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The anti-cancer activity of a first-in-class small molecule targeting PCNA

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Abstract

Purpose—Proliferating cell nuclear antigen (PCNA) plays an essential role in regulating DNA synthesis and repair and is indispensable to cancer cell growth and survival. We previously reported a novel cancer associated PCNA isoform (dubbed caPCNA), which was ubiquitously expressed in a broad range of cancer cells and tumor tissues, but not significantly in non-malignant cells. We found the L126-Y133 region of caPCNA is structurally altered and more accessible to

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protein-protein interaction. A cell permeable peptide harboring the L126-Y133 sequence blocked PCNA interaction in cancer cells and selectively kills cancer cells and xenograft tumors. Based on these findings, we sought small molecules targeting this peptide region as potential broad-spectrum anti-cancer agents.

Experimental Design—By computer modeling and medicinal chemistry targeting a surface pocket partly delineated by the L126-Y133 region of PCNA, we identified a potent PCNA inhibitor (AOH1160) and characterized its therapeutic properties and potential toxicity.

Results—AOH1160 selectively kills many types of cancer cells at below micromolar concentrations without causing significant toxicity to a broad range of non-malignant cells. Mechanistically, AOH1160 interferes with DNA replication, blocks homologous recombination-mediated DNA repair, and causes cell cycle arrest. It induces apoptosis in cancer cells and sensitizes them to cisplatin treatment. AOH1160 is orally available to animals and suppresses tumor growth in a dosage form compatible to clinical applications. Importantly, it doesn't cause significant toxicity at 2.5 times of an effective dose.

Conclusion—These results demonstrated the favorable therapeutic properties and the potential of AOH1160 as a broad-spectrum therapeutic agent for cancer treatment.

Keywords

PCNA; DNA repair; DNA replication; Novel antitumor agents

Introduction

Found in all eukaryotic cells as an evolutionarily conserved protein and widely used as tumor progression marker (1–3), proliferating cell nuclear antigen (PCNA) plays an essential role in regulating DNA synthesis and repair and is indispensable to cancer cell growth and survival (4). Therefore, it represents an attractive molecular target to develop broad-spectrum anti-cancer agents (5). A major interaction site in PCNA is the interdomain connecting loop that spans from amino acid M121 to Y133 (6). This loop is recognized by many PIP-box proteins including p21 (CDKN1A) (7), DNA polymerase δ (Pol δ) (8), and flap endonuclease 1 (FEN1) (9). Using 2D-PAGE, we previously reported that normal cells and tissues express an isoform of PCNA with a basic isoelectric point (referred to as nmPCNA) (10). In contrast, cancer cells express both the basic and, to a much higher level, a unique acidic isoform of PCNA (caPCNA) that is not significantly expressed in non-malignant cells (10–12). The isoelectric point differences between the two isoforms results from changes in the malignant cells' ability to post-translationally modify the PCNA polypeptide (13), and is not due to an mRNA splice variant or mutation within the PCNA gene. We mapped the caPCNA-specific antigenic site to a small eight amino acid peptide region (L126-Y133) within the interconnector domain of PCNA (10). Interestingly, the L126-Y133 region is only accessible to immunohistochemistry staining by both a polyclonal and a monoclonal antibody specific to this region in tumor cells (10), suggesting that this region is structurally altered and becomes more accessible for protein-protein interaction in tumor cells, which predominantly express the caPCNA isoform. Using a cell permeable peptide harboring this eight amino acid sequence to block PCNA interactions, we were able

to selectively kill neuroblastoma and breast cancer cells (11,14,15). Consistent with the tumor-associated expression pattern of caPCNA (10), the peptide doesn't cause significant toxicity to non-malignant cells, including human neural crest stem cells (14) and mammary epithelial cells (11).

We hypothesized that the distinct structure of the L126-Y133 region in caPCNA offers an attractive target for developing small molecules that specifically block caPCNA and are therefore selectively toxic to cancer cells. Leveraging this structural insight and the published PCNA crystal structure (PDB: 1U7B), we performed a virtual screen for compounds that target the binding pocket partly delineated by residues L126-Y133 in PCNA. Here, we report the identification of AOH39, a small molecule compound, which selective kills many types of cancer cells at a low micromolar concentration and the subsequent development of AOH1160, an analogue of AOH39, which has a significantly improved potency and therapeutic window. Mechanistically, AOH1160 interferes with the binding of 3,3',5-Triiodothyronine (T3), a known PCNA ligand (16), to PCNA. It interferes with DNA replication and blocks homologous recombination (HR) mediated DNA repair, leading to cell cycle arrest, accumulation of unrepaired DNA damages, and enhanced sensitivity to cisplatin treatment. Therapeutically, AOH1160 is orally available to animals and suppresses tumor growth without causing significant side effects in mice. In summary, our study demonstrated the feasibility of targeting PCNA, which is central to broad cellular processes and indispensable to the growth and survival of all cancer cells, without causing unacceptable toxicity. The favorable pharmacologic and therapeutic properties of AOH1160 demonstrate the potential of this compound as a broad-spectrum therapeutic agent for cancer treatment.

Materials and Methods

Identification of PCNA inhibitors by computer modeling

We performed a virtual screen of libraries of chemical structures based on the known crystal structure of the PCNA/FEN1 complex that is available from the RCSB protein database. We specifically focused on the binding pocket in PCNA delineated in part by residues between L126 and Y133 of PCNA (see Fig 2g). We screened chemical databases available at the Albany Molecular Research Institute (AMRI, Albany, NY), containing 300,000 chemical compounds available directly from AMRI in at least 2 mg quantities, and more than 6.5 million additional compounds which were available in similar quantities from external vendors. For more than 3 million drug-like compounds in the databases, we pre-computed multiple conformations and performed a combination of substructure and pharmacophore searches using tools in the MOE software (Chemical Computing Group, Montreal, Canada, MOE v2008.05). The initial virtual screen yielded more than 8000 hits. We further analyzed these hits by molecular docking studies using the computer program, Glide (Schrödinger, LLC, New York, NY, Impact v 50207) (17), and identified 57 compounds, (including AOH39), for acquisition and experimental testing.

