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A Stapled POL κ Peptide Targets REV1 to Inhibit Mutagenic Translesion Synthesis.

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Abstract

Stapled α-helical RIR (Rev1-interacting region) peptides of DNA POL κ bind more effectively to the RIR-interface of the C-terminal recruitment domain of the translesion synthesis DNA polymerase Rev1 than unstapled peptide. The tightest-binding stapled peptide translocates into cells and enhances the cytotoxicity of DNA damaging agents while reducing mutagenesis. Drugs with these characteristics could potentially serve as adjuvants to improve chemotherapy and reduce acquired resistance by inhibiting Rev1-dependent mutagenic translesion synthesis.

Keywords

Translesion synthesis (TLS); Staple peptide; chemoresistance; cytotoxicity; mutagenesis

Introduction

Intrinsic and acquired resistance to DNA damaging cancer drugs such as cisplatin poses a persistent clinical challenge, one that ultimately limits successful treatment of patients [Lippert et al. , 2008]. Recent evidence from mouse models has suggested that a possible strategy to both sensitize intrinsically resistant tumors to treatment and also reduce the acquisition of drug resistance by susceptible tumors during treatment would be to inhibit the major branch of mutagenic translesion synthesis (TLS) during chemotherapy [Doles et al. , 2010; Xie et al. , 2010; Xu et al. , 2013; Sail et al. , 2017; Wojtaszek et al. , 2019]. Translesion synthesis is a DNA damage bypass process where a group of low fidelity polymerases come together to tolerate a DNA lesion [Yamanaka et al. , 2017]. This pathway

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Author Contributions

N.C. conducted the ELISA, and cellular assays. S.D., M.S. and N.C. constructed the stapled peptide. C.A.H. ran the UV experiment. S.D. and G.H. ran the CD spectroscopy and FPA. G.L.V. and G.C.W. came up with the idea of RIR stapled peptide. N.C. and G.C.W. wrote the paper.

is critically dependent on two specialized TLS DNA polymerases: i) Rev1, a Y family DNA polymerase that has only a limited ability to insert nucleotides opposite lesions but possesses a critically important C-terminal domain (CTD) that recruits other TLS DNA polymerases and ii) POL ζ_4, a B family DNA polymerase that consists of the catalytic subunit REV3, the accessory subunit REV7, and two subunits shared with the POL δ replicative DNA polymerase, POLD2 and POLD3 [Korzhnev and Hadden, 2016].

Structural studies have suggested that an Achilles heel of the REV1/POL ζ_4-dependent pathway of mutagenic TLS could be Rev1's CTD. This 100 amino acid domain consists of an atypical four-helix bundle consisting of mixed parallel and antiparallel helices that has two distinct interfaces. One interacts with the RIR (Rev1-interacting region) peptides of POL κ POL η, POL ι, POLD3, and XRCC1, while the other interacts with REV7. Drugs that bind to either of these interfaces and thereby inhibit mutagenic TLS could potentially be used as adjuvants to improve the effectiveness of DNA damaging chemotherapy [Wojtaszek et al. , 2012a; Wojtaszek et al. , 2012b; Gabel et al. , 2013; Pustovalova et al. , 2016].

A rational strategy for obtaining such an adjuvant—which lacks small molecule-specific toxicity—is to develop a derivative of an RIR peptide that could bind to the RIR interface of REV1 CTD and interfere with the recruitment of other TLS DNA polymerases. These short α-helical RIR peptides interact with REV1 by inserting two conserved adjacent phenylalanines into preformed hydrophobic pockets in the RIR interface of CTD and make additional predominantly backbone contacts, a step important for TLS function [Ohashi et al. , 2009; Wojtaszek et al. , 2012b]. Normally only a small fraction of free RIR peptides would be in the α-helical conformation, but they can be stably locked into an α-helix by stapling as previously described [Verdine and Hilinski, 2012].

