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Small molecule targeting of PCNA chromatin association inhibits tumor cell growth

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Running title: *Inhibition of cell growth by PCNA targeting compounds*

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Abbreviations:

Con A, concanavalin A; FBS, fatal bovine serum; HuBrEC, human mammary epithelial cells; HuMSC, human bone marrow mesenchymal stem cells; HuPrEC, human prostate epithelial cells; HUVEC, human umbilical endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, Proliferating cell nuclear antigen; PCNA-I, PCNA inhibitor; PI, propidium Iodide.

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Abstract

Proliferating cell nuclear antigen (PCNA), a potential anti-cancer target, forms a homotrimer and is required for DNA replication and numerous other cellular processes. The purpose of this study was to identify novel small molecules that modulate PCNA activity to impact on tumor cell proliferation. An *in silico* screen of a compound library against a crystal structure of PCNA and a subsequent structural similarity search of the ZINC chemical database were carried out to derive relevant docking partners. Nine compounds, termed PCNA inhibitors (PCNA-Is), were selected for further characterization. PCNA-I1 selectively bound to PCNA trimers with a dissociation constant (Kd) of ~0.2-0.4 μM. PCNA-Is promoted the formation of sodium dodecyl sulfate (SDS)-refractory PCNA trimers. PCNA-I1 dose- and time-dependently reduced the chromatin associated PCNA in cells. Consistent with its effects on PCNA trimer stabilization, PCNA-I1 inhibited the growth of tumor cell of various tissue types with an IC50 of ~0.2 μM, whereas it affected the growth of non-transformed cells at significantly higher concentrations (IC50 ~1.6 μM). Moreover, uptake of BrdU was dose-dependently reduced in cells treated with PCNA-I1. Mechanistically the PCNA-Is mimicked the effect of PCNA knockdown by siRNA, inducing cancer cell arrest at both the S and G2/M phases. Thus, we have identified a class of compounds that can directly bind to PCNA, stabilize PCNA trimers, reduce PCNA association with chromatin, and inhibit tumor cell growth by inducing a cell cycle arrest. They are valuable tools in studying PCNA function and may be useful for future PCNA-targeted cancer therapy.

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Introduction

PCNA is a ubiquitously expressed protein conserved throughout evolution. It plays crucial roles in many vital cellular processes (Moldovan et al., 2007; Naryzhny, 2008; Stoimenov and Helleday, 2009). The human PCNA monomer is a 30-36 kDa protein consisting of 261 amino acid residues (Almendral et al., 1987). Functional PCNA is a homotrimer forming a ring structure, in which three monomers are joined together in an anti-parallel head to tail interaction (Gulbis et al., 1996; Kelman and O'Donnell, 1995; Naryzhny, 2008). To execute it's function, PCNA needs to be loaded to DNA by replication factor C (RFC) (Waga and Stillman, 1998) and interacts with numerous protein partners, including DNA polymerase δ, and DNA polymerase ε for DNA replication, DNMT1, HDAC1, and p300 for chromatin assembly and gene regulation, DNA mismatch repair protein Msh3/Msh6 for DNA repair, $p21^{(CIP1/WAF1)}$ for cell cycle control, and ESCO1/2 for sister-chromatid cohesion (Maga and Hubscher, 2003; Stoimenov and Helleday, 2009).

PCNA is synthesized in all stages of the cell cycle with a half-life of about 20 hours (Bravo and Macdonald-Bravo, 1987) and is elevated in early S phase to support cell cycle progression (Bravo and Macdonald-Bravo, 1987; Naryzhny, 2008). PCNA gene deregulation and posttranslational modulation are hallmarks of malignant cells. Tumor cells, regardless of their origins, express higher levels of PCNA (Celis and Olsen, 1994; Eltz et al., 2008; Kallakury et al., 1999; Kimos et al., 2004; Malkas et al., 2006; Miyamoto et al., 2006; Naryzhny, 2008; Naryzhny and Lee, 2007; Stoimenov and Helleday, 2009; Stuart-Harris et al., 2008; Zhong et al., 2008). Expression levels of PCNA correlate positively with other pathological indices in prostate cancer (Mulligan et

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al., 1997) and can serve as an independent prognosis marker (Miyamoto et al., 2006). Overexpression of PCNA is also a reliable biomarker for other tumor types (Cappello et al., 2006; Kimos et al., 2004; Stuart-Harris et al., 2008). These findings suggest that PCNA could be a valuable target for cancer therapy. In the present study, we have identified a series of novel compounds that directly bind to PCNA trimers, promote formation of stable PCNA trimers, reduce PCNA association with chromatin, and inhibit the growth of tumor cells of a variety of tissue origins.

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Materials and Methods

Reagents Compounds were provided by the University of Cincinnati Drug Discovery Center (UC-DDC, Cincinnati, OH) or purchased from Chembridge Co (San Diego, CA), ChemDiv (San Diego, CA), and Sigma-Aldrich (St Louis, MO). The recombinant His-PCNA (>95% pure) and antibody against Hus1 were purchased from Abcam (Cambridge, MA). Antibody against α-tubulin, PCNA siRNA, and scrambled siRNA control were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 transfection reagent and Alamar Blue were purchased from Invitrogen (Carlsbad, CA). Antibody against β-actin, propidium Iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A) and protease inhibitor cocktail were purchased from Sigma-Aldrich. Antibodies against PCNA (PC10) and histone 1 and BrdU cell proliferation assay kit were purchased from Cell Signaling Technology (Danvers, MA). The ECL Western Blotting Detection System was purchased from Millipore (Billerica, MA).

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Cells and culture The following cell lines were used in the study: 1) human cancer cells: LNCaP, 22Rv1, DU-145, LAPC-4, and PC-3 prostate cancer cells; MCF-7 and T47D breast cancer cells; A375 and MDA-MB435 melanoma cells; 2) mouse cancer cells: TRAMP-C2RE3 prostate cancer cells, B16 and K1735 melanoma cells, UV2237 fibrosarcoma cells, and CT-26 colon cancer cells; 3) primary human umbilical endothelial cells (HUVEC, PromoCell, Heidelberg, Germany), primary human bone marrow mesenchymal stem cells (HuMSC, PromoCell), primary human mammary epithelial cells (HuBrEC, Lonza, Walkersville, MD), primary human prostate epithelial cells (HuPrEC, Lonza), and primary mouse spleen lymphocytes (isolated in this laboratory), and primary mouse bone marrow stromal cells (isolated in this laboratory). Cells in exponential growth phase were harvested by treatment with a 0.25% trypsin - 0.02% EDTA solution, detached into RPMI 1640-10% fatal bovine serum (FBS), and resuspended in medium specific for different cells. Only suspensions of single cells with viability exceeding 95% were used.

Virtual screening A 3D representation of the UC-DDC drug-like chemical library was screened/docked against a model of PCNA derived from an x-ray crystal structure of human PCNA (Kontopidis et al., 2005). The PCNA trimer structure (PDB, 1VYJ) was prepared by adding missing atoms and minimizing energy of the all-atom model in explicit solvent (0.9% NaCl, pH 7.4) to remove steric clashes (Yasara Biosciences, 1220 Vienna, Austria), and verified using MolProbity (Hasinoff and Patel, 2009). The first round of screening involved individually docking of each compound structure into a rigid representation of PCNA using FRED (Openeye Scientific Software, Santa Fe, NM) under default settings. Top hits from 300,000 compounds were re-docked allowing the ligand to rotate freely within the binding site with Glide SP (Schrödinger, Portland, OR). Finally, the top 2,000 molecules were further docked using Glide XP (Schrödinger) at

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high-resolution settings as well as performing flexible-ligand, flexible-site (side chains) docking with Molegro (Molegro Bioinformatics, Denmark). The top 200 hits were selected for further evaluation in bioassays.