Development of a computer model for compound optimization

A computer model for compound optimization was initially built by the All-Around-Docking (AAD) methodology, which allows a small molecule to search the whole surface of the target protein for the binding site that has the lowest docking score by Schrödinger Glide (17). We further minimized the initial docking pose and refined the model by 50ns metadynamics simulation by the NAMD software (18). The free energy (ΔG) determined by the docking study relates to each compound's K_i by the Nernst equation for a system at chemical equilibrium: $\Delta G = -RT \ln(K_i)$, in which $R = 0.001987$ kcal/K/mol. One kcal/mol of difference in ΔG between two compounds at room temperature ($T = 300K$) translates into ~5.3 fold improvement in binding affinity measured by their K_i ratio.

Plasmids and Cell Lines

The human neuroblastoma cell lines: SK-N-DZ, SK-N-BE(2)c, SK-N-AS, and LAN-5, breast cancer cell lines: MDA-MB-436, MDA-MB-468, Hs578t, MCF7, HCC1937, and small cell lung cancer cell lines: H82, H524, and H526 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured in DMEM with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin. The MCF10A cell line was also obtained from ATCC and was cultured in the MEGM™ medium kit purchased from the Lonza Group Ltd. (Basel, Switzerland). Human PBMCs from a healthy donor were purchased from Sanguine BioSciences (Valencia, CA) and grown in RPMI1640 with 10% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 10 ng/ml IL-2. Human embryonic progenitor cell line 7SM0032 was acquired from Millipore (Billerica, MA) and cultured in the hEPM-1 Media Kit purchased from the same company. The human small airway epithelial cells (SAEC) and human mammary epithelial cells (hMEC) were both obtained from Lonza and were respectively cultured in the SAGM BulletKit and hMEC-MEGM BulletKit purchased from the same company. Glioblastoma stem cells (GSC) derived from newly diagnosed World Health Organization (WHO) grade IV glioblastoma tissues were cultured in DMEM/F12 medium supplemented with 20 ng/ml EGF, 20 ng/ml FGF, 5 μ g/ml heparin, 1×10^6 B27 (GIBCO/BRL), and 2 mM L-glutamine (19,20). Normal human neural stem cells (NSC) derived from primary human brain tissues were maintained in the same culture media (19,20). All cells were cultured in the presence of 5% CO₂ at 37°C.

The plasmid pCBASce expresses the rare cutting I-SceI meganuclease (21). The U2OS-derived cell lines, DR-GFP and EJ5-GFP, each contain a stably transfected reporter gene for DSB repair mediated by HR and end joining (EJ), respectively (22). These cell lines were cultured in DMEM media with 10% FBS at 37°C in the presence of 5% CO₂.

Cell growth and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays

To measure the effect of the compounds on cell growth, cells were seeded at 5×10^3 /ml or 3×10^4 /ml into a 96-well plate, depending on the cell lines. The GSC and NSC cells formed neurospheres. Other cells were allowed to attach. Cell growth was measured by the CellTitor-Glo assay (Promega, Madison, WI) according to the manufacturer's instruction after treatment with various concentrations of AOH39 or AOH1160 for 72 h. To measure

apoptosis, cells were seeded at 1×10^5 /ml onto a chamber slide. Once attached, cells were treated with 500 nM AOH1160 for 24 h. Cells were fixed and analyzed by a TUNEL assay using the TMR red *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN).

Cell Cycle Analysis

Cells were seeded at 1×10^5 /ml in a 6-well plate. Once attached overnight, cells were treated with or without AOH39 or AOH1160 for 6 or 24 h. After being fixed in 60% ethanol and stained with propidium iodide (PI), cells were analyzed by flow cytometry to determine the cellular PI fluorescence intensity. The flow cytometry data were analyzed by the FlowJo program to model various cell populations.

Double stranded DNA break repair assays

DR-GFP and EJ5-GFP cell lines were seeded at 2.5×10^4 cells/cm² in a 12-well plate. Once attached overnight, cells were transfected with the pCBASce plasmid that expresses I-SceI by Lipofectamine 2000 (Invitrogen). After incubation for 3 h, the media containing transfection complexes was aspirated and replaced with fresh media containing AOH39 or AOH1160. The HR and EJ-mediated DSB repair, indicated by the restoration of a functional GFP gene in the respective cell lines, were quantified by measuring the relative abundance of GFP-positive cells by flow cytometry 3 d after transfection.

Saturation Transfer Difference (STD) Nuclear Magnetic Resonance (NMR)

Recombinant human PCNA was purified and exchanged to D₂O-based phosphate buffer (15 mM), pH 7.2. Aliquots of 68 μ M PCNA stock were kept in a -80°C freezer. T3 purchased from Sigma (Saint Louis, MO) and AOH1160 synthesized in house were dissolved in D₆-DMSO at 5 mM and stored at -20°C freezer. The STD NMR experiments were carried out on samples composed of 1 μ M PCNA, 10 μ M Deuterated-DTT, and 2% D₆-DMSO with T3 and/or AOH1160 in 15 mM D₂O-based phosphate buffer. 4 μ M DSS was used as an internal reference to determine the reported ligand concentration in solution.