Materials and Methods

Stapled peptide synthesis

The POL κ RIR stapled peptide was synthesized on the 4-methylbenzhydrylamine (MBHA) rink amide resin (100-200 mesh, Novabiochem) using the standard Fmoc solid-phase peptide synthesis technique as described before [Kim et al. , 2011; Grossmann et al. , 2012]. Briefly, amino acids were coupled using COMU (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino—morpholino—carbenium hexafluorophosphate) and diisopropylethylamine (DIEA) in N-Methyl-2-pyrrolidone (NMP) for both standard and non-natural amino-acids in a custom-made 25 mL glass reaction vessel that is outfitted with a medium porosity frit and a "T"-bore for N_2 gas bubbling. The metathesis step was performed at a 0.15 mmol scale under constant nitrogen degassing, using 10mM of 1st generation Grubbs' catalyst in dichloroethane (DCE) in a 2 ml total solution volume, twice, for 2 hours at room temperature. Following olefin-metathesis, final Fmoc deprotection was carried out with piperidine in *N,N*-dimethylformamide and the peptide cleaved from the resin with a cleavage cocktail comprising TFA: TIS: H20 (95/2.5/2.5) within 3 hours. The peptide was consequently capped at its N-terminus with FITC (Fluorescein-5-isothiocyanate; 'Isomer I', [Molecular Probes] 9:1 isomer mixture) with an intervening β-alanine spacer that separated the label from the 1st amino acid of the peptide. FITC labeling enables detection of stapled peptide in cells, which appears as green foci.

The resultant crude peptide was purified and analyzed by reverse-phase HPLC using an Agilent 1200 HPLC system equipped with a DAD detector. Samples were loaded onto a Clipeus C18, 5 μm, 300 Å (150 × 4.6 mm) HPLC column Higgins Analytical, Inc.), which was equilibrated with 10% Eluent A (0.1% trifluoroacetic acid (TFA) in water). The peptides were eluted with Eluent B (0.1% TFA in acetonitrile) for 30 minutes at a 1.0 mL/min flow rate at 25 °C. The resultant elute was monitored for absorbance at 220 and 280 nm. The purified staple peptide fractions were combined, concentrated in a speed vacuum and stored as a lyophilized powder or as aqueous stock solution at −20 °C.

Stapled peptide detection was performed by electrospray ionization-mass spectrometry (ESI-MS) on an LC-MS system comprised of an Agilent 1260 LC, which is outfitted with a Poroshell 120 EC and C-18 column (2.7 μm pore, 3.0 × 50 mm, Agilent Technologies, Inc.). Agilent 6230 TOF system which houses an Agilent Jetstream ESI source is also connected to it. For peptide detection, Samples were run at a flow rate of 0.4 mL/min using a gradient of 0 to 95% of acetonitrile over 5 minute-window. High resolution mass spectroscopy data and HPLC traces of stapled peptide were obtained. Peptide quantification was carried out via UV absorbance.

Protein purification

The pET28b His-TEV hRev1 CTD plasmid containing 100 amino acids of the REV1 CTD was overexpressed in BL21RIL *Escherchia coli*, induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 18 °C for 18 hours until the A_{600} reached 0.4. Cells were pelleted at 4000g, 20 minutes at 4 °C and resuspended in 120 μl sodium hydrogen phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 20 mM BME and protease inhibitor cocktail). Cells were lysed using French press at 30 KPsi and incubated on ice for 15 minutes following the addition of 6 μl Benzonase. Cells were then spun at 10,000g for 25 minute at 4 °C and the supernatant was incubated with 5 ml of NiNTA resin for 1 hour at 4 °C before running it through size exclusion nickel affinity chromatography. A final protein purification step was carried out by applying the elute through an equilibrated (with 1X PBS and 1 mM DTT) Sephadex 75 column.

FLAG tagged purified POL κ protein was purchased from OriGene (Catalog # TP319806).

Circular Dichroism (CD)

The stapled peptide was dissolved at a 50 μM concentration in pure water, sonicated and centrifuged to remove insoluble peptides. Peptide concentration was determined by measuring UV absorbance at 280 nm. CD measurements were collected in a quartz cuvette with a 1 nm bandwidth and 0.1 cm path length at 20 °C using CD spectrometer (Jasco J-710). Other standard spectral-measurement parameters were 190-260 nm wavelengths, 0.5 nm step resolution and 10 accumulations at 20 nm/sec speed. Spectra units were normalized to the mean residue ellipticity, [θ] (deg cm^2 $dmol^{-1}$) using concentration determined by UV absorbance to account for minor variations.

