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Identification of the first small-molecule inhibitor of the REV7 DNA repair protein interaction

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ABSTRACT

DNA interstrand crosslink (ICL) repair (ICLR) has been implicated in the resistance of cancer cells to ICL-inducing chemotherapeutic agents. Despite the clinical significance of ICL-inducing chemotherapy, few studies have focused on developing small-molecule inhibitors for ICLR. The mammalian DNA polymerase ζ, which comprises the catalytic subunit REV3L and the non-catalytic subunit REV7, is essential for ICLR. To identify small-molecule compounds that are mechanistically capable of inhibiting ICLR by targeting REV7, high-throughput screening and structure–activity relationship (SAR) analysis were performed. Compound **1** was identified as an inhibitor of the interaction of REV7 with the REV7-binding sequence of REV3L. Compound **7** (an optimized analog of compound **1**) bound directly to REV7 in nuclear magnetic resonance analyses, and inhibited the reactivation of a reporter plasmid containing an ICL in between the promoter and reporter regions. The normalized clonogenic survival of HeLa cells treated with cisplatin and compound **7** was lower than that for cells treated with cisplatin only. These findings indicate that a small-molecule inhibitor of the REV7/REV3L interaction can chemosensitize cells by inhibiting ICLR.

1. Introduction

DNA interstrand cross links (ICLs) are a principal mechanism by which bifunctional chemotherapeutic drugs (e.g., cisplatin) eliminate cancer cells. Mammalian cells have extensive mechanisms for ICL repair (ICLR), which comprise complex networks of DNA damage response (DDR) processes such as nucleotide excision repair (NER), translesion synthesis (TLS) of DNA, and homologous recombination (HR).[1,2] Because cancer cells co-opt these mechanisms to promote their survival when challenged by ICL-inducing agents, each pathway involved in ICLR provides an attractive target for therapeutic intervention. However, few chemical inhibitors that target the DDR molecules required for ICLR are currently available, which poses a challenge in the optimal sensitization of ICL-inducing chemotherapies.

Several DDR molecules are essential for ICLR.[1] In mammalian cells, DNA polymerase zeta (Polζ) catalyzes the TLS step of ICLR.[2–6] Polζ is composed of a large 350-kDa catalytic subunit REV3L and a small 24-kDa non-catalytic subunit REV7/MAD2L2/MAD2B (hereafter designated as REV7).[7–9] Studies have identified that genetic elimination of REVL3 can chemosensitize cancer cells.[10–12] However, the large size of mammalian REV3L hinders the identification of Polζ inhibitors, because it is difficult to produce REV3L for enzymatic assays.[13] Therefore, we focused on REV7 as a target for inhibiting Polζ-mediated ICLR. REV7 is essential to recruit REV3L to the DNA replication fork.[14] The depletion of REV7 from a cell-free system derived from *Xenopus* egg extracts by immunogenic deprivation significantly delayed ICL removal from a plasmid DNA in the extracts.[15] Moreover, the genetic elimination of REV7 from cells markedly suppressed cell survival after cisplatin treatment.[4,16] These findings suggest that although REV7 has functions independent of Polζ,[17–22] it is a promising target for

Abbreviations: AlphaScreen, amplified luminescent proximity homogeneous assay screen; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FP, fluorescence polarization; GB1, protein G B1 domain; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HR, homologous recombination; HTS, high-throughput screening; ICL, interstrand crosslink; MBP, maltose binding protein; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PCR, polymerase chain reaction; TAMRA, tetramethyl-6-carboxyrhodamine; TLS, translesion DNA synthesis; WaterLOGSY, water-ligand observed via gradient spectroscopy.

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chemotherapeutic sensitization.[23,24] Because REV7 by itself does not have an enzymatic activity, we focused to inhibit the REV7/REV3L interaction.

2. Materials and methods

2.1. Materials

Chemical libraries for HTS were acquired from various sources. An additional batch of compound **1** was purchased from ChemBridge Corp. (San Diego, CA) for revalidation. All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless stated otherwise. Restriction DNA endonucleases and DNA ligases were purchased from New England Biolabs (Beverly, MA). SYBR green I stain was purchased from Life Technologies (Carlsbad, CA). The AlphaScreen assay kit was purchased from PerkinElmer (Waltham, MA). Taq DNA polymerases for PCR, the PCR purification kit, and the Plasmid Miniprep kit were purchased from Qiagen (Valencia, CA). COS7 and HeLa cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium containing 10% FBS. All cells were maintained in an incubator at 37 °C in a humidified atmosphere of 5% carbon dioxide.

2.2. Chemical synthesis

For synthetic procedure and characterization data of compounds **2**–**17**, see Supporting information.

2.3. Production of His-REV7(R124A)/biotin-AviTag-REV3L(1846–1898) pre-complex

The pETDuet1-REV7(R124A)/REV3L(1847–1898) plasmid[25] was digested with restriction enzymes NdeI and XhoI and purified to excise the region encoding REV3L(1847–1898). Concurrently, the region encoding the REV3L(1847–1898) was amplified by Taq PCR, using this parent plasmid as the template and 5′-ATACATATG AGTTCTGGACTAAACGACATATTCGAGGCACAGAAGATAGAGTGGCA CGAGGATATGTTGACACCAACTCCT-3′ and 5′-AATCTCGAGTTACT AGTCATGATCCAACAAAGTTGCC-3′ as the primers to generate a cDNA fragment encoding AviTag-REV3L(1846–1898), which contains an extra 3 nucleotides encoding REV3L(1846) inserted as a linker between the AviTag and REV3L(1847–1898). The cDNA was digested with NdeI and XhoI, ligated to the cut plasmid prepared above by using the Quick Ligation Kit (New England Biolabs), and cloned by transforming the *Escherichia coli* DH5α strain to generate the pETDuet1-REV7(R124A)/AviTag-REV3L (1846–1898) plasmid. The perfect match of sequence was confirmed by Sanger sequencing, using the Duet UP2 primer (TTGTACACGGCCGCATAATC).