All NMR experiments were carried out at 25°C on 700 MHz Bruker Avance III equipped with 5 mm triple resonance cryogenic probe. STD NMR spectra were acquired with transients 2880, spectral width 14ppm with 32k data points. The recycle delay was 3s. Selective saturation was composed of 50 gauss shaped pulses at field strength of 86 Hz, and the duration of each pulse is 50 ms with a 500 μ s delay between pulses. The spin lock filter used to suppress protein signal was optimized to 50 ms at a field strength of 5 kHz. The frequency for protein saturation was optimized to be 0.9 ppm, and the ligand signals were not disturbed with the employed selective saturation condition at this frequency. The reference spectrum was acquired with saturation irradiated at -30ppm . To eliminate potential artifacts, the saturation and reference experiments were acquired in an interleaved manner, and the finished experiments were separated into two 1D data sets for analysis. Two repeated STD experiments were carried out sequentially on the same sample with duration of 7 hrs 47 minutes for each experiment. The peak and noise intensity was measured using Bruker Topspin software, and the noise level in the range of 9 to 11ppm was used to estimate the error of the peak intensity. The STD effect was described using equation ($I_{\text{Ref}} - I_{\text{STD}}$)/

I_{Ref} , in which the I_{Ref} is the peak intensity from the reference experiment, and the I_{STD} is the peak intensity from an on-resonance saturation experiment.

Human thyroid hormone receptor beta (TR β) reporter assay

Reporter Cells constitutively expressing human TR β and containing a luciferase reporter gene functionally linked to a TR β -responsive promoter were purchased from Indigo Biosciences (State College, PA). Cells were treated by various concentration of T3, AOH39, or AOH1160 for 24 h. The effect of each compound on TR β activity was quantified by measuring luciferase reporter gene expression according to the manufacturer's instruction.

DNA combing analysis

A DNA combing assay was performed as described (23). Briefly, synchronized neuroblastoma cell (SK-N-BE(2)-C) or small cell lung cancer cells (H82 and H526) were incubated first with a thymidine analog, 5-Chloro-2'-deoxyuridine (CldU) for 10 minutes. The unincorporated CldU was washed away and cells were incubated with a second thymidine analog, 5-Iodo-2'-deoxyuridine (IdU), in the presence or absence of 0.2 μ M of AOH 1160 for 20 minutes. The cells were subsequently collected, spotted on microscope slides, and then lysed. The released DNA fibers were spread down the slides. The DNA was immunologically stained with fluorophore-conjugated antibodies specific for each analog. The stained CldU residues emitted green fluorescence and the stained IdU residues emitted red fluorescence. The rate of DNA replication fork extension before and after AOH1160 treatment was estimated by measuring the relative length of green- and red-stained DNA segments respectively, using the ImageJ software (National Institute of Health, Bethesda, MD).

Clonogenic Assay

Three hundred human SK-N-DZ NB cells were seeded onto a 60-mm tissue culture dish. Once attached overnight, cells were treated with or without various concentrations of cisplatin in the presence or absence of 500 nM of AOH1160 for 18 h. Cells were washed twice with growth medium and were cultured in fresh medium for 3 weeks to allow surviving cells to form colonies. The medium was changed every 3 d throughout the experiment. The colonies formed under each treatment conditions were counted after being stained with 0.5% crystal violet. To evaluate synergy between AOH1160 and cisplatin, combination indices (CI) based on the Bliss independence model [$CI = (E_A + E_B - E_A * E_B) / E_{AB}$] were calculated (24).

Western blot

Cells were dissolved into the Laemmli sample buffer on the plate. Whole cell extracts were sonicated, and the proteins in the lysate were resolved using a 4 – 12% SDS polyacrylamide gel, and the resolved proteins were blotted onto a nitrocellulose membrane. Antibodies specific to H2A.X, cleaved caspase-3, full-length caspase-3, or cleaved caspase-9 were purchased from Cell Signaling Technology (Danvers, MA). The anti- γ H2A.X antibody was purchased from Millipore. The membrane was blocked with 5% nonfat dry milk and incubated with individually with each of these antibodies diluted in the blocking buffer.

After incubation with peroxidase-conjugated secondary antibodies, the protein of interest was detected using an ECL kit purchased from Thermo Fisher Scientific (Waltham, MA).

Measurement of AOH1160 and metabolites in plasma

AOH1160 was incubated in plasma at 37°C. An aliquot of the reaction mixture was taken after various incubation times. The plasma concentration of AOH1160 was determined by liquid chromatography–mass spectrometry (LC-MS/MS). Briefly, LC/MS/MS analysis was performed using a Waters Acquity UPLC system (Milford, MA, USA) interfaced with a Waters Quattro Premier XE Mass Spectrometer. HPLC separation is achieved using a Kinetex 2.6 µm XB-C18 100 × 2.0 mm column (Phenomenex, Torrance, CA, USA) preceded by a Phenomenex C18 guard column (Torrance, CA, USA). The column temperature was maintained at 40°C. The mobile phase consisted of A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile). The gradient program includes 35% B (0min, 0.3ml/min), 54% B (4.0 min, 0.3ml/min), 74% B (5.4 min, 0.3ml/min), 95% B (6.4 min, 0.3ml/min), 35% B (6.5 min, 0.3ml/min), 35% B (10 min, 0.3ml/min). The electrospray ionization source of the mass spectrometer was operated in positive ion mode with a cone gas flow of 25 L/hr and a desolvation gas flow of 650 L/hr. The capillary voltage was set to 3.0 kV. The source temperature was 125 °C and the desolvation temperature was 480°C. A solvent delay program was used from 0 to 4.4 minutes and from 6.2 to 10.0 minutes to minimize the amount of the mobile phase to flow into the source. MassLynx version 4.1 software was used for data acquisition and processing.

Pharmacokinetic (PK) study of AOH1160 in animals

To characterize the bioavailability and pharmacological properties of AOH1160 *in vivo*, a dosing solution was prepared by dissolving AOH1160 (10 mg) under a continuous flush of nitrogen gas at 60 °C into the vehicle, consisting of Kolliphor HS 15 (383.57 mg) Poloxamer 407 (56.43 mg), Butylated Hydroxyanisole (1 mg), Butylated Hydroxytoluene (0.25 mg), and Propyl Gallate (2 mg). This formulation may be encapsulated in gelatin capsules and be given to large mammals (including dogs and humans) orally. For the mouse studies, the test compound (AOH1160) in the vehicle was diluted by drinking water and the vehicle to a final concentration of 4 mg AOH1160 per mL of 1;1 mixture of H₂O and the vehicle immediately before each dosing. The diluted dosing solution was administered *per os* (PO) to a group of male and female mice (40 mg/kg). At 0 (prior to dosing), 0.17, 0.33, 0.5, 1, 2, 4, 6, and 24 h after dosing, blood samples were collected from 3 male and 3 female mice by cardiac puncture. Following removal of blood cells, the plasma concentration of AOH1160 was determined by LC-MS/MS as described above. Data were acquired via multiple reactions monitoring. The oral PK was determined by a standard non-compartmental method.