Fluorescence polarization assay

Binding of the stapled peptide to the REV1 CTD was carried out in triplicate as described before [Grossmann et al. , 2012]. Briefly, the stapled peptide in a concentration range of 10 nM to 50 nM was incubated with two-fold dilution of purified REV1 CTD in 300 mM NaCl, 25 mM Tris (pH 8.8), 2 mM DTT, and 2% glycerol in the 384-well black flat-bottom plates for 45 minutes. Fluorescence polarization was measured on Spectramax-M5 plate reader, with excitation wavelength of 490 and emission wavelength of 519. K_D (dissociation constant) values were calculated by non-linear regression analysis using Prism software (GraphPad).

ELISA

To test whether the stapled peptide was able to inhibit REV1's interaction with POL K, 10 picomoles of His tagged-REV1 was incubated in 200 μL of PBS/BSA (50 mM potassium phosphate, 150 mM sodium chloride, pH 7.2 with 0.2% bovine serum albumin) in each well of a 96 well NiNTA HisSorb plate, which was covered with a cover film for one hour at room temperature on a gentle rocker. Negative control wells received no His tagged-REV1. Next, the stapled peptide in a concentration range from 0 to 15 μM was added to the NiNTA HisSorb plate pre-incubated with REV1 and was incubated at room temperature with gentle shaking for 20 minutes. After this, 18 picomoles of FLAG tagged POL κ in 225 μL of PBS was added to the wells, covered with a cover film and incubated for 30 minutes at room temperature. Positive control wells did not receive the stapled peptide. Then, the cover film from the NiNTA plate is removed and the plate was washed four times with PBS-Tween (PBS with 0.05% Tween 20) by filling each well with 250 μL of wash solution, letting it sit for 10-60 seconds, dumping out the wash solution, and tapping the plate upside down on some paper towels to dry. Finally, 200 μL of anti-FLAG M2-HRP antibody was diluted to 1:20,000 in PBS/BSA and added to each well of the plate, which was sealed with a cover film and incubated for one hour at room temperature on a gentle rocker. The NiNTA plate was then washed four times with PBS-Tween as before and 200 μL of SureBlue TMB substrate was added to each well and incubated for 20 minutes. The reaction was stopped by adding 50 μL 1 M hydrochloric acid and the absorbance of each well is read within ten minutes in a Tecan 10M plate reader, 25°C, at an OD of 450 nm.

Cell Culturing

HT1080 cells (purchased from ATCC) were grown at 37 °C with 5% CO_2 in RPMI 1640 (Gibco), 10% (vol/vol) FBS (HyClone), and 1% Penicillin-Streptomycin antibiotic (Corning). MEFs (wild type and Rev1 knockout) [Jansen et al. , 2006] and KP ($Kras^{G12D;p53^{-/-}}$) cells (kindly obtained from Jackson Lab, MIT) were also grown at 37 °C with 5% CO2, but in DMEM (Gibco), 10% (vol/vol) FBS (HyClone), and 1% Penicillin-Streptomycin antibiotic (Corning). All cells were trypsinized using 0.25% Trypsin-EDTA (Corning) for passaging or experimentation.

Immunofluorescence microscopy

HT1080 cells were grown on poly-L-lysine coated coverslips in 24-well plates in complete media until they reached at least 60% confluence. Plates were washed twice with PBS and

then stapled peptide at 6 μM concentration in OptiMEM media was added to the wells for 18 hours. Next, cells on the coverslip were first fixed with methanol for 20 minutes at −20 °C, followed by their rehydration, by washing twice them with PBS. Then, cells were incubated in 70% ethanol for 10 minutes at 4 °C and washed twice with PBS. Finally, Prolong Gold antifade reagent with DAPI (4′, 6-diamino-2-phenylindole, Molecular Probes) was mounted on cells and the coverslips were sealed with nail polish. Foci were examined in a Nikon H600L microscope. Randomly selected fields from 3 independent coverslips were examined.

Flow cytometry

HT1080 cells were grown in complete RPMI 1640 media, washed twice with PBS and then exposed to 6 μM stapled peptide for 18 hours in OptiMEM media. Cells were trypsinized, washed twice with PBS and resuspended in 1ml paraformaldehyde in PBS overnight at 4 °C. Next, cells were pelleted and resuspended in 1ml, 1× PBS and were run through BD BioScience Flow cytometer. Percent stapled-peptide positive cells were calculated by dividing the number of green cells by the total number of cells that were run through the cytometer.