PCNA binding assay The his-PCNA or rabbit IgG (control) was labeled with a reactive dye NT-647 using N-hydroxy succinimide (NHS)-ester chemistry using a kit (NanoTemper Technologies, Munich, Germany). PCNA-I1 at various concentrations was incubated with NT-647 labeled- PCNA or -IgG (100 nM) in a binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% NP-40 alternative). The binding of PCNA-I1 to the labeled proteins was measured by using the microscale thermophoresis technology (MST) in a Monolith NT.115 reader (NanoTemper) as detailed previously (Jerabek-Willemsen et al., 2011) using the Temperature Jump analysis. The dissociation constant (Kd) of the binding was calculated using nonlinear regression analysis (Prism 5, GraphPad Software Inc., La Jolla, CA).

Immunoblotting analysis Cells were scraped into a lysis buffer (1% Triton X-100, 20 mM Tris-HC1, pH 8.0, 137 mM NaC1, 10% glycerol, 2 mM EDTA, 1 mM PMSF, and a protease inhibitor cocktail) and analyzed by immunoblotting (Zhang et al., 2002). The immunoreactive signals were revealed using the ECL methods and visualized in an IS4000MM Digital Imaging System (Eastman Kodak, Rochester, NY).

PCNA trimer stability assay The assay was performed on both cell lysates (native PCNA source) and purified recombinant protein. Fifty μg PC-3 cell lysate or 0.1 μg His-PCNA was incubated with PCNA-Is or DMSO (0.1%, vehicle) in a reaction buffer

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(40 mM Tris-HCl, pH 7.5, 0.2 mg/ml BSA, 10 mM $MgCl_2$, and 10% glycerol). The reaction was stopped by the addition of the SDS-PAGE sample buffer without a reducing agent. The samples were resolved by SDS-PAGE without boiling and analyzed by immunoblotting.

Nuclear fractionation and PCNA chromatin association analysis Cells were lysed in buffer A (10 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, and protease inhibitors). Samples were pelleted by centrifugation (1500 x g, 2 min, 4^oC) and the resulting supernatant fraction collected designated as the NP-40-extractable (NP-E) fraction. The pellet was washed in buffer B (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, and protease inhibitors), resuspended and digested in buffer C (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.2 mM PMSF, and protease inhibitors) with 200 units/10^7 cells of DNase I for 30 min at 37^oC. After centrifugation at 13,000 x g for 5 min at 4^oC, the supernatant was collected as NP40 resistant (NP-R) fraction.

Cell growth assay Cell growth was assessed by MTT staining as described previously (Dong et al., 1991). Growth inhibition (%) by the compounds was calculated using the formula: (1 – A_{570} of treated/A_{570} of control) x 100 and IC50 (the concentration that inhibited cell growth by 50%) determined. For evaluation of lymphocyte growth, the alamar blue assay (Nakayama et al., 1997) was performed. Freshly prepared mouse spleen lymphocytes were stimulated for 72 hours with 2 μg/ml Con A. During the last 24 hours, 20 μl/well of alamar blue was added. The fluorescence intensity was measured at 530/580 (excitation/emission) in the FLUOstar Omega microplate reader (BMG LABTECH, Cary, NC).

Cell cycle analysis PC-3 cells were plated onto 60 mm plates at 2 x 10^5 cells/plate,

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treated, detached by trypsinization, and resuspended in 70% ethanol in PBS on ice. The cells were resuspended in PI staining solution (PBS containing 50 μg/ml PI, 100 μg/ml RNase A, and 0.05% Triton X-100) and incubated for 45 minutes at 37°C, followed by washing with PBS and flow cytometry analysis in an Epics-XL-MCL system (Beckman Coulter, Fullerton, CA). The 3 fractions (G0/G1, G2/M and S) were quantified by using the Synchronization Wizard of ModFit LT Flow Cytometry Cell-cycle Analysis software (Verity Software House, Topsham, ME).

BrdU cell proliferation assay PC-3 (2,500/well) and LNCaP (5,000/well) cells were seeded into 96-well-plates. After an overnight incubation, the cells were treated with increasing concentrations of PCNA-I1 for 48 hours. Eight hours prior to termination of the experiments, 10 μM BrdU was added to each well and the incorporation of BrdU into newly synthesized DNA was assessed by using an enzyme-linked Enzyme-linked immunosorbent assay (ELISA) kit following the manufacture's protocol. IC50 values were determined as described in "Cell growth inhibition assay" above.

Transfection of siRNA PC-3 cells were plated onto 60-mm plate at 2 x 10^5/plate in antibiotics-free medium and transfected with 200 pmol PCNA specific siRNA or control siRNA for 24 hours using Lipofectamine 2000. The cells were then starved in SFM for 24 hours, followed by stimulation with 5% FBS, and sampled at different times for analyses.

Statistical analysis Data shown are the mean ± SD. Differences between means were compared using the two-tailed Student's *t* test and were considered significantly different at the level of $p < 0.05$.

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Results

Identification of potential PCNA-Is The *in silico* docking computation using Glide and Molegro was performed to identify compounds that potentially bind to the interfaces between PCNA monomers (Fig. 1A). Two hundred compounds with the highest combined-docking scores were tested for inhibitory effects on cell growth. Two structurally similar compounds N'-[(1-hydroxy-2-naphthyl) methylene]-3-methyl-2-thiophenecarbo-hydrazide and N'-[(2-hydroxy-1-naphthyl) methylene]-1H-pyrazole-5-carbohydrazide), named as PCNA inhibitor 1 and 2 (PCNA-I1 and PCNA-I2), for which the IC50 was in the nM (PCNA-I1) or nM to low μM (PCNA-I2) range, were identified (detailed below in Table 1). The docking analysis suggested that PCNA-I1 binds to Arg146 through an O-N hydrogen bond (H-bond, 2.2 Å distance) of one PCNA monomer and to Asp86 through a N-O H-bond (2.0 Å distance) of the adjacent monomer. A strong nonpolar interaction is also predicted between the lipophylic aroyl hydrazone of a naphthol on PCNA-I1 and carbons dominated by Lys110 of the adjacent monomer (Fig. 1A). To further explore the structure-activity relationships (SAR) with the goal of improved intrinsic inhibitory activity and/or permeability, a structure similarity search was performed with PCNA-I1 as the template against the ZINC database (http://zinc.docking.org/). Eight additional compounds, PCNA-I3 through PCNA-I10 (Fig. 1B) were identified and selected for further characterization detailed below.

The binding of PCNA-Is to PCNA To establish that PCNA-Is bind directly to PCNA and determine the affinity of the binding, MST analysis was performed. PCNA-I1 bound to NT-647-labeled recombinant PCNA with a Kd of 0.41 ± 0.17 μM (Fig. 2A) but not rabbit IgG (Fig. 2B), indicating that PCNA-I1 selectively bound to PCNA. The native gel electrophoresis analysis, followed by immunoblotting, revealed that NT-647-labeld

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PCNA was in a trimer form (Fig. 2C), indicating that PCNA-I1 bound directly to PCNA trimers. The binding of PCNA-I1 to PCNA was validated by using Surface Plasmon Resonance (SPR) technology. The kinetic binding constants were analyzed using the BIAevaluation software in the iterative model of the best fit for the interaction parameters in a Langmuir 1:1 binding, which resulted in Kd values of 0.14 μM for PCNA-I1 (Supplementary data, Fig. 1A). Therefore, PCNA-I1 bound to PCNA with a Kd of 0.14 to 0.41 μM. In addition, the SPR analysis showed that PCNA-I3 bound to PCNA with a Kd of 0.17 μM (Supplementary data, Fig. 1B).