In vivo tumor model

All experiments involving live animals were carried out in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health. The protocol (#11034) was reviewed and approved by the City of Hope Institutional Animal Care and Use Committee. A breeding colony of ES1^c/SCID mice, originally provided by Dr. Philip M. Potter of the St. Jude Children's Research Hospital, was maintained at the City of Hope. SK-N-BE(2)c and

SK-N-AS neuroblastoma cells were suspended in Matrigel (BD Biosciences) at $5 \times 10^7/\text{ml}$ and $1 \times 10^8/\text{ml}$, respectively, after they were harvested and washed twice in PBS. MDA-MB-468 breast cancer cells and H82 small cell lung cancer cells were suspended in Matrigel at $2 \times 10^7/\text{ml}$ after they were harvested, washed, and mixed with Matrigel in the same manner. 0.1 ml of suspended cells was subcutaneously injected into the right flank ES1^e/SCID mice. For each xenograft model, mice were randomly divided into two groups, each receiving a daily dose of 40 mg/kg AOH1160 or an equivalent amount of vehicle by gavage throughout the entire experiment starting on the fifth day after tumor cell injection. Mice were monitored twice weekly for any sign of side effects. The weight of the animals was measured as an indicator of compound toxicity. At the end of the experiment, tumors were isolated from sacrificed mice and analyzed by immunohistochemistry staining with antibodies specific for phosphor-Chk1 and $\gamma\text{H2A.X}$ as described (25).

Results

Identification and characterization of AOH39

To identify small molecule compounds that target the PCNA and FEN1 interface, we started with the known crystal structure of the PCNA/FEN1 complex that is available from the RCSB protein database. To improve the likelihood of identifying novel small molecules that specifically target caPCNA, we focused our virtual screen on the binding pocket in PCNA delineated by residues from L126 to Y133 and screened databases consisting of more than 6.8 million chemical structures available at the AMRI. A set of 57 compounds identified by the virtual screen was acquired and further tested in a cell viability assay (See Fig S1–S3 for details on screen and compound triage). AOH39 (Fig 1a and S3) was selected for further development due to its anti-cancer activity and selectivity. As shown in Fig 1b and 1c, AOH39 is toxic to multiple neuroblastoma and breast cancer cell lines with IC_{50} ranging from 1.3 to 3.4 μM . It's much less toxic to non-malignant cells including human peripheral blood mononuclear cells (PBMC), human embryonic progenitor cells with neural crest mesenchyme properties (7SM0032), and immortalized human mammary epithelial cells (MCF10A) (Fig 1b & 1c), with IC_{50} s between 15.4 μM and more than 100 μM on these cells. Based on the selectivity of AOH39 seen in these cellular studies, we decided to choose its scaffold to further develop a selective anti-cancer agent.

To explore possible mechanisms by which AOH39 exerts its anti-tumor activity, we performed cell cycle analysis and found that AOH39 treatment caused cell cycle arrest of cancer cells at the S and, to a larger degree, G2/M phases (Fig 1d), suggesting an interference of DNA replication and repair. As early as 24 h after treatment by AOH39, cancer cells start to die through apoptosis as indicated by the rise of a sub-G1 cell population. The cell cycle arrest by AOH39 treatment coincides with enhanced intracellular $\gamma\text{H2A.X}$ levels, indicating an accumulation of double stranded DNA breaks (DSB) (Fig 1e). DSB, if not resolved in time, are lethal to cells. Cells deal with DSB mainly through end joining-mediated (EJ-mediated) DNA repair pathways during the G1 phase and homologous recombination-mediated (HR-mediated) pathways during the S and G2 phases (26,27) of the cell cycle. Reporter cell lines have been established to monitor each of these DNA repair pathways (22). These cells lines each contain a GFP reporter cassette disrupted by an

insertion of recognition site(s) for the rare cutting endonuclease I-SceI. Introduction of exogenous I-SceI creates DSB(s) within the reporters. Each reporter is designed such that repair of the I-SceI-induced DSB(s) by a specific pathway can result in restoration of the GFP cassette: HR for DR-GFP and EJ for EJ5-GFP. The relative abundance of GFP-positive cells determined by flow cytometry, therefore, reflects the efficiency of the respective DSB repair pathways in these reporter cell lines. Using these characterized reporter cell lines, we observed that AOH39 treatment inhibited HR-mediated DNA repair, without exerting any statistically significant effect on EJ (Fig 1f). Collectively, these results suggest that AOH39 interferes with DNA synthesis and HR-mediated DNA repair, causing accumulation of DNA damage and S and G2/M cell cycle arrest.

Identification of AOH1160, a potent AOH39 analogue

To improve the anti-tumor potency of AOH39 while preserving its favorable selectivity, we synthesized and tested a series of AOH39 analogues (not shown). One analogue (AOH1160) derived from substituting the methylene group linking the two benzene rings in AOH39 with an ether oxygen (Fig 2a) is significantly more potent than AOH39 in killing cancer cells with IC_{50} s ranging 0.11 μ M to 0.53 μ M on multiple neuroblastoma, breast cancer, and small cell lung cancer cell lines (Fig 2b–2d). AOH1160 is not significantly toxic to non-malignant cells, including human PBMCs, mammary epithelial cells, small airway epithelial cells, and 7SM0032 cells, up to a concentration of at least 5 μ M. It is also slightly less toxic to non-malignant cells than AOH39. The combined improvements in potency and selectivity lead to a significant improvement in the therapeutic window (Fig 1b–1c and 2b–2d). Importantly, AOH1160 inhibited the growth of glioblastoma stem cells without significantly affecting the growth of normal neural stem cells (Fig 2e). Consistent with the predominant expression of caPCNA in many cancers (10,12), the selectivity of AOH1160 between malignant and non-malignant cells is broad based. The median concentration to achieve 50% growth inhibition (GI_{50}) is about 330nM (Fig S4) in the 60 cell lines of the NCI60 panel (28). Although AOH1160 and AOH39 share certain structural similarities with T3 and T2AA, both known PCNA ligands with significant thyroid hormone (TR) activities (16), neither AOH1160 nor AOH39 showed any thyroid hormone activity in a TR reporter assay (Fig 2f).