Cytotoxicity assay

Cytotoxicity was assessed by using the Cell-titer glo luminescence stain that monitors cell viability by quantifying the amount of ATP in the media, which is directly proportional to the number of metabolically active (viable) cells. Briefly, 5,000 cells were plated in complete media in each well of a 96-well plate for 24 hours, followed by the addition of increasing doses of cisplatin. The next day, serum-free OptiMEM media containing 6 μM stapled peptide replaces complete media and incubates over cells for 18 hours. Complete media inhibits translocation of the stapled peptide into cells, as the serum proteins tend to bind to the stapled peptide. Cell-titer glo Luminescence stain was then added to the equilibrated plates per the manufacturer's instructions and luminescence was measured on the Tecan Spark 10M plate reader. Relative luminescence, which indicates relative survival of metabolically active cells, was calculated by dividing the luminescence of treated samples with untreated controls.

HPRT mutagenesis assay

For the hypoxanthine-guanine phosphoribosyl transferase (HPRT) mutagenesis assay, HT1080 cells were first grown in HAT (complete media with 100 μM Hypoxanthine, 0.4 μM Aminopterin and 16 μM Thymidine) media for 14 days to remove spontaneous *Hprt* mutants. After HAT selection, cells were exposed to cisplatin at 0.6 μM concentration for 24 hours. Then, in OptiMEM media, stapled compound was added to cells. After 18 hours hours of stapled peptide treatment, cells were trypsinized and washed with PBS. 600 cells were plated in triplicate in 6-well plates to determine clonal efficiency from the cisplatin and stapled peptide treatment in a colony survival assay (supplementary figure 10). Rest of the cells were plated in complete media to recover for 8 days. Then, 500,000 recovered cells per treatment were plated in six 10 cm dishes in 6-TG media to allow proliferation of $HPRT^+$ cells. After 15 days, colonies were fixed (50% methanol and 10% glacial acetic acid) and stained (0.02% Coomassie brilliant blue R-250 stain in methanol: acetic acid: water in a

ratio of 46.5:7:46.5 (v/v/v)) counted after 14-20 days. The HPRT mutation frequency was calculated as a ratio of the number of HPRT mutants to the number of surviving colonies plated at the time of selection [Silva et al. , 2005].

Results and Discussion

We synthesized two stapled versions of the 10 amino acid POL κ RIR peptide (WT) by introducing pairs of μ-methyl,μ-pentenylglycine residues at two different *i* and *i*+4 positions [Walensky et al. , 2004], followed by ruthenium-catalyzed ring-closing olefin metathesis to form the staple (Staple A and B) (Fig. 1a). Additional stapled variants were constructed in parallel that contained alanines instead of the conserved phenylalanines (Staple $A^{FF\ to\ AA}$ and Staple $B^{FF\ to\ AA}$). FITC was added at the N-termini through a β-alanine linker (Supplementary Fig. 1).

Circular dichroism (CD) spectroscopy measurements of molecular ellipticity showed that Staple A and B, as well as their stapled variants, have minima at 208 and 222 nm consistent with an α-helical conformation, while the unstapled WT and $WT^{FF\ to\ AA}$ peptides exhibit minima around 200 nm, indicative of an unstructured peptide (Fig. 2a). Fluorescence polarization studies showed that Staple B peptide binds most efficiently to the REV1 CTD, followed by Staple A, and WT. The FF→AA derivatives of the stapled peptides interact much more weakly, indicating that Staple A and B are binding at the intended interface, while the $WT^{FF\ to\ AA}$ peptide does not bind detectably (Supplementary Fig. 2).

Noting that the preformed pocket in the Rev1 CTD might be able to bind a larger hydrophobic group, we replaced the conserved Phe578 residue with naphthylalanine (Staple P) and cyclohexylalanine (Staple K) (Fig. 1b). These second-generation stapled peptides showed a significantly enhanced binding to the REV1 CTD (Fig. 2b). In an ELISA assay, Staple K at IC50 (6.6 μM) successfully inhibited the REV1 CTD from interacting with POL κ protein, while the other peptides did not inhibit the interaction at the same concentration (Supplementary Fig. 3–6). This observation suggested that the Staple K could potentially inhibit the interaction of RIR-containing polymerases with REV1 in living cells.