The plasmids described above and pBirA+ were co-transformed into BL21(DE3) (Novagen, Madison, WI) with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) selection. Cells were grown in LB media at 37 °C to a density of 0.6 at 600 nm, induced with 1 mM IPTG, and 50 μM biotin was added. Cells were grown for another 6 h at 25 °C. Then, cells were harvested by centrifugation and suspended in lysis buffer (50 mM HEPES-NaOH, pH 7.8, 100 mM NaCl, and 20 mM imidazole) and disrupted using a microfluidizer. The clarified supernatant was applied to a Nickel column (GE Healthcare Life Sciences, Marlborough, MA) and washed with a buffer containing 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, and 40 mM imidazole. The bound protein was eluted by a linear gradient of imidazole in the same buffer. Fractions containing the protein of interest were pooled and dialyzed in a buffer containing 25 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, and 10% glycerol. The overall yield was approximately 4 mg protein per liter of culture. A 100-μL sample was desalted and eluted by using 50% acetonitrile (Honeywell Burdick & Jackson, Morristown, NJ) plus 2% formic acid (Sigma–Aldrich) and then loaded onto a nanospray emitter tip (New Objective, Woburn, MA) for static ionization, followed by time-of-flight analysis (Waters LCT Premier XE Mass Spectrometer). The Waters MaxEnt1 software was used for deconvolution to determine average mass of the sample, which matched that of the His-REV7(R124A)/biotin-AviTag-REV3L(1846–1898).

2.4. Preparation of MBP-REV7(R124A)

cDNA encoding full-length wild-type REV7 was amplified by Taq PCR, using the primers 5′-ATACCATGGATCATCATCATCATC ATCATCATCATACCACGCTCACACGACAAG-3′ and 5′-ATAGTCGA CTTATTTATCATCATCATCTTTGTAATCGCTGCCTTTATGAGCGCGC-3′. The amplicon containing a 5′-octahistidine tag and a 3′-FLAG tag (for possible use for the AlphaScreen and pull-down assays) was digested with *Sal*I-HF and *Nco*I-HF (New England Biolabs), ligated into the *Sal*I/*Nco*I site of a pMAL-c5X vector (New England Biolabs) by using the Quick Ligation kit (New England Biolabs), and transformed into the *E. coli* DH5α strain (Invitrogen) according to the manufacturer's instructions. Positive clones of the MBP-His-REV7-FLAG expression vector were used as templates to generate the MBP-His-REV7(R124A)-FLAG mutant vector by site-directed mutagenesis, using the GeneArt Site-Directed Mutagenesis System (Invitrogen) and the primers 5′-CATGTGGAG-CAGCTGCTCGCAGCCTTCATCCTGAAGATC-3′ and 5′-GATCTTCAG-GATGAAGGCTGCGAGCAGCTGCTCCACATG-3′. The sequence of the mutated vector was subsequently confirmed by Sanger sequencing, using an MBP sequencing primer (5′-GATGAAGCCCTGAAA-GACGCGCAG-3′).

The plasmid pMAL-His8-REV7(R124A)-FLAG was transformed into BL21(DE3) (Novagen). Cells were grown in LB media at 37 °C to a cell density of OD0.6 at 600 nm and induced with 1 mM IPTG. Cells were further grown for another 3 h at 37 °C. Cells were harvested by centrifugation, suspended in lysis buffer (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, and 10% glycerol), and disrupted by using a microfluidizer. After clearing the lysate by centrifugation, amylose resin (New England Biolabs) was added and the mixture was stirred for 1 h at 4 °C. The mixture was added into an empty chromatography column, and the resin was allowed to settle by gravity. After washing with the lysis buffer, the bound protein was eluted with 10 mM maltose (Sigma–Aldrich) in lysis buffer.

2.5. Hit identification by AlphaScreen HTS

HTS was conducted by using the AlphaScreen assay to analyze the bioactive (5760 compounds) and kinase inhibitor-like scaffold (2650) compounds chemical library in a solution consisting of 100 nM His-REV7(R124A)/REV3L(1846–1898) protein and 10 nM C-terminal biotin-tagged REV3L(1875–1895) peptide (ANILKPLMSPPSREEIMATLL) in the AlphaScreen buffer (25 mM HEPES, 100 mM NaCl, pH 7.4, 0.1% BSA, and 0.05% Tween-20). Briefly, 5 μL of the assay solution was transferred into each well of a white OptiPlate-384 (PerkinElmer) by using a Matrix WellMate liquid dispenser (Thermo Scientific, Waltham, MA). Then, 20 nL of the test compounds in DMSO were transferred in duplicate with a pin tool (V&P Scientific, Inc., San Diego, CA), by using a Biomek (Beckman Coulter, Indianapolis, IN), into the solution to give a final drug concentration of 40 μM in each well. The negative control in each plate was DMSO, and the positive control was unlabeled REV3L(1875–1895) peptide as a self-competitor. After incubating the plates overnight at room temperature, AlphaScreen nickel chelate (Ni-NTA) acceptor beads (PerkinElmer) diluted 1:100 in 10 μL