Mechanism of action

To gain further structural insight into the binding of AOH39 and AOH1160 to PCNA, we implemented an in-house computer program based on the All-Around-Docking (AAD) methodology (17) to model the best binding site and the binding pose of AOH39 and AOH1160. In contrast to the virtual screen strategy that focused on the binding pocket delineated by L126 and Y133, the AAD approach allows a small molecule to search the whole surface of the target protein for the binding site that has the lowest docking score. We first validated our AAD docking method by modeling the binding of T3, which had been co-crystallized with PCNA (PDB: 3vkx). The T3 model pose predicted by our program is only 0.47 Å in root mean square deviation (RMSD) from what's indicated by the crystallographic study of the T3/PCNA complex, indicating that our calculation fits well with crystallographic results. Using this program, we found that AOH39 and AOH1160 bind to the same binding pocket as T3 does on PCNA (Fig 2g). Our model also indicated that the binding free energy (ΔG) of AOH1160 and AOH39 to PCNA are -5.54 kcal/mol and -4.62

kcal/mol respectively, indicating approximately a 5-fold improvement in binding affinity of AOH1160 to PCNA over that of AOH39 (see Methods). The calculated difference in PCNA binding affinity agrees well with the 6 – 7 fold increase in compound potency observed in cell viability assays (Fig1b–1c and 2b–2d).

To verify whether AOH1160 competes with T3 in binding to PCNA, Saturation Transfer Difference (STD) NMR experiments (29) were carried out to observe STD of T3 (50 μ M) in the absence and presence of AOH1160. In a STD experiment, the saturated proton magnetization of protein is transferred to the protons of a ligand if the ligand binds to protein, and thus the signal intensity of protein-bound ligands is reduced compared to that of unbound ligands. The STD values of T3 were consistent with the binding pose of T3 on PCNA observed in the crystal structure (data not shown). Addition of AOH1160 to reach an expected concentration of 16 μ M caused reduction of STD values of T3 (Fig 2h), suggesting AOH1160 interferes with T3 binding to PCNA. A further increase of AOH1160 concentration in the sample did not reduce T3 STD further, likely because the maximum concentration achievable of AOH1160 under this experimental condition is approximately 14.5 μ M as determined by 1D NMR spectra.

Although the AOH1160 binding site forms part of the PCNA interface with PIP box proteins such as Fen1, AOH1160 doesn't seem to block Fen1 or a PIP consensus peptide from binding to PCNA in a fluorescent polarization (FP) assay or in a co-immunoprecipitation assay (data not shown). Therefore, AOH1160 likely works via a different mechanism from T2AA or T3, even though they all bind to the same PCNA pocket. Whereas T2AA or T3 causes DNA replication fork stress by inhibiting PCNA interaction with PIP box proteins (16), AOH1160 might exert its effect by changing the subtle dynamics between PCNA and its binding partners. Mutagenesis analyses of PCNA function have shown that mutations that affect a fine balance between different PCNA-partner interactions often cause a stronger effect on the processivity of DNA replication than mutations that block PCNA interaction (30). To measure the effect of AOH1160 on DNA replication fork extension, we treated synchronized S-phase cells with CldU, a modified thymidine analogue, in the absence of AOH1160. After washing away the unincorporated CldU, cells were incubated with another modified thymidine analogue, IdU, in the presence or absence of AOH1160. The rate of DNA replication fork extension before and after AOH1160 treatment was estimated by measuring the relative length of CldU-incorporated DNA strands and adjacent IdU-incorporated DNA strands, respectively. The average lengths of the CldU-incorporated DNA strands are similar before AOH1160 treatment in the control and experimental cells, indicating that DNA replication forks extended at similar rates in these two cell populations (green bars in Fig 3). After the addition of AOH1160, the experimental cells treated by AOH1160 contain significantly shorter IdU-incorporated DNA strands than the untreated control cells (red bars in Fig 3), indicating that AOH1160 interferes with the extension of preexisting DNA replication forks in multiple cancer cell lines.

AOH1160 induces cell cycle arrest, accumulation of DNA damage, and apoptosis at below micromolar concentrations

Like AOH39, AOH1160 causes cell cycle arrest (Fig 4a), increases γ H2A.X levels (Fig 4b), and promotes apoptosis, as indicated by the increase in the sub-G1 population (Fig 4a) in neuroblastoma and small cell lung cancer cells. The increase in apoptosis, confirmed by a TUNEL assay (Fig 4c), in cancer cells coincides with activation of caspase-3 and caspase-9, suggesting the involvement of these two caspases in AOH1160-induced apoptosis (Fig 4b). Consistent with its lack of toxicity to non-malignant cells in a cell viability assay, AOH1160 doesn't significantly change the cell cycle profiles of the non-malignant 7SM0032 or SAEC cells (Fig 4a). Nor does it increase intracellular γ H2A.X level (Fig 4b) or induce apoptosis in 7SM0032 cells (Fig 4c).