We next tested the ability of the unstapled and stapled peptides to translocate into cells. At 6 μM concentration and 18-hour incubation in optimum media, Staple K formed discrete green foci in nuclei of cells, Staple P formed some foci and the unstapled and Staple B, failed to form visible foci (Fig. 3a, Supplementary Fig. 7). The translocation of the stapled peptide was further confirmed by FACS analysis, with *ca.* 85% of cells exhibiting FITC fluorescence under the same conditions (Fig. 3b).

Staple K had only a minimal effect on the viability of $Rev1^{+/+}$ and $Rev1^{-/-}$ MEFs (Mouse Embryonic Fibroblasts) (Fig. 4a and 4b). In contrast, Staple K sensitized $Rev1^{+/+}$ MEFs to killing by ultraviolet (UV) light but had no effect on the sensitivity of $Rev1^{-/-}$ MEFs to UV radiation (Fig. 4a and 4b), an important observation indicating that Staple K increases cell death in response to DNA damage by interfering with Rev1 function rather than by an off-target effect. Consistent with this conclusion, Staple K also sensitizes MEFs to killing by three additional DNA damaging agents—cisplatin, BPDE and MMS—that are known to

cause greater killing of REV1-deficient cells (Supplementary Fig. 8). The Staple K peptide also sensitizes two additional cancer cell lines to killing by the widely used chemotherapeutic agent cisplatin (Supplementary Fig. 9).

Importantly, our observation that Staple K additionally decreases the frequency of HPRT mutagenesis in response to cisplatin treatment indicates that Staple K is acting at least in part by interfering with the REV1/POL ζ_4-dependent pathway of mutagenic TLS (Fig. 4c). It seems most likely that the effect is due to the stapled POL κ RIR peptide preventing both the RIR containing polymerases, including POLD3, a component of POL ζ_4, from interacting with the RIR interface of the Rev1 CTD. Our study describes an example of a class of drug to inhibit Rev1-dependent mutagenic translesion synthesis that could potentially be used as an adjuvant to improve DNA damaging chemotherapy by increasing cell killing while reducing acquired resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Financial Interests: G.L.V. has equity stake in Hydrocarbon-stapled peptide technology licensed to Aileron Therapeutics.

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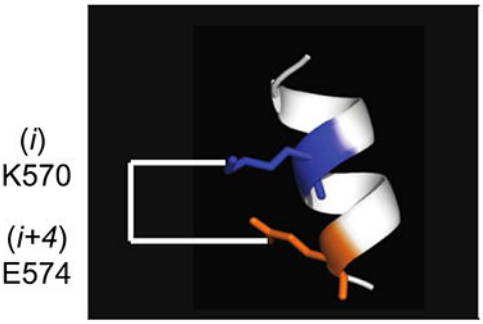
A

	566									575
Wild Type	S	F	F	D	K	K	R	S	E	R
Staple A	S	F	F	*	K	K	R	*	E	R
Staple B	S	F	F	D	*	K	R	S	*	R
Wild Type$^{FF\ to\ AA}$	S	A	A	D	K	K	R	S	E	R
Staple A$^{FF\ to\ AA}$	S	A	A	*	K	K	R	*	E	R
Staple B$^{FF\ to\ AA}$	S	A	A	D	*	K	R	S	*	R



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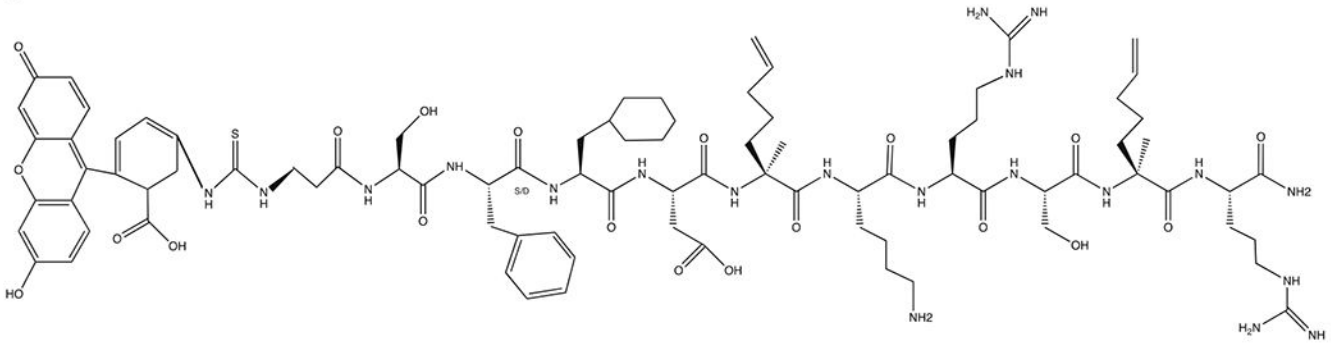