of the AlphaScreen buffer were added by using the Wellmate liquid dispenser. After 1 h, streptavidin AlphaScreen donor beads (PerkinElmer) diluted 1:100 in 10 μL of the AlphaScreen buffer were added. One hour later, the AlphaScreen signal was read by using an EnVision plate reader (PerkinElmer). Data were processed by using in-house programs in Pipeline Pilot (Accelrys, v.7.0.1). Hit compounds showing more than a 50% decrease in the AlphaScreen signal were further validated by establishing a dose response in triplicate, using another batch of the compound. Nonspecific inhibitors of the AlphaScreen signal generation were identified and eliminated by assaying the hit compounds on AlphaScreen for biotin-tagged hexahistidine. Compounds that nonspecifically denatured the His-REV7(R124A)/REV3L(1846–1898) protein were identified and eliminated by the thermal shift assay. After this selection process, only compound **1** was left for further evaluation.

2.6. Thermal shift assay

For the thermal shift assay, a mixture of a 5 μM solution of His-REV7(R124A)/REV3L(1846–1898) protein in buffer (25 mM HEPES, 100 mM NaCl, pH 7.3) and a 1:2000 dilution of SYPRO orange dye (Sigma) was prepared. To a 96-well plate, 47.5 μL of the protein solution was added with either 2.5 μL DMSO or 2.5 μL compound **1** (from a 10 mM DMSO stock) to yield a final concentration of 500 μM. Then, 20 μL of the mixture was added in duplicate to a 384-well MicroAmp optical reaction plate (Applied Biosystems, Foster City, CA). The plate was covered with a clear film and centrifuged for 1 min at 1500 rpm. The plate was incubated for 4 h at room temperature and then analyzed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The temperature was increased from 25 °C to 95 °C at the rate of 1 °C/min, and fluorescence was monitored for the SYPRO orange dye (λ_{ex} 492 nm and λ_{em} 610 nm). The Prism software (GraphPad, La Jolla, CA) was used to generate a Boltzmann sigmoid fit on a plot of fluorescence versus temperature to calculate the melting temperature at the inflection point of the curve.

2.7. Pull-down assay

A mixture of REV7(R124A)/AviTag-REV3L(1846–1898) (1 μM) and DMSO (0.5%), compound **1** (50 μM), or REV3L(1875–1895) peptide (5 μM) in 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween 20 (total 100 μL) was incubated by vibrating at 1500 rpm over WellMate (Eppendorf, Hamburg, Germany) at 4 °C for 20 h. Each treatment was performed in duplicate. Washed streptavidin-conjugated agarose beads (10 μL, settled, Cell Signaling Technologies, Danvers, MA) were added to the mixture and incubated for 4 h. Beads were then washed with the same buffer as used above (5× with 500 μL of buffer), and bound proteins were eluted by heating at 85 °C for 10 min with the SDS-loading buffer, analyzed by the NuPAGE gel system (10% Bis-Tris, MES running buffer; Thermo Fisher Scientific, Waltham, MA), and imaged by using the Oriole Gel Stain (Bio-Rad, Hercules, CA).

2.8. Fluorescence polarization assay

The assay solution consisted of 200 nM MBP-His8-REV7(R124A)-FLAG protein and 10 nM C-terminal TAMRA-tagged REV3L(1875–1895) peptide in a buffer consisting of 35 mM HEPES, 10% glycerol, 0.01% Triton X-100, pH 7.3. Each compound was serially diluted from a 10 mM stock solution in DMSO in the assay solution in a 96-well plate. In each plate, the negative control was DMSO and the positive control was the unlabeled REV3L(1875–1895) and REV3L(1875–1887) peptide as a self-competitor. After 4 h, 20 μL of the assay mixture was transferred in triplicate into each well of a black 384-well plate. The florescence signal was measured by using an EnVision plate reader (PerkinElmer) with 555-nm excitation (38-nm bandpass) and 632-nm emission (45-nm bandpass) filters and a D595fp/D635 dichroic mirror. FP values were calculated in millipolarization (mP) units. Absence of the autofluorescence background was concurrently confirmed for each compound in the same assay plate.

2.9. WaterLOGSY NMR

All NMR experiments were performed at 298 K (25 °C), using a Bruker Avance 600-MHz spectrometer equipped with a TCI cryogenic gradient probe. Compound **7** was dissolved in DMSO-d_6 at a concentration of 10 mM. For NMR experiments, 10 μL of each compound was dissolved in a solution containing 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 10% D_2O, or buffer containing the MBP-His8-REV7(R124A)-FLAG protein (10 μM), or MBP protein to yield a final concentration of 200 μM for each compound. One-dimensional (1D) ^{1}H-WaterLOGSY NMR spectra (mixing time of 1.2 s)[26] were recorded in the presence and absence of the protein described above.

2.10. Cell toxicity assay

HeLa cells (1000 cells/well) were seeded into 96-well plates and allowed to attach overnight. Next day, the culture medium was carefully removed and fresh medium containing compound **7** or DMSO vehicle control was added in triplicate. After 3 days, the alamarBlue reagent (Thermo Fisher Scientific) was added as per manufacturer's instructions. After 3 h, the florescence signal was read by using an EnVision plate reader (PerkinElmer) with 510-nm excitation and 590-nm emission filters. Cell viability was calculated by normalizing the signal value with that of cells treated with the DMSO vehicle control.

