Anti-tumor effects of a novel small molecule targeting PCNA chromatin association in prostate cancer

Kelsey L. Dillehay§, Shan Lu§, and Zhongyun Dong¶,2

¶Department of Internal Medicine, §Department of Pathology and Molecular Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 46267

Running title: Targeting PCNA for cancer therapy

Key words: Proliferating cell nuclear antigen, DNA damage, apoptosis, autophagy, therapy, prostate cancer

1Financial support: This work was supported in part by the National Institutes of Health National Cancer Institute grants: R01-CA131137-01A1 (to Z. Dong), the Millennium Scholar Funds from the University of Cincinnati Cancer Center (to S. Lu and Z. Dong), and the Dean’s Bridge Funding of College of Medicine (to Z. Dong).

2To whom requests for reprints and other correspondences should be addressed at the Department of Internal Medicine, University of Cincinnati College of Medicine, 3125 Eden Ave., Rm 1308, Cincinnati, OH 45267. Phone: 513-558-2176; Fax: 513-558-6703; e-mail: dongzu@ucmail.uc.edu.

Conflicts of interest: None
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 (wildtype 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.
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). While the 5 year survival rate of patients with localized prostate cancer is 100%, the prognosis for the 10-20% 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 which is loaded onto chromatin by replication factor C (RFC) (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). Additionally, 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 comprised of 261 amino acids and contains two globular domains, providing the trimeric ring sixfold symmetry (15). The toroid structure of PCNA is evolutionally
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 physiological functions, as demonstrated in studies in which formation of the PCNA trimer was disrupted via a single mutation of tyrosine 114 (Y114A) (18). This suggests that alterations to PCNA trimer structure and/or stability will affect PCNA function.

PCNA is synthesized during all stages of the cell cycle; however, the rate of PCNA synthesis is increased 2-3 fold during early S phase (5, 19, 20). Furthermore, PCNA is present in two distinct populations, free PCNA and chromatin associated PCNA; the latter is the functional form of PCNA (21). 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 (22). For these reasons, PCNA is a reliable diagnostic and prognostic biomarker (22-29).

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 (30). Of those nine compounds PCNA-I1 was most potent. In this study, we show that treatment with PCNA-I1 induces DNA damage and programmed cell death and reduces clonogenicity of human prostate tumor cells. Furthermore, treatment with PCNA-I1 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 (Bar Harbor, ME) and used in the study when they were 8-10 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 National Institute of Health. 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 (St. Louis, MO). Antibodies against pChk2 (T68), p53, phosphor-p53 (S15), PCNA, cleaved PARP, and LC3B were purchased from Cell Signaling Technologies (Danvers, MA). Antibody to H2AX (S139) was purchased from Epitomics (Burlingame, CA). Bcl-2 antibody was purchased from Santa Cruz (Dallas, TX). Alexa Flour secondary antibodies were purchased from Invitrogen (Grand Island, NY).
Cells and Culture. LNCaP and PC-3 cells were obtained from ATCC (Manassas, VA) 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. Based on 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 1-3 minute treatment with a 0.25% trypsin – 0.02% EDTA solution and resuspended in the specified medium. Only suspensions of singles cell with viability exceeding 95% (ascertained by trypan blue exclusion) were used.

Clonogenic Assay. Colony formation was assessed following a previously published protocol (31). Briefly, single cell suspensions of LNCaP and PC-3 cells were seeded into 6 well plates at 1 x 10^3 cells/well and allowed to adhere overnight. The cells were treated with 1 μM PCNA-I1 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 greater than 50 cells were viewed and counted under a stereomicroscope. The plating efficiency (PE) and surviving fraction (SF) were calculated (31).

Western Blot Analysis. LNCaP and PC-3 cells were seeded into 6-well plates at 5 x 10^5 cells/well and treated as described in the results. The cells were lysed using a lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM 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, Billerica, MA) and visualized using the Kodak IS4000MM Digital Imaging System (Carestream Health, Rochester, NY).

**Immunofluorescence.** Cells were seeded into a chamber slide at $2 \times 10^4$ cells/well. After an overnight incubation, the cells were treated with 1 μM PCNA-I1 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, Sterling Heights, MI). The number of foci/cell was determined using ImageJ (National Institute of Health, Bethesda, MD).

**Annexin V staining.** Cells were seeded into 10 cm plates at $5 \times 10^5$ cells/plate. After an overnight incubation, the cells were treated for 48 hours with 1 μM PCNA-I1, trypsinized, and collected in their respective media, stained using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA), and analyzed by flow cytometry for FITC Annexin V and
propidium iodide (PI) using the Epics-XL-MCL system (Beckman Coulter, Fullerton, CA). Data was analyzed using FCS Express (De Novo Software, Los Angeles, CA).