AOH1160 inhibits HR-mediated DSB repair and sensitizes cancer cells to cisplatin

Like AOH39, AOH1160 blocks DNA repair in DR-GFP, but not in EJ5-GFP cells, indicating that it selectively inhibits HR-mediated DNA repair (Fig 4d). HR-mediated DNA repair plays an important role in repairing cross-linked DNA caused by chemotherapeutic drugs, such as cisplatin (31,32). We performed a clonogenic assay to investigate whether the AOH1160 would increase cancer cells' sensitivity to cisplatin. We treated SK-N-DZ neuroblastoma cells with or without various concentrations of cisplatin in the presence or absence of 500 nM AOH1160 for 18 h. Cells were washed and cultured in fresh medium in the absence of either agent for 3 weeks to allow colony formation. As shown in Fig 4e, SK-N-DZ cells are more sensitive to cisplatin treatment in the presence of AOH1160 than in its absence. The combination index of AOH1160 and cisplatin at the respective concentrations of 500 nM and 3 μ M is about 0.55, demonstrating the potential synergy of combining AOH1160 with conventional chemotherapeutic drugs in treating cancer patients. Similar synergy of AOH1160 and cisplatin was also observed on inhibiting SK-N-AS NB cells (Fig S5).

AOH1160 is orally active and inhibits tumor growth in animals

Given the potency and the favorable therapeutic properties of AOH1160, we tested its efficacy *in vivo*. AOH1160 was found to be sensitive to cleavage by the carboxyl esterase, ES-1, which is highly expressed in rodent blood, but not significantly expressed in the blood of higher mammal species (Fig 5a). Therefore, AOH1160 is not stable in rodent plasma due to ES-1-mediated by amide hydrolysis (Fig 5b). However, the compound is stable in the plasma of canine, monkey, and human, as well as in the plasma of the Es1^{-/-}/SCID mice, which are partially deficient in ES-1 expression (33), confirming that ES-1 overexpression is responsible for the rodent-specific metabolism of the compound (Fig 5b). Therefore, we performed all *in vivo* studies in ES1^{-/-}/SCID mice, which are commonly used to study drugs that are ES-1 substrates (33,34). Analysis of the compound's physiochemical properties indicates that AOH1160 has a low solubility, but high permeability, a property shared by about 30% of all approved drugs (35). It has reasonably good solubility in certain polar solvents including ethanol and in non-ionic oil-in-water solubilizers such as Kolliphor HS 15. Based on these properties, we developed a hot melt formulation with Solutol HS 15 and Poloxamer 407 for oral delivery (see Materials and Methods for recipe). Antioxidants

acceptable for regulatory applications were incorporated into this formulation (e.g., butylated hydroxytoluene, butylated hydroxytoluene, and propyl gallate) to ensure that this oral formula is compatible with accepted clinical application. The test compound (AOH1160) in the dosing formula was administered *per os* (PO) to a group of male and female mice. We took blood from 3 male and 3 female mice at each of the time points shown in order to determine the pharmacokinetic profile of the compound (Fig 5c). AOH1160 is available to the animal through the oral dosing route, and has a half-life of about 3.5 h *in vivo*. When dosed at 40 mg/kg, it reaches a peak concentration (C_{MAX}) well above the calculated IC_{50} of most cancer cell lines in a cell viability assay. Metabolites derived from AOH1160 hydroxylation were observed in animal plasma, suggesting involvement of the cytochrome P-450-dependent oxidation.

We tested *in vivo* activity of AOH1160 in ES1[®]/SCID mice bearing xenograft tumors derived from the neuroblastoma SK-N-AS and SK-N-BE2(c) cells, as well as from breast cancer and small cell lung cancer cells. We administered AOH1160 to mice at 40 mg/kg once daily by oral gavage. The compound treatment significantly reduced tumor burden (Fig 6 top) in comparison to the control groups that were given vehicle only. We also monitored the weight loss of the animals throughout the experiment as an indication of toxicity. AOH1160 did not cause any death or significant weight loss in the experimental animals (data not shown). No significant toxicity was observed in a comprehensive repeated dose toxicity study, in which mice were dosed once daily up to 100 mg/kg for two weeks (Supplemental Data). These *in vivo* properties of AOH1160 demonstrate conceptually the therapeutic potential of this compound in cancer treatment.

We previously showed that perturbation of PCNA function by a synthetic peptide causes accumulation of DNA damage and subsequent activation of Chk1 signaling to deal with the unrepaired DNA damages (25). To determine whether the DNA damage marker γ H2A.X and phosphor-Chk1 may be used as response markers for AOH1160 treatment, we analyzed the xenograft tumors harvested from mice treated by AOH1160 or vehicle only by Immunohistochemistry. Strong focal staining of γ H2A.X and phospho-Chk1 was observed in AOH1160 treated tumors. Cell disintegration was often observed at or around sites showing positive staining of γ H2A.X and phospho-Chk1. Overall, the tumors from AOH1160 treated mice are less dense than from the control mice. These observations demonstrated the potential utility of γ H2A.X and phosphor-Chk1 as response markers in therapy.

Discussion

The ultimate challenge of developing an anti-cancer therapy is to selectively destroy cancer cells, while sparing normal tissue. Most early chemotherapeutic or radiotherapeutic agents target DNA structures or mitotic spindles. Although they kill cancer cells effectively, these drugs cause significant side-effects. When used for the treatment of childhood cancers, these drugs may give rise to secondary malignancy as well. Following the success of Gleevec (18), many therapeutic agents targeting specific oncogenic signaling components have reached the clinic over the past 15 years (36–41). Whereas these so-called target-based therapies in general cause less severe side-effects than early chemotherapeutic agents, resistance often

develops to target-based drugs (42–44) through accumulation of mutations within the target genes or by activation of alternate survival pathways. We hypothesized that one way to prevent such acquired drug resistance, which is inherent in the adaptive and heterogeneous nature of cancers is to target “hub” proteins which are capable of influencing the activity of broad cellular processes that are essential to the growth and survival of all cancer cells. The key is to target crucial processes, such as the DNA replication/repair process, without causing unacceptable side-effects in non-malignant cells. To a large extent, the success of this strategy depends on the identification of cancer specific features of essential “hub” proteins and cellular processes.

