to androgen depletion *in vivo*. *Cancer Res* 1995;55:4438–45.
38. Kajiwara T, Takeuchi T, Ueki T, Moriyama N, Ueki K, Kakizoe T, et al. Effect of Bcl-2 overexpression in human prostate cancer cells *in vitro* and *in vivo*. *Int J Urol* 1999;6:520–5.
39. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, et al. Molecular mechanisms of cisplatin resistance. *Oncogene* 2012;31:1869–83.
40. Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, et al. Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res* 1996;56:556–62.
41. O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, et al. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. *Cancer Res* 1997;57:4285–300.
42. Branch P, Masson M, Aquilina G, Bignami M, Karran P. Spontaneous development of drug resistance: mismatch repair and p53 defects in resistance to cisplatin in human tumor cells. *Oncogene* 2000;19:3138–45.
43. Hengstler JG, Pilch H, Schmidt M, Dahlenburg H, Sagemuller J, Schiffer I, et al. Metallothionein expression in ovarian cancer in relation to histopathological parameters and molecular markers of prognosis. *Int J Cancer* 2001;95:121–7.
44. Siddik ZH, Hagopian GS, Thai G, Tomisaki S, Toyomasu T, Khokhar AR. Role of p53 in the ability of 1,2-diaminocyclohexane-diacetate-dichloro-Pt(IV) to circumvent cisplatin resistance. *J Inorg Biochem* 1999;77:65–70.
45. Zenz T, Benner A, Dohner H, Stilgenbauer S. Chronic lymphocytic leukemia and treatment resistance in cancer: the role of the p53 pathway. *Cell Cycle* 2008;7:3810–4.
46. Gewirtz DA. The four faces of autophagy: implications for cancer therapy. *Cancer Res* 2014;74:647–51.
47. Su M, Mei Y, Sinha S. Role of the crosstalk between autophagy and apoptosis in cancer. *J Oncol* 2013;2013:102735.
48. Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, et al. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis* 2013;4:e838.
49. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005;5:726–34.

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