As an approach to help to define potential impacts of the binding of PCNA-Is on stability of PCNA trimer structure, we used SDS-PAGE separation analysis of PCNA pretreated with PCNA-Is and analyzed by immunoblotting. The his-tagged recombinant PCNA was incubated with DMSO (vehicle) or PCNA-Is (1 or 10 μM). PCNA-I5 has the structure almost identical to PCNA-I4 and, hence, was not included. As shown in Fig. 2D, approximately 2-5% of PCNA was present as the trimer form under the experimental conditions. After incubation with PCNA-Is, PCNA-I1 and PCNA-I2 in particular, the amount of PCNA in the trimer form was significantly elevated. Similarly, the treatment with PCNA-Is enhanced the amount of PCNA trimers by natural PCNA present in a lysate of PC-3 cells (Fig. 2E). To determine specificity of this effect of PCNA-Is, the trimer stability assay was performed to examine trimer formation by the 9-1-1 proteins as a control. The 9-1-1 protein complex (approximately 110 kD) is another member of the clamp family proteins and a heterotrimer formed by Rad9, Rad1, and Hus1. The 9-1-1 protein complex also encircles DNA and is involved in DNA repair (Park et al., 2009; Song et al., 2007; Song et al., 2009; Xu et al., 2009). PC-3 cell lysate was subjected to the treatment with 1 or 10 μM of PCNA-Is and analyzed by immunoblotting using an

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antibody against Hus1. As shown in Fig. 2F, a high level of Hus1 was detected in the PC-3 cell lysate. However, the treatment with PCNA-Is did not detectably induce 9-1-1 trimer formation. Taken together, these data indicated that the PCNA-Is bind directly and selectively to PCNA trimers, which will potentially stabilize the trimer structure of PCNA.

Effects of PCNA-I1 on the association of PCNA with chromatin Association with chromatin is a prerequisite for PCNA to execute its functions. We therefore determined whether treatment of cells with PCNA-I1 alters the association of PCNA with chromatin. PC-3 cells were treated for various times with 1 μM of PCNA-I1 and lysed in a buffer containing 0.5% NP-40 (Savio et al., 1996). PCNA in the NP-40 extractable (NP-E) and NP-40 extraction resistant (NP-R) fractions was analyzed by immunoblotting to identify the free and chromatin-associated PCNA with α-tubulin (free form) and histone 1 (chromatin protein) as loading controls, respectively. As shown in Fig. 3A, PCNA in NP-E fraction (the free-form PCNA), was not significantly altered in cells treated by PCNA-I1 for up to 8 hours but reduced in cells treated for 16 hours. In contrast, the level of PCNA in NP-R fraction (the chromatin-associated PCNA) was reduced in 1-2 hours in cells treated with PCNA-I1. A more significant reduction of the chromatin-associated PCNA was observed in cells treated with PCNA-I1 for 8 hours. Therefore, the reduction of the chromatin-associated PCNA occurred much earlier than did the free form PCNA. We next analyzed this effect of PCNA-I1 in cells treated for 8 hours with increasing concentrations of PCNA-I1. Data in Fig. 3B show that the effects of PCNA-I1 on reduction of chromatin-associated PCNA were dose-dependent and could be observed in cells treated with the compounds at concentrations of 0.5 μM or higher. The same treatment did not significantly alter the level of free form PCNA. The similar reduction of

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chromatin-associated PCNA by PCNA-I1 was also observed in other cell lines examined, including LNCaP, HeLa, and A375 cells (Fig. 3C).

Inhibitory effects of PCNA-Is on cell growth The growth-inhibitory effects of PCNA-I1 and PCNA-I2 were tested on a panel of human and mouse tumor cell lines, as well as primary cells and immortalized cells, of various tissue origins. As summarized in Table 1, PCNA-I1 inhibited growth of all tumor cells, regardless of tissue origins, with IC50 values at nM levels. CT26-R100 and CT26-R500 cells (Killion et al., 1993), which overexpress P-glycoprotein and exhibit the multidrug resistance (MDR) phenotype, were more sensitive than their parental CT26 cells to PCNA-I1 (Table 1). PCNA-I2 was 3-5 times less potent than PCNA-I1 in suppressing growth of most cell lines examined (Table 1). PCNA-I1 also inhibited growth of primary cultures of bone marrow mesenchymal stem cells, endothelial cells, lymphocytes, mammary epithelial cells, and prostate epithelial cells (Table 1). However, the potency of PCNA-I1 on growth of all normal cells was significantly lower than that on tumor cells. As revealed in Table 1, the IC50 of PCNA-I1 on normal cells were approximately 9 times higher than that for tumor cells ($p < 0.001$). Next, we further determined effects of other PCNA-Is (PCNA-I3 to PCNA-I10, except for PCNA-I5) on growth of PC-3 and LNCaP cells (Table 2). It was noteworthy that, with the exception of PCNA-I3, all compounds identified through the structural similarity search were less potent than PCNA-I1 and PCNA-I2 in suppressing tumor cell growth (Table 2). Importantly, the potencies of growth inhibition by the compounds correlated closely with those of their effects on PCNA trimer stability (Fig. 2D and Fig. 2E).

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Effects of PCNA-Is on cell cycle distribution Given the pivotal roles of PCNA in cell cycle regulation, the effects of the compounds, PCNA-I1 in particular, on cell cycle progression were determined. PC-3 cells were starved in SFM for 24 hours, which partially synchronized the cells in G1 phase, then stimulated with 5% FBS in the absence or presence of 1 μM PCNA-I1. As expected, the serum starvation led to accumulation of cells in G1 phase at all times examined. The G1 arrest was rescued upon serum stimulation, leading to a significant reduction of cells in G1 phase and an increase of cells in S and G2/M phases in the first 24 hours (Fig. 4A and 4B). The cell cycle progressed smoothly over the next 48 hours. By 72 hours, the distribution of cells treated with serum in all phases of the cell cycle was similar to the culture without serum stimulation (Fig. 4A and 4B). A significantly different cell distribution profile was observed in cultures treated with PCNA-I1. PCNA-I1 partially attenuated the serum-stimulated G1 decrease at 24 hours but led to significant G1 reductions at 48 and 72 hours. On the other hand, the number of cells in S and G2/M phases gradually increased over the 72 hours, resulting in an accumulation of cells in S and G2/M phase of the cell cycle (Fig. 4A and 4B). A similar S and G2/M arrest was also observed in cells treated with other PCNA-Is (Supplementary data, Fig. 2). Therefore, the cell cycle distribution analysis indicated that treatment of PC-3 cells with PCNA-Is led to G1 phase accumulation during the first 24 hours and an S and G2/M phase arrest by 72 hours.

Effects of knocking down endogenous PCNA protein on cell cycle distribution were determined to validate S and G2/M arrest induced by PCNA-Is. As shown in Fig. 4C and 4D, the transfection of PC-3 cells with PCNA-specific siRNA, but not the control scrambled siRNA, led to an accumulation of cells in the S and G2/M phases of the cell cycle over the 72 hour incubation, mimicking the effects of the treatment with PCNA-Is.

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However, the partial G1 phase accumulation induced by PCNA-Is was not observed, possibly due to the timing of the sampling (72 hours after the transfection). The knocking down of PCNA protein by the siRNA was confirmed in a parallel set of samples and shown in Fig. 4E. The PCNA protein level in PCNA-siRNA transfected cells was reduced by 50% at 0 hour, 50% at 24 hours (the first time point of sampling for flow cytometry analysis and 72 hours after the transfection), 60% at 48 h, and 80% at 72 hours, respectively (Fig. 4E). These data strongly suggest that the S and G2/M arrest induced by the PCNA-Is could be caused by their interference with PCNA function.

To further validate effects of PCNA-I1 on DNA replication, PC-3 and LNCP cells were treated with increasing concentrations of PCNA-I1 for 48 hours and uptake of BrdU into cellular DNA was measured. Data in Fig. 5 show that incorporation of BrdU in both cell lines was dose-dependently inhibited by the treatment of PCNA-I1 with IC50 values of 0.51 and 0.45 μM in PC-3 and LNCaP cells, respectively, which is consistent with their IC50 values in the growth inhibition assay (Table 1).