2.11. ICL repair assay

COS7 cells (80,000 cells/well) were initially seeded into 24-well cluster plates and allowed to settle. The medium was removed after 5 h and replaced with 200 μL of a medium containing 1.5× the final concentration of each compound or DMSO vehicle control. Transfection mixtures (100 μL) containing pGL-ICL[27] (10 ng) or pGL4.50 (2.5 ng; Promega, Madison, WI), pRL-TK (1 μg; Promega), 4 μL of FuGENE 6 (Promega), and 95 μL of OptiMEM (Invitrogen) were immediately prepared according to the manufacturer's instructions and added to each well. Cells were detached by adding 100 μL of trypsin 5 h after the transfection, and then cells were resuspended in 200 μL of medium containing 1.5× the final concentration of compound **7** or DMSO vehicle control. All samples were then re-dispensed (70 μL/well) in quadruplicate into a white opaque 96-well cluster plates. After the cells were incubated for another 13 h, the medium was removed, and the signals of both firefly and *Renilla* luciferase reporters were assayed by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The ICL repair activity was calculated as previously described.[27]

2.12. Cisplatin sensitization by the clonogenic survival assay

HeLa cells (450 cells/well) were seeded into 6-well plates and allowed to attach overnight. Each indicated final concentration of cisplatin was added. After 24 h, compound **7** at a final concentration of 75 μM or DMSO vehicle control was added to the wells, and plates were incubated for another 24 h. The culture medium was replaced with fresh medium, and cells were cultured for another 6 days. Cells were gently rinsed twice with PBS, fixed with 3.7% formaldehyde (w/v), and stained with 0.5% crystal violet

(w/v). Colonies containing more than approximately 40 cells were counted. Each treatment was performed in triplicate. The survival rate was reported as 1 for the average of colony numbers in wells that received the vehicle control of cisplatin [0.9% sodium chloride (w/v)].

3. Results

3.1. Structure-based design was unsuccessful for designing a REV7 inhibitor

REV7 binds to short motifs of REV3L,[28,29] which are essential for resistance to cisplatin.[30,31] Crystal structures of the complex of REV7 with the REV3L(1847–1898) fragment have been determined. The most notable feature of this interaction is that a portion of the REV3L(1847–1898) containing a PxxxxP motif is inserted into the bridge-like 'safety-belt' structure of REV7.[25,32,33] In contrast, no structures of uncomplexed REV7 have been solved, likely due to the intrinsically disordered state of this protein. ^{15}N-HSQC NMR spectra of the GB1-fused[34] REV7 protein revealed peaks in only the GB1 region, and there were only faint peaks clustered in the region of the unstructured portion (data not shown), indicating that the uncomplexed REV7 portion is almost unstructured. This property of REV7 precludes the use of a structure-based approach. In fact, none of compounds selected by virtual screening of approximately 450,000 compounds using Glide docking to the REV7 structure of the complex were active in the binding inhibition assay (next section). Therefore, we used a non-rational screening approach to identify a hit compound that inhibits the REV7 interaction.

3.2. Hit identification by HTS

Despite the small size of REV7, we were unable to produce the native REV7 protein in a standard bacterial expression protocol. Although the R124A mutation of REV7 can stabilize the protein without losing the interaction to REV3L(1847–1898),[35] expression of the REV7(R124A) protein was not still productive enough to yield large amounts. Thus, to enable running HTS, we had to take an approach using REV7(R124A)/REV3L(1847–1898) complex that can be produced in large amounts. A hexahistidine (His)-REV7 (R124A) was expressed and purified concurrently with REV3L (1847–1898).[25] Titration of the His-REV7(R124A)/REV3L(1847–1898) complex with a biotin-tagged REV3L(1875–1895), but not with another biotin-tagged unrelated peptide of similar length [Frizzled-7(544–566)[36]], generated a strong signal in the amplified luminescent proximity homogenous assay screen (AlphaScreen), which detects the proximity of a His-tag to biotin (Fig. 1A). The fact that the structure of REV7(R124A)/REV3L(1847–1898) complex[25] does not harbor an additional site to adopt another REV3L peptide suggested that part of the REV3L(1847–1898) fragment could be exchanged with the exogenous REV3L(1875–1895) peptide in this assay condition. When a mixture of His-REV7(R124A)/REV3L (1847–1898) complex and REV3L(1875–1895)-biotin was titrated with the non-tagged REV3L(1875–1895), the signal in the AlphaScreen assay was reduced in a dose-dependent manner by non-tagged REV3L(1875–1895) but not by non-tagged Frizzled-7 (544–566) (Fig. 1B). These findings indicate that the interaction of biotin-tagged REV3L(1875–1895) to the His-REV7(R124A) was inhibited specifically by the self-competition. Therefore, this assay should identify a compound that inhibits the interaction of His-REV7 with the REV3L(1875–1895)-biotin. We performed HTS of approximately 8400 compounds by using this protocol. After removing false-positive hits, only compound **1** (Fig. 1C) was left. HTS of approximately 6500 more compounds did not yield additional true hits.