**LNCaP tumor xenograft model.** The xenograft LNCaP tumor model was detailed in our previous study (32). 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, Greensboro, SC) were placed under the skin. The wound was closed with a metal clip (Autoclip, Clay Adama, Parsippany, NJ) which were removed in 2 weeks after the surgery. Tumor size was measured twice a week using calipers. Tumor volume (mm^3) was calculated according the formula: (width^2 x Length)/2.

**Therapy procedure.** Tumor-bearing mice were randomized two groups and intravenously injected 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 (33). Experiments were terminated 6 weeks after the therapy intervention. Tumors were weighed and sampled for histology examination.

**Immunohistochemical analysis.** Formalin fixed tumor tissue was embedded in paraffin and cut into 4 μm sections and immunohistochemically stained as detailed previously (34). Briefly,
tissue sections were deparaffinized in xylene followed by rehydration. Antigen was retrieved in Target Retrieval Solution (Dako, Carpinteria, CA). 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, Grand Island, NY) 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, Madison, WI) 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, Sterling Heights, MI).

**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, Waterbury, CT). Data is represented as mean ± SD.

**Statistical Analysis.** Data from each assay were expressed as means ± SD. Statistical differences between 2 groups were determined by the Student’s 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 BrdU incorporation in cells, and induced S and G2/M arrest (30). Since 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 Figure 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 (Figure 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 (Figure 1B). The numbers of γH2AX foci were elevated by 2.4 and 4.5 fold in LNCaP cells and PC-3 cells (Figure 1B and 1C), respectively. The PCNA-I1-triggered expression of γH2AX was further elevated at 48 and 72 hours, revealed by immunoblotting (Figure 1D).

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

Given the DNA damaged inflicted by treatment with PCNA-I1, we analyzed the effects of PCNA-I1 on apoptosis by using Annexin V staining and flow cytometry (Figure 2A). Treatment with PCNA-I1 for 48 hours reduced the percentages of viable cells in both LNCaP (Figure 2A upper panel and B) and PC-3 (Figure 2A lower panel and C) cells (Annexin V-/PI-). PCNA-I1 treatment increased the percentages of dead cells (Annexin+/PI+) and apoptotic cells
(Annexin V+/PI-) (Figures 2A, B, and C). There was no significant increase in necrotic cells (Annexin V-/PI+) (Figure 2A, B and C). We next examined the effects of PCNA-I1 on expression of the anti-apoptotic 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 (Figure 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 γH2AX. Moreover, expression of cleaved apoptotic protein PARP was also significantly elevated (Figure 2E). Furthermore, the combination treatment increased the percentage of necrotic cells (Annexin V-/PI+) and dead cells (Annexin V+/PI+) compared to cisplatin treatment alone (Figure 2F), confirming the recent findings that inhibiting PCNA function sensitizes cells to DNA damage and cell death-induced by cisplatin (35, 36).

**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 (Figure 3A and B).
contrast, there was a significant increase in the number of LC3B puncta present in PC-3 cells treated with PCNA-I1 (Figure 3A and B). The differential expression of LC3B in LNCaP and PC-3 cells was further determined using immunoblotting. While an increase in LC3B-I was observed in LNCaP cells treated with PCNA-I1, there was no expression of LC3B-II (Figure 3C). In contrast, treatment with PCNA-I1 increased the expression of LC3B-II at all time points in PC-3 cells (Figure 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 (31). The untreated PC-3 cells formed 247 ± 28 colonies, which is approximately 2 times more than those formed by LNCaP cells (109 ± 25) (Figure 4A and B). The colonies formed by PC-3 cells were also significantly larger than those formed by LNCaP cells (Figure 4A). Despite differences in colony formation efficiencies between the 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 (Figure 4C). The treatment with PCNA-I1 also significantly reduced the sizes of the colonies (Figure 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 Figure 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) (Figure 5B). The body weights were not significantly different between vehicle- and PCNA-I1-treated mice (Figure 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 growth of LNCaP tumors and did not cause significant toxicity to the host.

Immunohistochemical analysis of tumor lesions showed that treatment with PCNA-I1 reduced expression of PCNA by approximately 26% (p<0.01, Figure 5D and E) and increased the number of apoptotic cells (TUNEL staining) by approximately 5 Fold (p<0.01, Figure 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 (30). In the present study, PCNA-I1, which is most potent among the nine PCNA-Is, was chosen for further investigation to determine the effects of 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 has been shown to increase susceptibility to DNA damage, resulting in the formation of double strand breaks, the activation of ATM (37) and potentially cell death. The inhibitory effects of PCNA-I1 on DNA replication and the observed S-G2/M phase arrest (32) 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 γH2AX 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. Analysis of programmed cell death demonstrated that the PCNA-I1-mediated inhibition of DNA replication (30) 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 anti-apoptotic 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 (38). This expression of Bcl-2 has been shown to confer resistance 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 (38, 39). Therefore, the observed decrease in Bcl-2 expression upon treatment with PCNA-I1 suggests 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 (40). However combination treatment of PCNA-I1 and cisplatin synergistically increased γH2AX, phospho-p53, and cleaved PARP expression and the percentage of apoptotic cells compared to 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 (35, 36). 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 (40-43). Tumor cells with p53 mutations are often resistant to cytotoxic drugs, such as cisplatin (44-46). 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 (47). While autophagy is traditionally thought of as a cell-survival pathway, it has been demonstrated that excessive or prolonged autophagy results in “autophagic death” that occurs either independent of or in conjunction with apoptosis (48-50). 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 γH2AX 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 xenograft 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 immunohistochemical 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 (30). 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 cells death in two lines of human prostate cancer. The potential pathways leading to cell death induced by PCNA-I1 are summarized in Figure 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, since PCNA is required and is overexpressed in almost all cancer cells. The therapeutic implications for PCNA-I1 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-I1 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 (30). 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.