Discussion

PCNA is required for DNA synthesis and repair as well as numerous other pivotal cellular activities. It is one of very few proteins universally overexpressed in all types of tumors and, hence, a potentially valuable target for cancer therapy. The present study, through a combination of the computational virtual docking screen, ligand-binding assays, biochemical assays, and bioassays on cell growth, has identified a series of small molecule compounds that directly bind PCNA trimers, reduce chromatin-

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associated PCNA in cells, inhibit cell growth through induction of S and G2/M arrest, and attenuate DNA replication in cells.

Structural analysis reveals that PCNA-I3 is most similar to PCNA-I1 since thiophenes are generally phenyl-like in character, differing primarily in the shift of the hydroxyl functions from the 1 to 3 position. Indeed, SPR analysis confirmed that the two compounds bound to PCNA with very similar Kd values (0.14 μM and 0.17 μM, respectively). PCNA-I2 reverses the acylhydrazone and hydroxyl positions from PCNA-I3 leaving the naphthyl ring more perpendicular to the acylhydrazone chain, resulting in an approximate 3-5 fold loss in activity. PCNA-I2 also differs in that it has a pyrazole ring, which although relatively non-basic, is smaller and capable of being an H-bond donor relative to the aryl groups of PCNA-I1 and 3. The weaker cell growth-inhibitory activity of PCNA-I2 is possibly due to its lower cell permeability, which is predicted in a computer simulation analysis using QikProp software (Schrödinger, LLC, New York, NY). PCNA-I8 is nearly identical to PCNA-I3 except for the methyl group at the imine carbon of the hydrazone and the halogen substitution. This methyl group may induce greater deplanarization of the overall structure and a consequent twofold loss of activity. Comparison of PCNA-I9 to PCNA-I8 suggests a loss of activity by addition of H-bonding functions in the aroyl moiety, a trend consistent with the comparison of PCNA-I7 to PCNA-I1, with the latter pair suggesting some size restrictions in this moiety also. The leap from the naphthol scaffolds in PCNA-I1, 2, 3, 7, 8, and 9 to the 4-hydroxy-2-quinolone scaffolds in PCNA-I4, 6, and 10 is suggested by the good potency observed with hydroxyl positions in PCNA-I1 and PCNA-I3 on either side of the hydrazone chain. Overall the dominant pharmacophore is a lipophylic aroyl hydrazone of a naphthol (Fig.

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1C). This template will serve as the basis for further analog selection, wherein substitutions of the naphthyl and aroyl moieties will be further explored.

The majority of PCNA is present as the trimer form in the nucleoplasm, but only the chromatin-associated PCNA trimers are functional. Loading of PCNA trimers to DNA requires the RFC complex (Waga and Stillman, 1998). Extensive interactions with RFC open the PCNA ring and the engagement of the RFC:PCNA with the primer-template junctions of DNA results in ATP hydrolysis, closing of the ring, and release of the sliding clamp on DNA (Bowman et al., 2004). Since the PCNA-Is directly bind to PCNA trimers at the junction of the interfaces (by the docking analysis), which may stabilize the trimer structure of PCNA (implicated by the SDS-PAGE analysis data), it is possible that the PCNA-I-complexed PCNA becomes relatively insensitive to the RFC loading, resulting in the reduction of chromatin-associated PCNA. On the other hand, it has been shown that Lys110 is required for chromatin association and formation of double homotrimer of PCNA through its interaction with Arg5 on the other homotrimer (Kim and Lee, 2008; Naryzhny et al., 2005). The docking analysis suggested that PCNA-Is interact with Lys110, which would potentially interfere with the formation of the double homotrimers or recognition of DNA replication and repair foci. It should be mentioned that although the effects of PCNA-Is on PCNA trimer stability correlate with their inhibitory potencies on cell growth, they might not directly correlate with the Kd values of the compound to PCNA.

In addition to the reduction of the chromatin-associated PCNA, treatment with PCNA-I1, particularly after a longer duration, also leads to a significant downregulation

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of total PCNA. Since that treatment with PCNA-I1 does not lead to acceleration of PCNA degradation (data not shown) and that PCNA is synthesized mainly in early S phase to support cell cycle progression (Bravo and Celis, 1980; Bravo and Macdonald-Bravo, 1987; Naryzhny, 2008), it is very possible that this downregulation of total PCNA by the compounds is due indirectly to the blockade of cell cycle progression.

PCNA, through its interaction with many cell cycle regulatory proteins, such as CDKs, cyclins, p21, Cdc25 (Helt et al., 2004; Kawabe et al., 2002; Luo et al., 1995), plays a crucial role in cell cycle regulation. Therefore, it was not surprising that treatment with the PCNA-Is or PCNA knock-down led to cell cycle arrest. A minor differential effect on cell cycle distribution was observed in the first 24 hours in cells treated with PCNA-Is and knocking down PCNA expression. Whereas the PCNA-Is induced a partial G1 arrest during the first 24 hours, this effect was not observed in experiments knocking down PCNA expression. Given that flow cytometric analysis on the siRNA-transfected cells was started at 48 hours after the transfection when PCNA in the cells was already significantly reduced, it is very likely that a transient G1 arrest occurred in the first 48 hours. Indeed, it has been reported by others that transfection of antisense oligodeoxynucleotide of PCNA inhibited the progression of cells through G1 to S phase (Yang et al., 2002).

One of the major dose-limiting factors for successful chemotherapy of cancer is toxicity of the therapeutic drugs. In contrast to many chemotherapeutic drugs used in clinics, the PCNA-Is show more profound inhibitory effects on tumor cells versus normal cells, which would provide a significant therapeutic window. This differential sensitivity was reported previously in studies knocking down PCNA expression by antisense

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oligonucleotides (Sakakura et al., 1994). The differential sensitivity is likely due to higher demands of PCNA in tumor cells. In addition to their constantly growing and requiring more PCNA, tumor cells are genetically more instable, because of accumulation of gene mutations, and require more functional PCNA for DNA repair to survive, and, therefore, are more vulnerable to a PCNA targeting agent.

The PCNA-Is identified in our studies (not in the pharmacophore) show a structural similarity of iron-binding site with some iron chelators, suggesting that they might be able to bind to iron (Merlot et al., 2010; Richardson and Milnes, 1997). Iron is required for many cellular processes and iron chelators have been shown to inhibit tumor cell replication and to induce apoptosis (Merlot et al., 2010; Richardson and Milnes, 1997). However, the findings that the inhibitory effects of PCNA-Is on cell growth and cell cycle distribution are recapitulated in cells by knocking down PCNA using siRNA and that the Kd of PCNA-I1 to PCNA correlates its IC50 in tumor cell growth inhibition strongly suggest these PCNA-binding compounds inhibit cell growth through an attenuation of PCNA function. Importantly, many chemotherapeutic compounds, such as doxorubicin (Hasinoff and Patel, 2009), cisplatin (Baliga et al., 1998), curcumin (Jiao et al., 2009), as well as some typical iron chelators (Rao et al., 2009), are all capable of iron-binding, but were shown to inhibit cell growth and induce apoptosis through mechanisms independent of iron-chelating.

The binding and stabilization of protein structures by small molecule ligands are unique but not uncommon phenomenon. Recently, a group of compounds were shown to bind to and stabilize transthyretin tetramers at Kd of nM to μM (Alhamadsheh et al.,

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2011). The present studies have identified a series of novel compounds that bind to PCNA, promote stable PCNA trimer formation, reduce chromatin-associated PCNA, and inhibit tumor cell proliferation through induction of S and G2/M cell cycle arrest. These unique compounds are the first in class and provide a novel pharmacological tool for studying PCNA functions and show promise as lead compounds in the development a novel PCNA-targeting cancer therapy.