Two additional assays were performed to confirm that compound **1** is a true inhibitor of the REV7-REV3L(1875–1895) interaction. In a thermal shift assay, Compound **1** showed a clear shift in the denaturation point of the His-REV7(R124A)/REV3L(1847–1898) complex (Fig. 1D), suggesting that compound **1** exchanged with REV3L(1847–1898) in the complex but was not nonspecifically bound on the complex. To verify this, a His-REV7(R124A)/biotin-AviTag-REV3L(1846–1898) complex was generated. The complex was incubated with compound **1** or non-tagged REV3L (1875–1895) and a pull-down assay using streptavidin-conjugated beads was performed. The isolation of His-REV7(R124A) was significantly inhibited (Fig. 1E), suggesting that both compound **1** and REV3L(1875–1895) can exchange with the biotin-AviTag-REV3L(1846–1898) in the complex, resulting in the release of His-REV7(R124A) from the beads.

3.3. Structure–activity relationship (SAR) of the hit scaffold

Next, we studied the SAR of the chemical scaffold of compound **1**. Because the HTS method that uses a protein complex is atypical to evaluate the inhibitors of protein–protein interactions, we wished to use an uncomplexed REV7(R124A) protein to study SAR, which does not require large-scale protein production. In a recent study, the MBP-fused REV7 protein was produced and used for functional studies.[13] The MBP conjugation improves protein stability and increases fluorescence anisotropy because of the increase in overall protein size (from 24 kDa to 67 kDa). Thus, we produced the MBP-REV7(R124A) protein and titrated it with a TAMRA-tagged REV3L(1875–1895) peptide in a fluorescence polarization (FP) assay. The EC_{50} of the dose–response curve was approximately 75 nM (Fig. 2A). When a mixture of MBP-REV7 (R124A) and REV3L(1875–1895)-TAMRA was titrated by the non-tagged REV3L(1875–1895), a dose-responsive competition was observed, which indicated the self-competition (Fig. 2B). This finding confirms that the FP competition assay can be used to evaluate inhibitors of the interaction between REV7(R124A) and REV3L (1875–1895)-TAMRA.

Analogs of compound **1** were synthesized and assayed by the FP competition assay to study SAR (Fig. 2C and D). None of compounds in which the furan ring was replaced with a different hydrophobic group (compounds **2**–**5**) were active. Changing the orientation of the ring to a 3-furan configuration neither yielded an active compound (compound **6**), highlighting the importance of the 2-furan moiety. Among examined, the only tolerated substitution on the furan ring of compound **1** was a 5-methyl group (compound **7**), which yielded higher activity (IC_{50} = 78 μM) than compound **1** in the FP assay. A series of amide substitutions on the furan ring yielded compounds **8**–**10** that were inactive, whereas the morpholino analog (compound **11**) showed some activity. Therefore, a 5-methyl substitution was made on the furan ring to explore the acetylpiperidinemethyl moiety of compound **7**. Removing the entire moiety (compound **12**) eliminated activity. Comparable activity was observed on substituting the piperidine ring by an azetidine (compound **13**) or a pyrrolidine ring (compound **14**) or shifting the position of the acetyl group (compound **15**). However, replacing the acetylpiperidine moiety with a phenyl (compound **16**) or pyridine (compound **17**) ring yielded inactive compounds, which supports the notion that the *N*-acyl-heterocycle moiety plays a role in activity.

3.4. Direct REV7 binding of the lead compound

Compound **7** was chosen for further evaluation because of its potency and a symmetric structure that makes the NMR spectra simple, and thereby feasible for the water-ligand observed via gradient spectroscopy (WaterLOGSY)[26] study. To verify whether