References


Table 1. PCNA-I1 did not affect hematology profiles

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

Blood was collected from 4 mice per group by submandibular puncture following the described treatment. Data shown are mean ± SD from 4 mice.
Figure Legends

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 μM PCNA-I1 for 24, 48 and 72 hours was analyzed by western blot. β-Actin was used as a loading control. B, LNCaP and PC-3 cells were plated in chamber slides and treated with 1 μM PCNA-I1 for 24 hours. PCNA and γH2AX expression were visualized using fluorescently labeled secondary antibodies. Nuclei were stained with DAPI and visualized using 40x magnification. C. The number of γH2AX foci in LNCaP and PC-3 cells treated with 1 μM PCNA-I1 were counted using ImageJ. D. γH2AX expression was determined in LNCaP and PC-3 treated with 1 μM PCNA-I1 at 24, 48 and 72 hours by western blot analysis. β-Actin was used as a loading control. *, p < 0.05, **, p < 0.01, *** p < 0.001.

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 μM PCNA-I1 for 48 hours. The percentage of normal, necrotic, apoptotic, and dead cells were plotted for B, LNCap and C, PC-3 cells. D, the expression of Bcl-2 in LNCaP and PC-3 cells treated with 1 μM 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 μM PCNA-I1 and 5 μM 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 μM...
cisplatin alone or in combination with 1 μM PCNA-I1 for 48 hours. *, p < 0.05, **, p < 0.01, *** p < 0.001.

**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 M PCNA-I1 for 24 hours. LC3B puncta were visualized using a fluorescently labeled secondary antibody. Nuclei were stained with DAPI and visualized using 40x 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. β-Actin was used as a loading control. **, p < 0.01.

**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 μM 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) x 100. *, p < 0.05, **, p < 0.01.

**Figure 5. Administering PCNA-I1 intravenously inhibits prostate tumor growth in vivo.** 2 x 10⁶ 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 two 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 we then stained with H&E, PCNA, and TUNEL with DAPI counterstain and visualized at 40x 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.

**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 which 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.
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PCNA-I1
pChk2 (T68)
62 kDa
p-p53 (S15)
53 kDa
p53
53 kDa
β-Actin
45 kDa

B

LNCaP
Control
PCNA-I1
PC-3
Control
PCNA-I1

C

γH2AX foci/cell

D

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

γH2AX
15 kDa
β-Actin
45 kDa
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>LNCaP Control</th>
<th>PCNA-I1</th>
<th>PC-3 Control</th>
<th>PCNA-I1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC3B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

LC3B foci/ cell

Control | PCNA-I1

**

C

<table>
<thead>
<tr>
<th></th>
<th>LNCaP 24</th>
<th>48</th>
<th>72</th>
<th>PC-3 24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA-I1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LC3B-I</td>
<td>(16 kDa)</td>
<td></td>
<td></td>
<td>(16 kDa)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3B-II</td>
<td>(14 kDa)</td>
<td></td>
<td></td>
<td>(14 kDa)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4

A

Control  PCNA-I1

LNCaP

PC-3

B

Control  PCNA-I1

Colony Number

0  100  200  300  400

LNCaP  PC-3

C

Surviving Fraction (%)

0  50  100  150  200

LNCaP  PC-3
Figure 5

A. Tumor volume (mm$^3$)
- Vehicle
- PCNA-I1

B. Tumor Weight (g)
- Vehicle
- PCNA-I1

C. Body Weight (g)
- Vehicle
- PCNA-I1

D. H&E, PCNA, TUNEL, DAPI

E. PCNA positive cells
- Vehicle
- PCNA-I1

F. TUNEL cells/field
- Vehicle
- PCNA-I1
Molecular Cancer Therapeutics

Anti-tumor effects of a novel small molecule targeting PCNA chromatin association in prostate cancer

Kelsey L. Dillehay, Shan Lu and Zhongyun Dong

Mol Cancer Ther Published OnlineFirst September 24, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0522

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.