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Conducted experiments: Zongqing Tan, Matthew Wortman, Kelsey L. Dillehay, Chris R. Evelyn, Shanna L. Smith

Contributed new reagents or analytic tools: Matthew Wortman, William L. Seibel, and Zhongyun Dong

Performed data analysis: Matthew Wortman, William L. Seibel, Chris R. Evelyn, Shanna L. Smith, Linda H. Malkas, Yi Zheng, and Zhongyun Dong

Wrote or contributed to the writing of the manuscript: Matthew Wortman, William L. Seibel, Linda H. Malkas, Yi Zheng, Shan Lu, and Zhongyun Dong

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Footnote

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Legend to the Figures

Fig. 1. Chemical structures of PCNA-Is

A. An *in silico* docking image of the binding of PCNA-I1 to PCNA at the interface of two monomers. B. Structures of PCNA-I1 through PCNA-I10. C. The predicted pharmacophore of PCNA-Is.

Fig. 2. Biochemical analyses of PCNA-I binding to PCNA

A. PCNA-I1 binds to purified human PCNA protein with a Kd of ~ 407 +/- 168 nM (-6.390 +/- 0.174 Log M). B. PCNA-I1 does not bind to the negative control purified rabbit IgG protein. Data in both panel A and B were baseline-subtracted and analyzed as described in the Materials and Methods. Closed circles in panel A and open circles in panel *B* represent the mean +/- SEM measurements from three separate single point experiments. C. NT-647-PCNA was resolved in a native gel electrophoresis and analyzed by immunoblotting. The panels D and E showed elevation of PCNA trimer in recombinant PCNA (D) and cell lysate (E) treated with PCNA-Is. The recombinant His-PCNA (0.1 ug/reaction) or PC-3 cell lysates (50 ug/reaction) was incubated at room temperature for 3 hours with DMSO (0.1%, a vehicle control) or PCNA-Is (1 or 10 μM). The reactions were stopped by addition of SDS-PAGE sample buffer, resolved by SDS-PAGE without the sample boiling pre-treatment, and analyzed by immunoblotting using the PC10 PCNA antibody. F. A duplicate set of samples of PCNA-Is-treated PC-3 cell lysate was analyzed by immunoblotting using an antibody to Hus1.

Fig. 3. Effects of PCNA-I on PCNA association with chromatin.

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PC-3 cells were treated with 1 μM of PCNA-I1 for various times (A) or for 8 hours with various concentrations of PCNA-I1 (B). The NP-40 extractable and NP-40 resistant fractions of protein were analyzed by immunoblotting using PC10 PCNA antibody or using an antibody to histone 1 (chromatin-associated protein control) or α-tubulin (NP-40 extractable free form PCNA control). C. MCF-7, HeLa, or LNCaP cells were treated for 8 hours with 1 μM of PCNA-I1. The NP-40 extractable (NP-E) and NP-40 resistant (NP-R) fractions were analyzed as described for PC-3 cells in A and B. Data shown were from one representative experiment of 3.

Fig. 4. Effects of PCNA-Is on cell cycle progression

A and B. PC-3 cells were plated onto 60-mm plates at 2 x 10^5/plate. After an overnight incubation, the cells were starved for 24 hours in serum-free medium (SFM). The starved cells were then cultured in fresh SFM or stimulated in the medium supplemented with 5% FBS and sampled 24, 48, or 72 hours later for flow cytometry analysis. C and D. PC-3 cells were plated onto 60-mm plates at 2 x 10^5/plate in antibiotics-free medium. After an overnight incubation, the cells were transfected for 24 hours with PCNA specific siRNA or a scrambled siRNA control using Lipofectamine 2000. The cells were then starved for 24 hours in SFM, followed by stimulation with fresh medium supplemented with 5% FBS. Cells were sampled 24, 48, or 72 hours later for flow cytometry analysis. The profile files (A and C) were from one representative experiment of 5. Data in B and D were mean ± SD of 5 experiments. E. Cells were transfected as described in C and D and sampled for western at immediately after the 24-hour starvation (0 hour) or 24-72 hours after FBS stimulation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Fig. 5. Effects of PCNA-I1 on DNA replication in cells

PC-3 and LNCaP cells were plated into 96-well plate. After an overnight incubation, the cells were treated with PCNA-I1 for 48 hours with10 μM BrdU added to each well during the last 8 hours. The BrdU incorporated into DNA was detected by using an ELISA kit. The data shown were mean ± SD of three independent experiments.

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Table 1. IC50 values of PCNA-I1 and PCNA-I2 (μM)

Species	Tissue origin	Cell line	PCNA-I1	PCNA-I2
		Tumor cell lines		
Human	Breast	MCF-7	0.15	1.01
		T47D	0.15	N.D.
	Prostate	PC-3	0.24	0.97
		DU145	0.16	1.19
		22Rv1	0.18	N.D.
		LAPC-4	0.30	1.79
		LNCaP	0.14	0.56
	Melanoma	A375	0.16	3.75
		MDA-MB435	0.29	N.D.
Mouse	Prostate	TRAMP-C2RE3	0.20	N.D.
	Melanoma	B16	0.14	
		N.D.		
		K1735	0.05	N.D.
	Fibrosarcoma	UV2237	0.25	N.D.
	Colon cancer	CT26-P	0.13	N.D.
		CT26-R100	0.08	N.D.
		CT26-R500	0.07	N.D.
	Mean ± SD		0.17 ± 0.07	N.D.
		Normal cells		
Human	Blood vessel	HUVEC	1.54	N.D.
	Bone marrow	Mesenchymal Stem cells	0.99	N.D.
	Breast	Epithelial cells	1.67	N.D.
	Prostate	Epithelial cells	2.00	N.D.
Mouse	Bone marrow	stroma cells	1.90	N.D.
	Spleen	Lymphocytes	1.50	N.D.
	Mean ± SD		1.60 ± 0.36	N.D.

Cells were plated into 96-well plates at 1000 to 5000 cells/well. After an overnight incubation, the cells were treated for 4 days with various concentrations (up to 10 μM) of PCNA-I1 or PCNA-I1. The live cells were stained with MTT and counted as described in Materials and Methods. IC50 values were derived from growth inhibition curves. Data shown for tumor cells are mean of 5 to 7 experiments and for primary cells are mean of 3 experiments. The means of IC50 for tumor cells and normal cells were 0.17 ± 0.07 and 1.60 ± 0.36, respectively ($p < 0.0001$).

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Table 2. IC50 values of PCNA-Is (μM)

PCNA-Is	PC-3	LNCaP
PCNA-I3	0.44	0.26
PCNA-I4	3.00	0.68
PCNA-I6	>10.0	>10.0
PCNA-I7	3.60	2.20
PCNA-I8	1.00	0.68
PCNA-I9	2.20	0.71
PCNA-I10	>10.0	2.20

The growth inhibitory effects of the compounds were determined by the MTT assay and IC50 values were derived from growth-inhibitory curves as detailed in the legend to Table 1. Data shown are mean of 2 independent experiments.