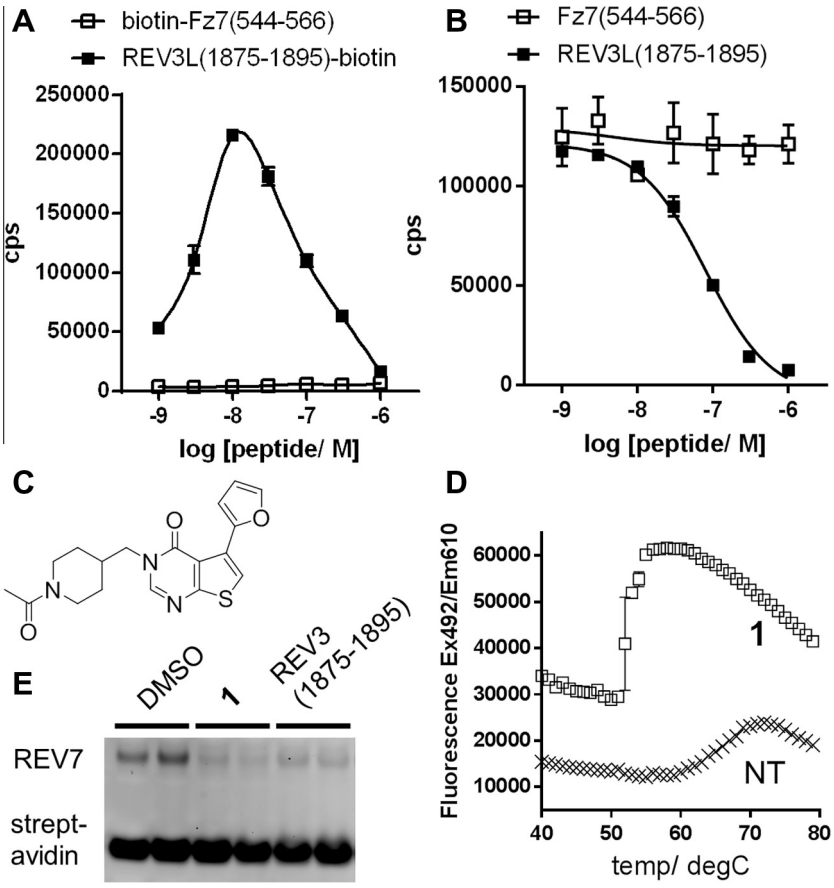

Figure 1. HTS identification of a REV7 inhibitor by AlphaScreen assays. (A) Dose response of indicated biotin-tagged peptide titrated over His-REV7(R124A)/REV3L(1847–1898) in AlphaScreen. A hooking peak characteristic to AlphaScreen assay is observed only for REV3L(1875–1895)-biotin peptide. (B) Dose response of competition by the indicated peptide titrated over a mixture of His-REV7(R124A)/REV3L(1847–1898) and REV3L(1875–1895)-biotin peptide. IC_{50} for REV3L(1875–1895) is read as 80 nM. An unrelated peptide of similar length [Fz7(544–566)] served as a negative control. Error bars represent standard deviation ($n = 2$). Hit identification and validation by independent assays. (C) Structure of the hit compound **1**. (D) Destabilization, but not nonspecific denaturation, of His-REV7(R124A)/REV3L(1847–1898) protein by compound **1** measured by thermal shift assay. (E) Dissociation of His-REV7(R124A)/biotin-AviTag-REV3L(1847–1898) protein complex (1 μM) by compound **1** (50 μM) or REV3L(1875–1895) peptide (5 μM), on streptavidin-conjugated agarose beads. DMSO was used as a vehicle control for compound **1**. Each treatment was duplicated, as shown by the bars.

compound **7** directly binds to REV7(R124A), WaterLOGSY NMR spectra of compound **7** were recorded in the absence and presence of MBP-REV7(R124A) or MBP. Except for the negative resonance peaks of buffer salts, almost no other resonances appeared in the absence of MBP-REV7(R124A). However, positive peaks that matched those from the ^{1}H NMR of compound **7** appeared in the presence of MBP-REV7(R124A) (Fig. 3) but not MBP (data not shown), indicating that compound **7** directly binds to REV7 (R124A) but not MBP. The relative size of several peaks in Water-LOGSY spectra were smaller than those in the ^{1}H NMR (Fig. 3, arrowheads), which suggests that those hydrogens are exposed out from the interface of the REV7(R124A)/compound **7** complex. However, the attempt to determine structural detail of interaction of compound **7** to REV7 was not successful, due to failure of crystallization of REV7/compound **7** complex.

3.5. Functional activity of the lead compound

At a concentration approximately at IC_{50} in the FP assay, compound **7** decreased HeLa cell growth but did not completely eliminate the cells (Fig. 4A). Thus, this concentration was used to validate compound **7** for inhibiting the DDR in cells. A luciferase plasmid containing an ICL between the promoter and luciferase-coding regions was used (Fig. 4B) to test ICLR activity.[27] This plasmid also contains the SV40 origin sequence and thus is replicated in mammalian cells expressing the SV40 large-T antigen (e.g., COS7, HEK293T), resulting in removal of the ICL during replication and the restoration of luciferase activity. COS7 cells were transfected with this plasmid. Compound **7** suppressed luciferase activity in cells transfected with the ICL plasmid, after normalizing with those in cells transfected with the corresponding non-ICL plasmid, indicating that compound **7** inhibits ICLR (Fig. 4C). Finally, a cell-survival assay was performed to validate the chemotherapeutic potential of compound **7**. HeLa cells were first treated with cisplatin and then compound **7** or vehicle control, and the number of cell colonies was counted. The normalized number of colonies was lesser for cells treated with cisplatin and compound **7** than for cells treated with cisplatin only (Fig. 4D), which suggests a chemosensitization effect.

4. Discussion

We did not observe a sharp SAR of the compound **1** scaffold for REV7 inhibition, which suggests that the scaffold might not be enough capable for antagonizing entire REV3L(1875–1895). A previous study identified that the PxxxxP motif of REV3L(1880–1885) is the main region that interacts with REV7.[28] In this interaction, a helix structure of REV7 binds with the PxxxxP motif, and the bridge structure of REV7 stabilizes the PxxxxP motif interaction.[25] However, the PxxxxP motif might not be sufficient for a tight REV7 interaction. A small helical motif in the REV3L(1887–1893) fragment is not the main region binding to REV7, but might increase