Figure 1:

Construction and characterization of POL κ RIR Stapled peptides. (a) Sequences of wild type, Stapled A, Stapled B peptides and their corresponding Phe to Ala mutants (in blue). Red periods indicate location of staple, which confers helicity shown on right. (b) Unstapled structure of stapled peptide K, showing location of cyclohexylalanine.

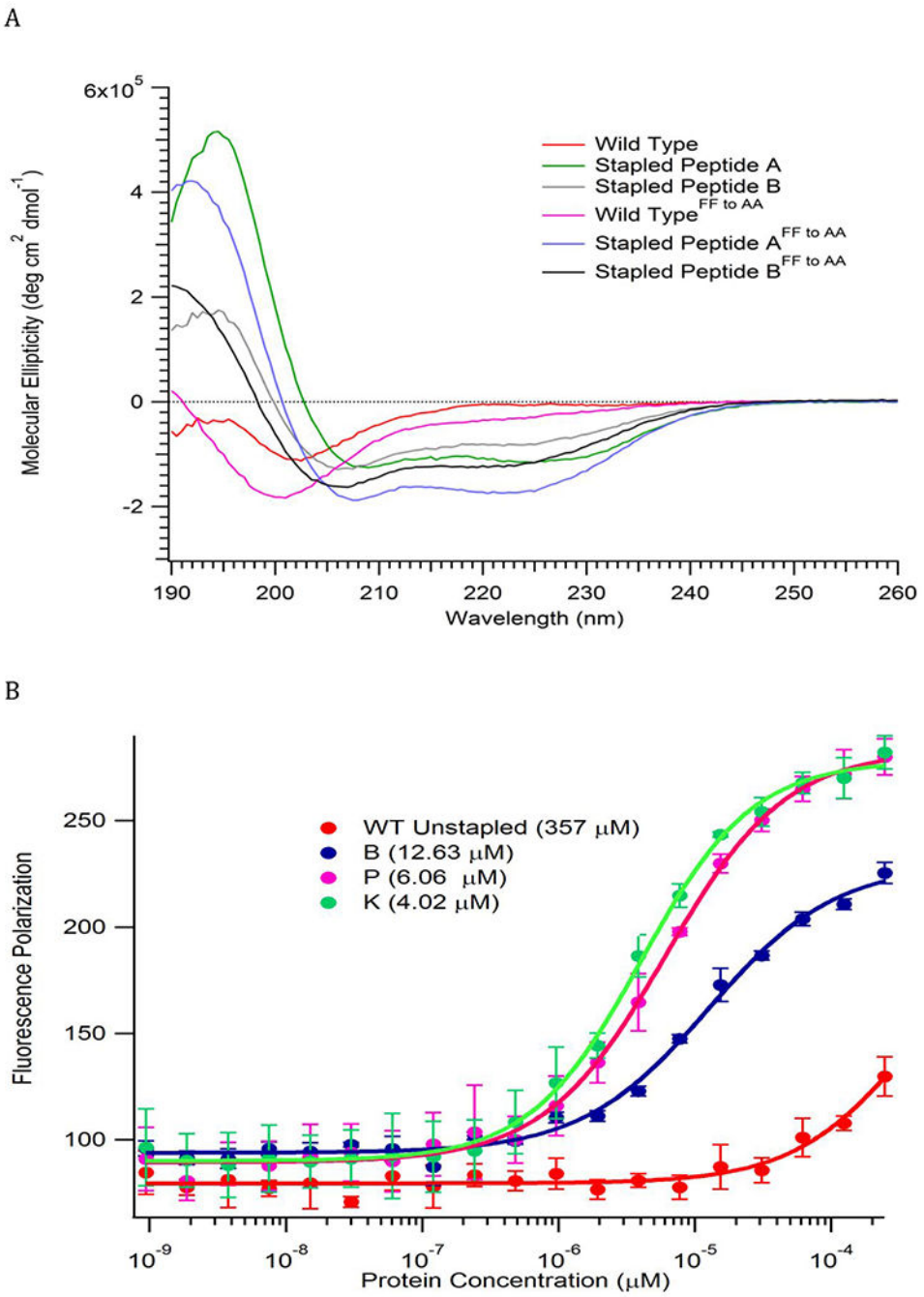

Figure 2:

Characterization of POL κ RIR Stapled peptides. (A) CD analysis reveals enhanced α-helicity of stapled versus the unstapled peptides (B) Stapled peptides K and P bind REV1 CTD with highest affinity in a fluorescence polarization assay.