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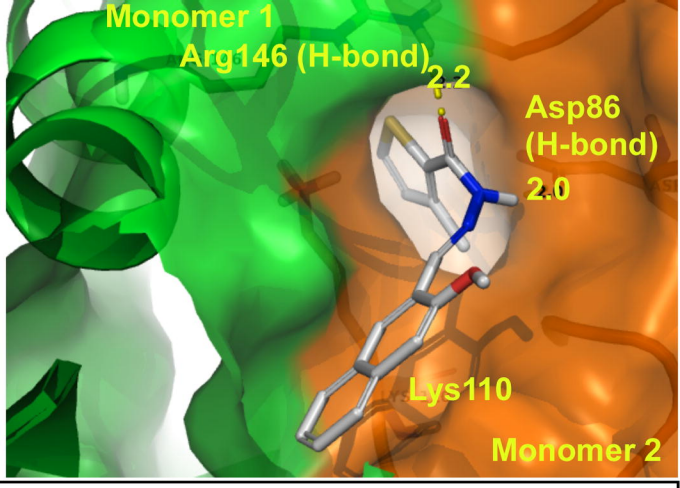
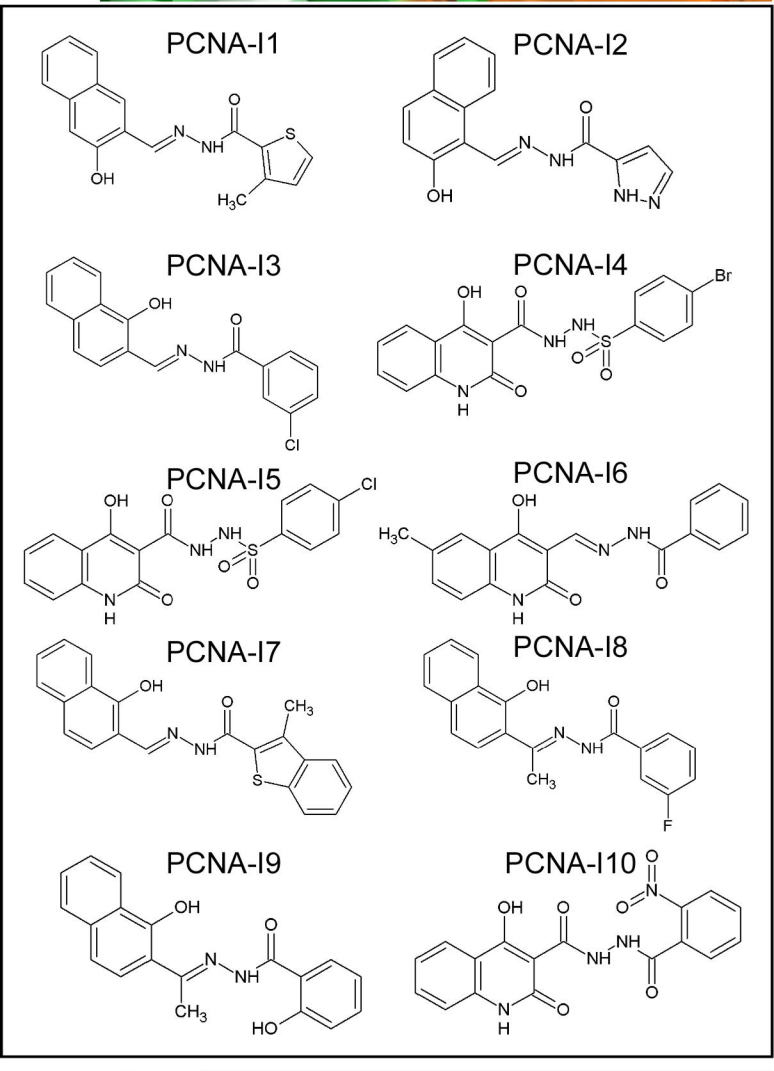
A


Monomer 1
Arg146 (H-bond)
2.2
Asp86
(H-bond)
2.0
Lys110
Monomer 2

B


PCNA-I1
PCNA-I2
PCNA-I3
PCNA-I4
PCNA-I5
PCNA-I6
PCNA-I7
PCNA-I8
PCNA-I9
PCNA-I10

C

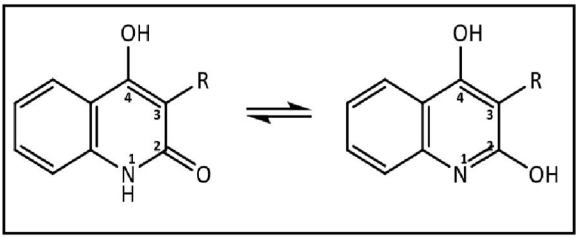


Figure 1

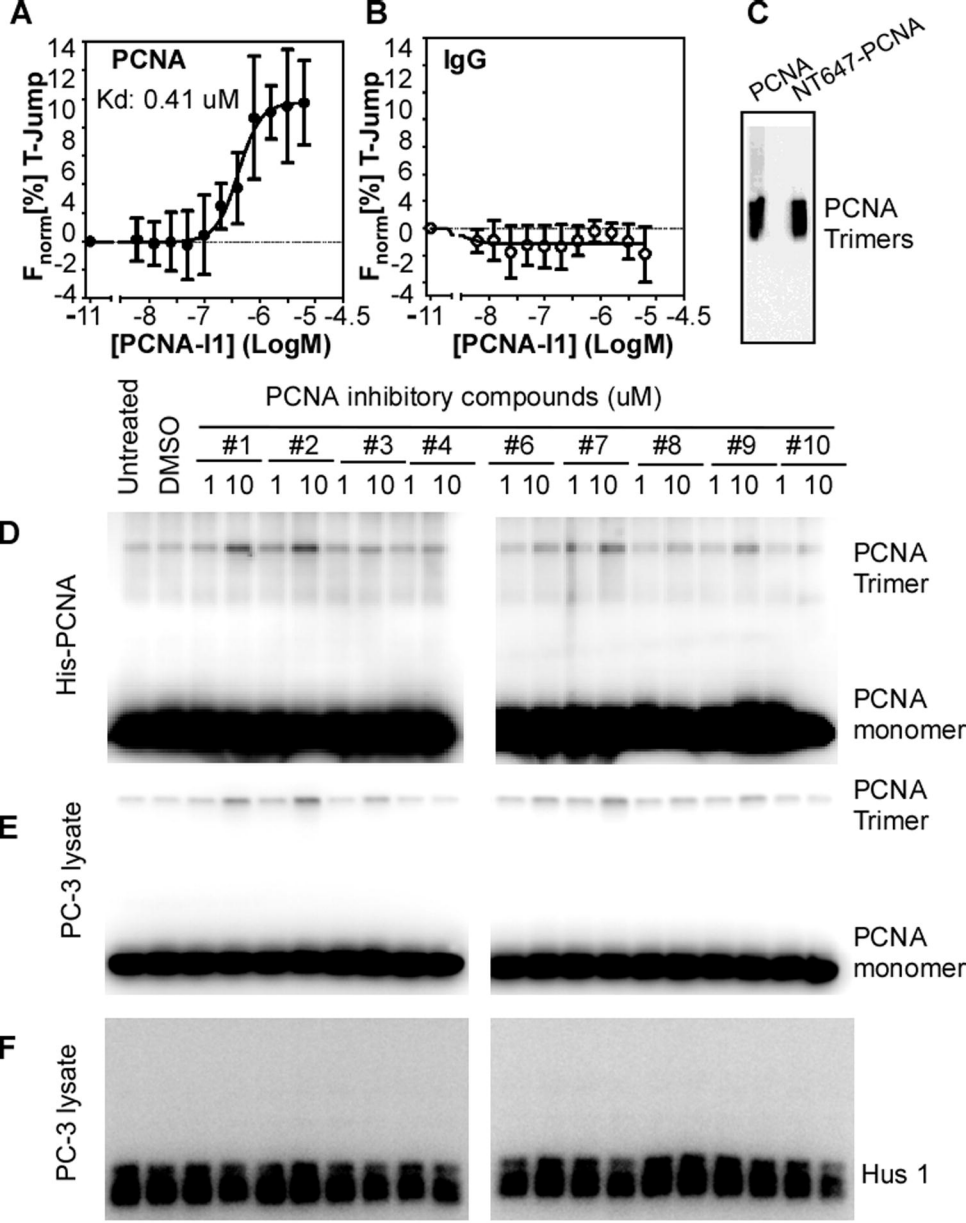

A
PCNA
Kd: 0.41 uM
F_{norm}[%] T-Jump
[PCNA-I1] (LogM)
B
IgG
F_{norm}[%] T-Jump
[PCNA-I1] (LogM)
C
PCNA
NT647-PCNA
PCNA Trimers
PCNA inhibitory compounds (uM)
Untreated
DMSO
#1 #2 #3 #4 #6 #7 #8 #9 #10
1 10 1 10 1 10 1 10 1 10 1 10 1 10 1 10 1 10
D
His-PCNA
PCNA Trimer
PCNA monomer
E
PC-3 lysate
PCNA Trimer
PCNA monomer
F
PC-3 lysate
Hus 1