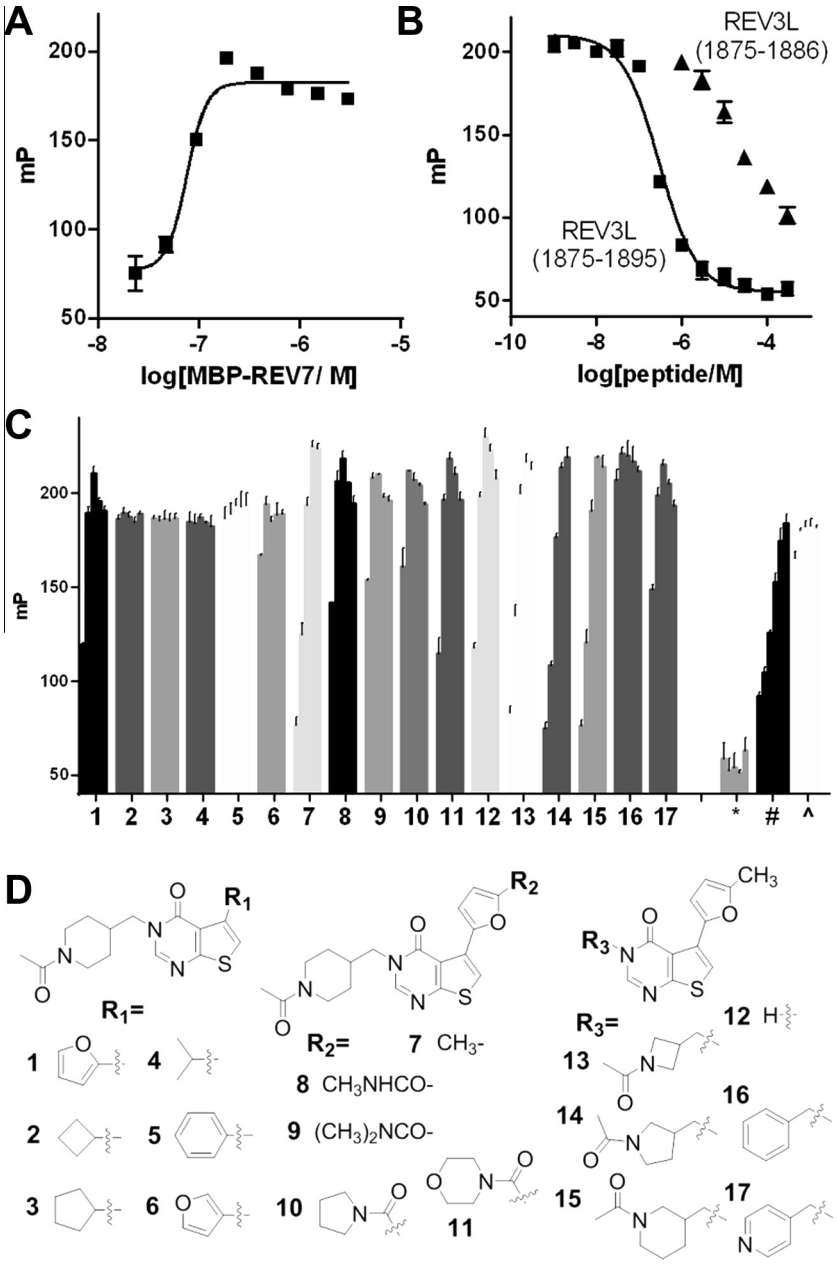

Figure 2. SAR study by fluorescence polarization assays. (A) Dose response of MBP-REV7(R124A) titrated over REV3L(1875–1895)-TAMRA peptide in fluorescence polarization. EC_{50} is read as 75 nM. (B) Dose response of self-competition by indicated peptide titrated over a mixture of MBP-REV7(R124A) (200 nM) and REV3L(1875–1895)-TAMRA peptide (10 nM). IC_{50} is read as 0.3 μM [REV3L(1875–1895): square] and 30 μM [REV3L(1875–1886): triangle]. Error bars represent standard deviation ($n = 3$). (C) Dose response of competition by indicated compound titrated over a mixture of MBP-REV7(R124A) and REV3L(1875–1895)-TAMRA peptide. The 17 compounds, REV3L (1875–1895) (*), REV3L(1875–1886) (#), and DMSO (^) were assayed all together for direct side-by-side comparison at 300, 100, 30, 10, and 3 μM (from left to right for each compound). DMSO was used as a vehicle control for the compounds. The mP value of DMSO treatment represents 0% inhibition and that of REV3L(1875–1995) peptide does 100% inhibition. Error bars represent standard deviation ($n = 3$). IC_{50} of compound **7** is read as 78 μM. (D) Structure of the compounds **1**–**17**.

REV7 association by promoting a structural transition of REV7 from a disordered to an ordered state. Indeed, the competition by the REV3L(1875–1886) peptide (i.e., the PxxxxP motif only) was merely 30 μM (Fig. 2B), which was 100 times weaker than that of the REV3L(1875–1895) peptide (Fig. 2B), regardless of the fact that the REV3L(1885–1895) peptide by itself (i.e., the helical motif only) showed no competition (not shown). It is possible that compound **1** scaffold antagonizes the PxxxxP motif but not the entire REV3L (1875–1895) sequence, given that the IC_{50} of compound **7** (78 μM, Fig. 2C) is only 2–3 times weaker than that of the REV3L (1875–1886) PxxxxP peptide. Thus, it is likely that the structure transition of REV7 to adopt compound **7** is not successful.

Numerous studies have reported the Polζ-independent functions of REV7, such as cell cycle regulation by inhibiting APC[37,38] and resection of double-strand DNA breaks.[19,21] It is reasonable to assume that an inhibitor of the REV7/REV3L interaction might be inert to REV3L-independent (i.e., Polζ-independent) functions. However, given the intrinsically disordered nature of uncomplexed REV7, a compound that induces a global change in the structure of REV7 may also allosterically inhibit REV7 interactions with other REV7 partners for Polζ-independent functions. Given this possibility, the suppressive effect of compound **7** on cell growth (Fig. 4A) might be in part through mechanistic inhibition of multiple functions of REV7. From a chemotherapeutic standpoint, it will be