Amino acid region L126-Y133 of PCNA is evolutionarily conserved, and this region of PCNA lies at the center of a variety of essential cellular processes, including DNA replication, cell cycle control, and DNA damage repair (4). These processes are of fundamental importance to the proliferation and survival of cancer cells. Consequently, inhibition of PCNA is viewed as an effective way to suppress tumor growth. Several attempts have been made in recent years to block various aspects of PCNA function (7,16,45–48). Based on our previous discovery of the caPCNA and nmPCNA isoforms, the subsequent studies pointing to structural distinction between these two isoforms, the distinct accessibility of PCNA binding partners to the L126-Y133 region of caPCNA and nmPCNA (10), and the ability of a cell permeable peptide containing the L126-Y133 octapeptide to selectively block PCNA interaction with its binding partners and to selectively kill neuroblastoma and breast cancer cells without causing significant toxicity to non-malignant cells (11,14,15,49), we focused our attention on targeting the L126-Y133 region of PCNA and successfully identified a series of small molecule compounds, including AOH39 and AOH1160 that bound this region. Both compounds are chemically novel in the drug discovery space; their scaffold has never been linked to any biological activity by others according to our search of science and patent databases. These compounds, and especially AOH1160, have remarkably favorable therapeutic properties. To our knowledge, AOH1160 is the first small molecule PCNA inhibitor that is orally available and inhibits tumors *in vivo* without causing significant toxicity after being systematically administered to animals. Therefore, successful translation of this compound to the clinic may lead to a new class of broad-spectrum anti-cancer drug, and significantly improve current cancer treatment options. Given the limitations of toxicity studies in mice, particularly with regard to the symptom of nausea and vomiting, further toxicity and PK studies in a non-rodent mammal species (in dogs in this case) will be conducted. Such a study will also enable us to evaluate marrow suppression and to better estimate possible PK profiles in human. In addition to the potential of AOH1160 to serve as an effective monotherapeutic agent, its ability to sensitize cancer cells to treatment by DNA damaging agents is expected to significantly improve the efficacy and reduce the dose-limiting side-effects of traditional chemotherapies, likely radiotherapy as well, used in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Proliferating cell nuclear antigen (PCNA) plays an essential role in regulating DNA synthesis and repair and is indispensable to cancer cell growth and survival. We previously discovered that the L126 - Y133 region of PCNA was structurally altered in cancer cells and tumor tissues. By targeting a surface pocket partly delineated by the L126 - Y133 region, we identified a novel PCNA inhibitor, AOH1160, which selectively kills a broad range of cancer cells at a below micromolar concentration, but is not associated with significant toxicity to non-malignant cells. This compound interferes with DNA replication and blocks homologous recombination mediated DNA repair, leading to cell cycle arrest, accumulation of unrepaired DNA damages, and enhanced sensitivity to cisplatin treatment. It is orally available to animals and suppresses tumor growth without causing significant side effects in mice. These findings demonstrated the potential of this compound as a novel therapeutic agent warranting clinical investigation for cancer treatment.

Figure 1. Identification and characterization of a novel PCNA ligand, AOH39

(a) Chemical structure of AOH39 (patent pending). The indicated human (b) neuroblastoma cell lines and (c) human breast cancer cell lines were cultured in the presence of various concentrations of AOH39 for 72 hours. The non-malignant 7SM0032 human embryonic progenitor cell line, (black diamonds in panel b), the immortalized, but non-transformed MCF10A cells (black circles in panel c), and PBMCs (black circles and black squares in panels b and c respectively) isolated from a healthy human donor were also cultured in the presence of the same AOH39 treatment. Cells cultured in the absence of AOH39 were used as control. Cell growth was measured by a CellTitor Glo assay (Promega). The average of

luminescence signals from triplicate samples normalized to the control for each cell line was graphed plus/minus standard deviations. (d) The SK-N-DZ cells were treated with 3 μ M AOH39 for 0, 6, or 24 h. Cells were fixed and stained with PI. The cellular PI fluorescence intensity was analyzed by flow cytometry. The flow cytometry data were analyzed by the FlowJo program to model various cell populations. (e) The SK-N-DZ cells were treated with 3 μ M AOH39 for 0, 6, or 24 h. Total cell extracts were analyzed by western blot, using antibodies specific to γ H2A.X or total H2A.X. (f) The DR-GFP and EJ5-GFP cell lines were transiently transfected with the pCBASce plasmid that expresses the I-SceI meganuclease. Three hours after transfection, cells were treated with or without 4 μ M AOH39 in fresh growth medium. The HR and EJ-mediated DSB repair events, indicated by the restoration of a functional GFP gene in the respective cell lines, were quantified by measuring the relative abundance of GFP-positive cells by flow cytometry. Results from triplicates for each cell line with (light bars) or without (dark bars) AOH39 treatment were averaged and graphed plus/minus standard deviations.

Figure 2. Development of AOH1160, a potent AOH39 analogue

(a) AOH1160 structure (patent pending). The sole substitution of an ether oxygen in AOH1160 for the corresponding methylene group in AOH39 is indicated by a red dashed box. The indicated (b) human neuroblastoma cell lines, (c) breast cancer cell lines, and (d) small cell lung cancer cell lines were cultured in the presence of various concentrations of AOH1160. The non-malignant (b) 7SM0032 cells and human PBMCs, (c) human mammary epithelial cells (hMEC), and (d) human small airway epithelial cells (SAEC) were also cultured under the same AOH1160 treatment. Cells cultured in the absence of AOH1160 were used as control. Cell growth was measured by a CellTitor Glo assay (Promega). The average of luminescence signals in triplicates normalized to the control for each cell line was graphed $\pm$ S.D. e) Three normal neural stem cell lines (NSC005-007) and three glioblastoma stem cell (GSC) lines, PBT003, PBT017, and PBT707, respectively representing three of the four glioblastoma subtypes (classical, proneural, and mesenchymal) (50) were treated with DMSO or 1 μ M of AOH1160 for 72 hours. Shown are cell growth related to the control cells $\pm$ S.D. f) TR β reporter cells were treated with various concentrations of T3, AOH39, or AOH1160 for 24 h. The effect of compounds on TR β activity was examined by measuring the relative luminescence units (RLU) in a luminescence plate reader. Grey: Signals from T3-treated cells and black: overlapping signals from AOH39 or AOH1160-treated cells. g) Computer modeling of small molecule binding to PCNA. The model was initially built using the AAD methodology (see text) and further refined by 50ns metadynamics simulation. Shown are small molecules (in stick) and PCNA surface around the binding pocket. The loop residues of L126-Y133 of PCNA are colored in blue to green. Top panel: AOH39 is shown as colored sticks and T3 as grey sticks. Bottom panel: AOH1160 is shown as colored