Figure 2

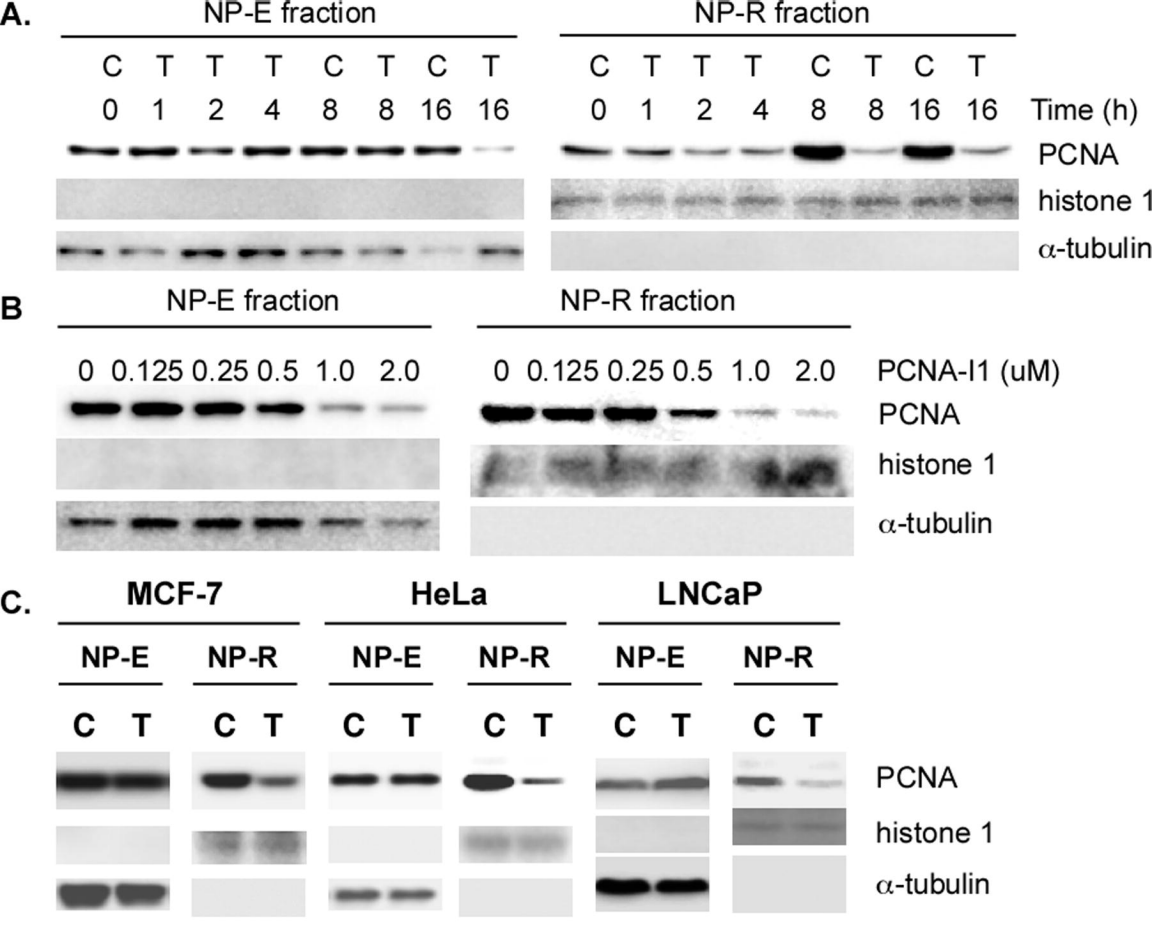
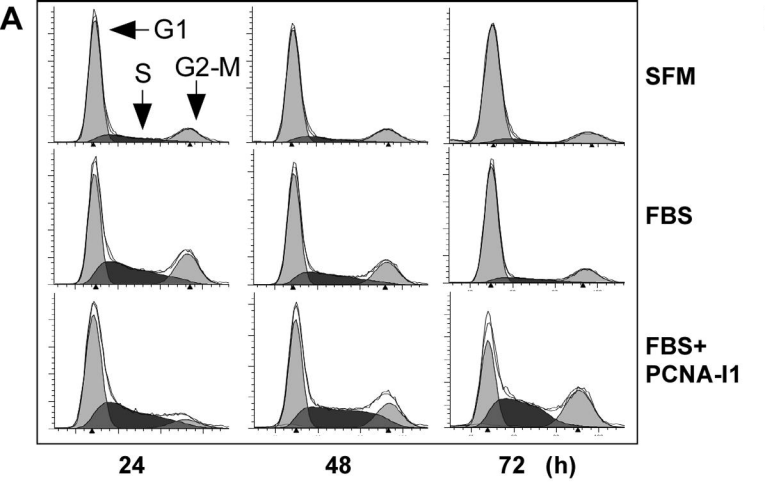
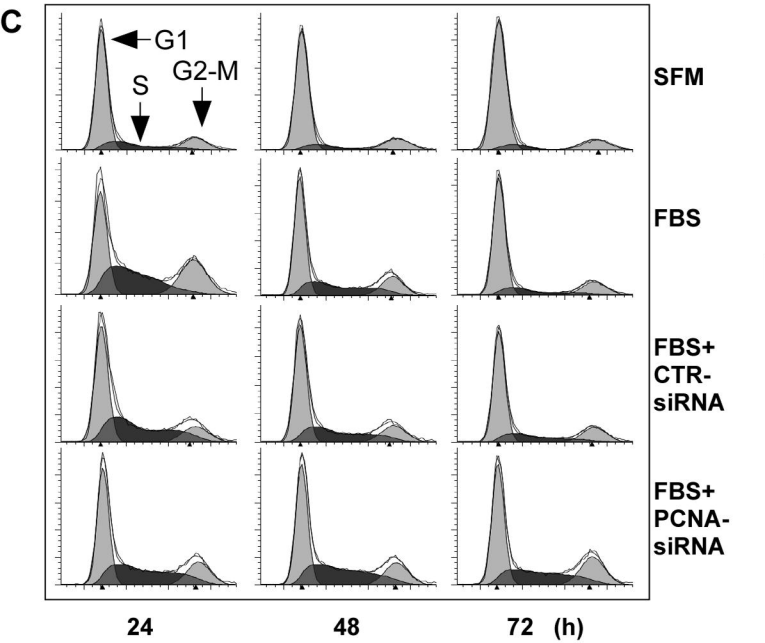
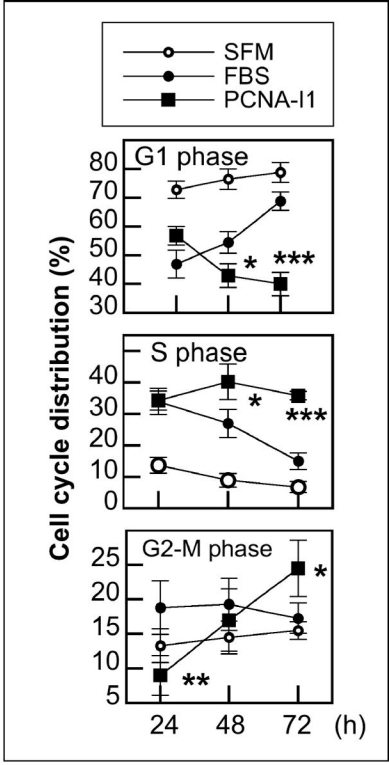
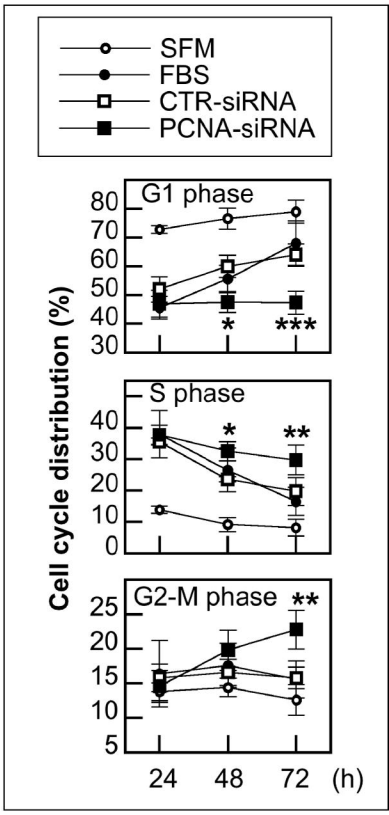