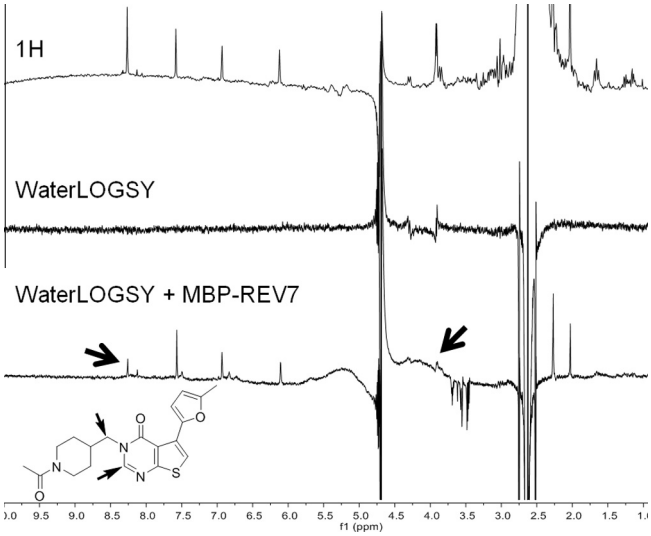

Figure 3. Direct binding of the compound to REV7(R124A). Spectra ^{1}H NMR, and WaterLOGSY NMR with or without MBP-REV7(R124A), for compound **7**. See text for detail. Arrowheads: peaks smaller in the WaterLOGSY NMR with MBP-REV7(R124A) than in the ^{1}H NMR, and their assignments (left below).

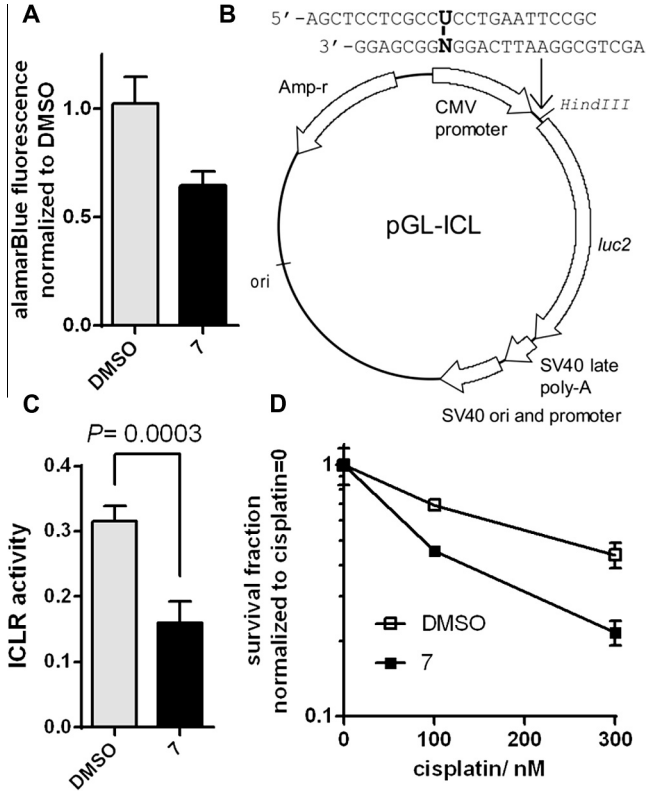

Figure 4. Functional activity of the compound. (A) Viability of HeLa cells after compound 7 treatment (75 μM) measured by alamarBlue reagent, normalized by DMSO treatment. (B) Reporter plasmid used in the ICLR assay. (C) ICLR activity measured in COS7 cells transfected with the plasmid (B) and treated with compound **7** (75 μM) or DMSO. Statistical analysis was performed by two-tailed unpaired *t*-test. (D) Clonogenic survival of HeLa cells that were treated with indicated concentration of cisplatin followed by compound **7** (75 μM) or DMSO. Data is indicated as survival fractions normalized by that of no cisplatin group for each treatment. DMSO was used as a vehicle control, same volume of the compound solution. Error bars represent standard deviation (n = 3 (B, D), 4 (C)).

intriguing to compare inhibition of Polζ-independent REV7 functions and that of Polζ-specific REV7 function for ICLR and the chemotherapeutic sensitization. The functional validation of REV7 by genetic elimination of REV7 may not address this objective, because this approach is not capable to distinguish particular REV7 interactions and functions. An approach in which the specific interaction site of REV7 is mutated would be the most plausible approach to validate specific REV7 functions, but interaction sites for Polζ-independent REV7 functions have not been identified to date. Hence, another approach is required to determine whether Polζ-independent REV7 functions are druggable target.

Author contributions

MLA performed all chemical syntheses, FP assays, thermal stabilization assays, and additional HTS for ~6500 compounds; and analyzed the data. NDA performed virtual screenings, initial HTS for ~8400 compounds to identify compound **1**, and thermal stabilization assays. BJE generated the expression plasmid for MBP-REV7(R124A), optimized the FP assay, and performed the ICLR assay. YH and RH generated the His-REV7(R124A)/biotin-AviTag-REV3L(1847–1898) and MBP-REV7(R124A) proteins. MV performed the WaterLOGSY analysis. AI performed cell growth and clonogenic assays. ETM attempted HSQC NMR analysis of the REV7/compound **7** complex. SK, KH, and HH generated the plasmid for expressing His-REV7(R124A)/REV3L(1847–1898), produced this protein, validated the interaction of REV3L(1875–1895) to uncomplexed REV7, and attempted to crystallize the REV7/compound **7** complex. NF generated the plasmid for expressing His-REV7(R124A)/AviTag-REV3L(1846–1898), developed the AlphaScreen and FP assays, performed the pull-down assay, conducted the project, analyzed the data, and wrote the manuscript. All authors have approved the final version of the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.07.026.

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