sticks and AOH39 as grey sticks. h) STD NMR experiments using 1 μ M of PCNA. The T3 compound structure is shown on top along with proton labels. The inserted table shows the reduction in STD $\pm$ errors for each of the T3 proton positions in the presence of AOH1160 (see Methods).

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Figure 3. Inhibition of replication fork extension by AOH1160

Synchronized (a) SK-N-BE(2)c neuroblastoma cells and (b) small cell lung cancer cells (H82 and H526) were sequentially incubated in the presence of CldU (green) and IdU (red) before and after AOH1160 treatment, respectively. Cells sequentially incubated with the same two nucleotide analogues but without AOH1160 were used as control. Shown in the left of panel (a) are representative images of the labeled DNA strands from cells treated with or without AOH1160. The lengths of CldU (green) and IdU (red) incorporated DNA segments measured from more than 30 independent DNA strands in the indicated cells treated with or without AOH1160 were averaged and graphed with standard deviations in the

right of panel (a) and in panel (b). The p values were determined by the Student's t-test on the lengths of IdU labeled DNA segments (red bars) between each AOH1160 treatment and the untreated control.

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Figure 4. Induction of cell cycle arrest, DNA damage, and apoptosis by AOH1160

(a) The normal cells (7SM0032 and SAEC) or cancer cells (SK-N-DZ and H524) cells were fixed, stained by PI, and analyzed by flow cytometry after being treated with 500 nM AOH1160 for the indicated time. (b) Extracts from the indicated cancer and normal cells treated by 500 nM AOH1160 for the indicated time were analysis by western analysis. (c) TUNEL analysis. 7SM0032 or SK-N-DZ cells treated by 500 nM AOH1160 for 24 h were fixed on slides. Cell apoptosis was analyzed by a TUNEL assay. Left panel: The TMR fluorophore (red) attached to the free ends of DNA indicates cells undergoing apoptosis. The blue indicates DAPI stained nuclei. Right panel: The abundance of apoptotic cells relative to the total number of cells in six randomly selected fields were averaged and graphed plus/minus standard deviations. The dark and light bars represent results from 7SM0032 and SK-N-DZ cells respectively. (d) Inhibition of HR-mediated DSB repair. The DR-GFP (black) and EJ5-GFP (grey) cell lines were transiently transfected by the pCBASce plasmid that

encodes the I-SceI meganuclease. Three hours after transfection, cells were treated with various concentrations of AOH1160 in fresh growth medium. Cells treated with DMSO were used as control. The HR and EJ-mediated DSB repair events, indicated by the restoration of a functional GFP gene in the respective cell lines, were quantified by measuring the relative abundance of GFP-positive cells by flow cytometry. Results of triplicate samples from each cell line and treatment condition relative to those from the control were averaged and graphed plus/minus standard deviations. (e) Enhanced sensitivity to cisplatin by AOH1160. Human SK-N-DZ NB cells were treated with or without various concentrations of cisplatin (CPT) in the presence or absence of 500 nM of AOH1160 for 18 h. Cells were washed twice with growth medium and were cultured in fresh media for 3 weeks to allow colony formation. The colony counts in dishes treated by cisplatin without AOH1160 (black) were normalized to the colony counts in dishes without cisplatin or AOH1160 treatment. The colony counts in dishes treated by both cisplatin and AOH1160 (red) were normalized to the colony counts in dishes treated by 500 nM AOH1160 only. The relative number of colonies in triplicates for each treatment condition were averaged and graphed plus/minus standard deviations. * indicates $p < 0.01$.

Figure 5. AOH1160 metabolism and *in vivo* PK

a) Illustration of carboxyl esterase-mediated cleavage of AOH1160. b) AOH1160 was incubated in plasma of the indicated species at 37°C. An aliquot of the reaction mixture was taken after various incubation times. AOH1160 concentrations, determined by liquid chromatography–mass spectrometry (LC-MS/MS), as a percent of the input concentration were graphed. c) AOH1160 was administered orally to ES1^o/SCID mice. The plasma concentrations of AOH1160 from 3 male and 3 female mice at each time points were averaged and graphed $\pm$ SD. The insert contains PK parameters determined by a standard non-compartmental method.

Figure 6. Inhibition of tumor growth by AOH1160 *in vivo*

(a) Mice bearing the indicated xenograft tumors of NB (SK-N-BE(2)c and SK-N-AS), breast cancer (MDA-MB-468), and small cell lung cancer (H82) were given vehicle only or 40 mg/kg of AOH1160 once daily starting on the fifth day after tumor implantation. Tumor sizes were measured by a dial caliper each week. Tumor volumes ($0.4 \times L \times W^2$) were averaged and graphed. Black cycles represent mice treated with vehicle only and grey triangles represent mice treated with 40 mg/kg AOH1160. * indicates $p < 0.01$. (b) The levels of phosphor-Chk1 (pChk1) and γ H2A.X in SK-N-BE(2)c derived tumor samples were analyzed by immunohistochemistry. Shown are representative images taken from two tumors treated by vehicle only and two tumors by 40 mg/kg AOH1160.