A.
NP-E fraction
NP-R fraction
C T T T C T C T
0 1 2 4 8 8 16 16
C T T T C T C T
0 1 2 4 8 8 16 16
Time (h)
PCNA
histone 1
α-tubulin
B
NP-E fraction
NP-R fraction
0 0.125 0.25 0.5 1.0 2.0
0 0.125 0.25 0.5 1.0 2.0
PCNA-I1 (uM)
PCNA
histone 1
α-tubulin
C.
MCF-7
HeLa
LNCaP
NP-E
NP-R
NP-E
NP-R
NP-E
NP-R
C T C T C T C T C T C T
PCNA
histone 1
α-tubulin

Figure 3


A
G1
S
G2-M
SFM
FBS
FBS+
PCNA-I1
24
48
72 (h)


C
G1
S
G2-M
SFM
FBS
FBS+
CTR-
siRNA
FBS+
PCNA-
siRNA
24
48
72 (h)


B
SFM
FBS
PCNA-I1
G1 phase
S phase
G2-M phase
Cell cycle distribution (%)
24
48
72
(h)


D
SFM
FBS
CTR-siRNA
PCNA-siRNA
G1 phase
S phase
G2-M phase
Cell cycle distribution (%)
24
48
72
(h)

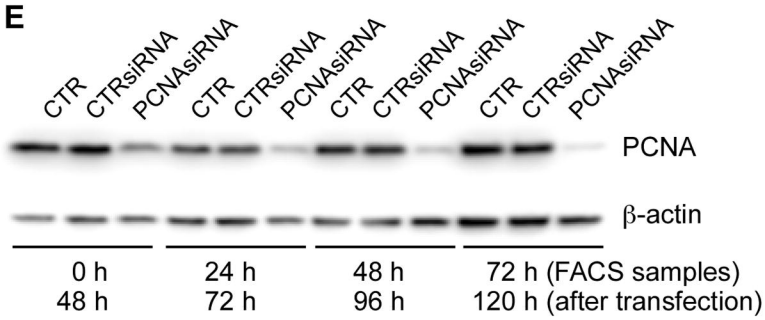

E
CTR
CTRsiRNA
PCNAsiRNA
PCNA
β-actin
0 h
48 h
24 h
72 h
48 h
96 h
72 h (FACS samples)
120 h (after transfection)

Figure 4

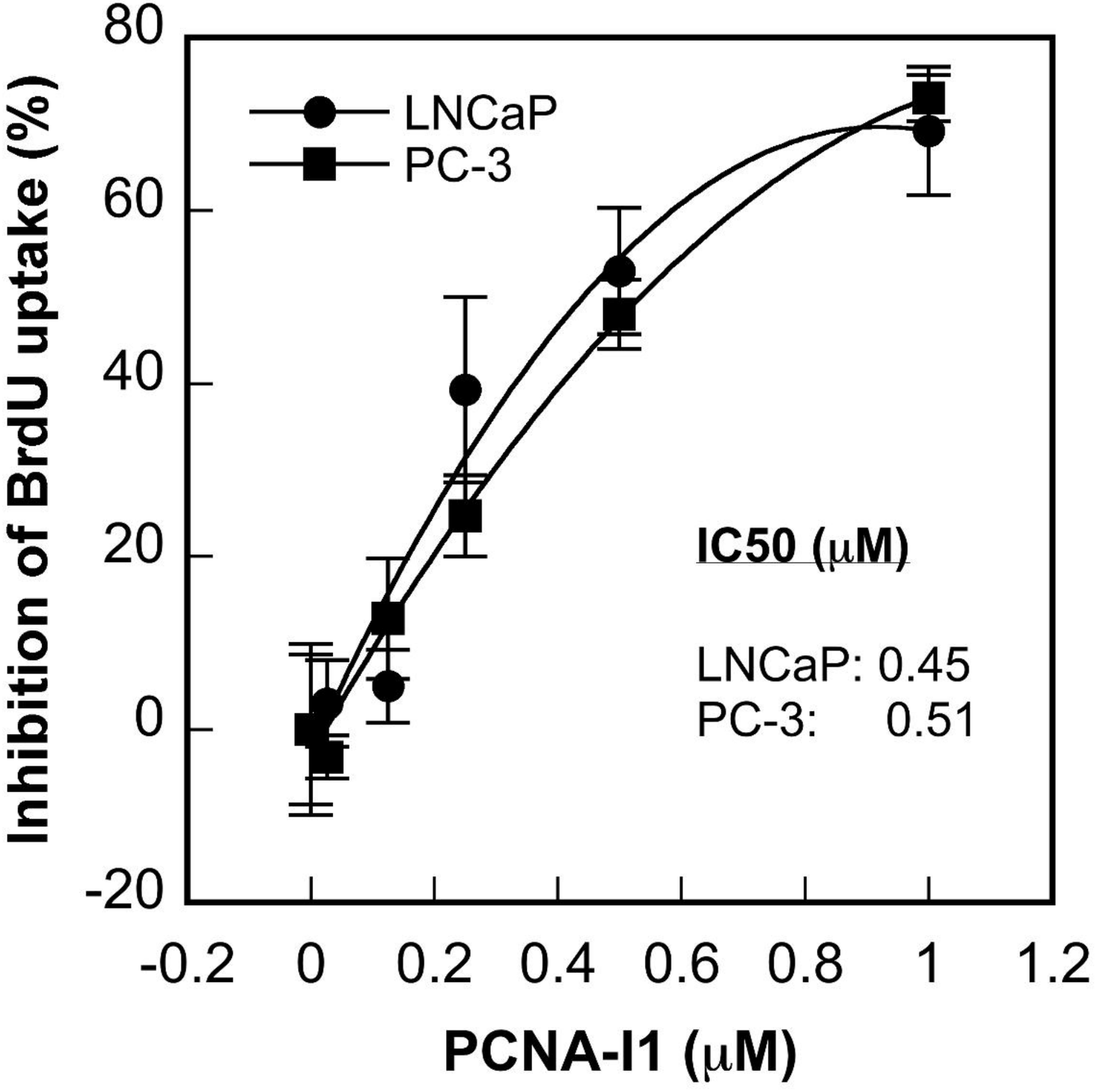

80
60
40
20
0
-20
Inhibition of BrdU uptake (%)
LNCaP
PC-3
IC50 (μM)
LNCaP: 0.45
PC-3: 0.51
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
PCNA-I1 (μM)

Figure 5