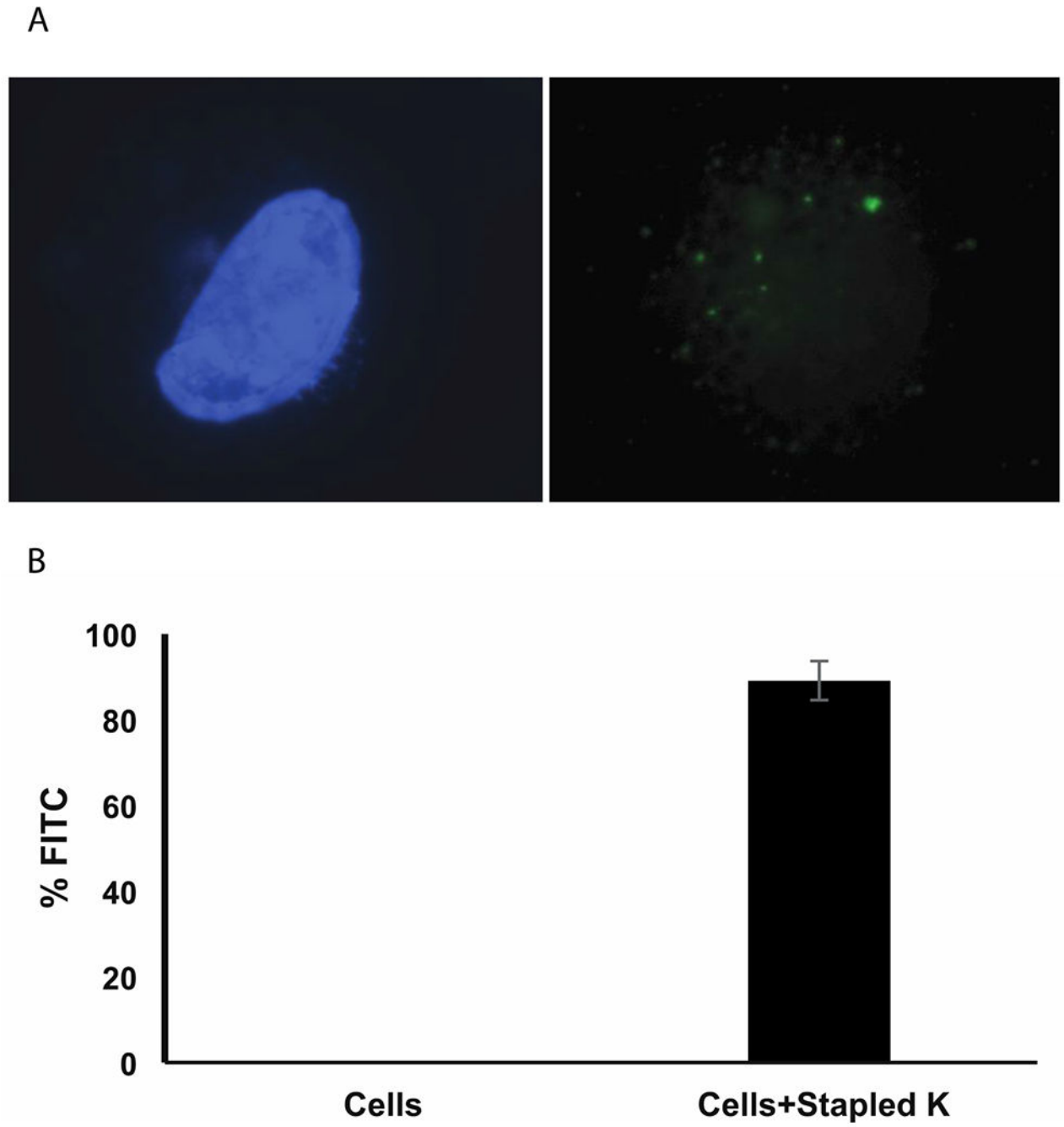

Figure 3:

Stapled peptide K translocates into human cells in serum free media (A) Discrete foci formation of Stapled peptide K in HT1080 cells. Left panel shows blue DAPI (4',6-diamidino-2-phenylindole) stained nuclei. Right panel shows green FITC (Fluorescein-5-isothiocyanate)-labeled stapled peptides as green foci. (B) Percentage of FITC positive HT1080 cells quantified in a flow cytometer.

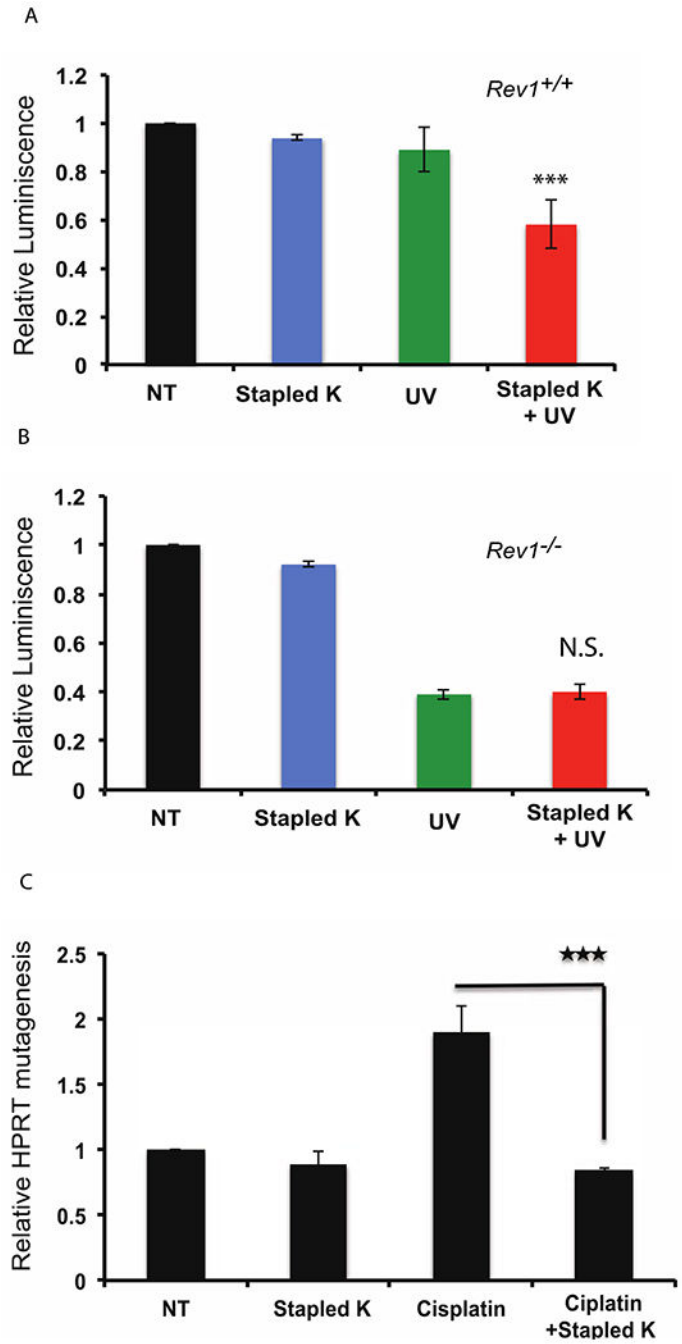

Figure 4:

Stapled peptide K peptide enhances cytotoxicity in human cells (4A-4B). Stapled peptide K has minimal effect on *Rev1*$^{+/+}$ (Fig. 4 A) and *Rev1*$^{-/-}$ (Fig. 4B) MEF cells, but increases UV-induced cytotoxicity only in *Rev1*$^{+/+}$ cells (Fig. 4A) in a Cell Titer Glo (CTG) assay. 4C shows relative increase in *HPRT* mutagenesis in HT1080 cells. n=6; error bars represent SD; statistical significance was determined by Student's two-tailed *t* test: *** $P<0.001$.