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SPRTN Metalloprotease as a Target to Sensitize Cancer Cells to Crosslinking Chemotherapy

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Vitae

Cole J. Turner was born on September 23, 2002, to parents Lori and Chris Turner. He was raised in Peterborough, New Hampshire, where he graduated Contoocook Valley Regional High School in 2020. Cole entered Colby College as a part of the first cohort of the Pulver Science Scholars program in Fall 2020 and began conducting research in the Rice Lab studying DNA-protein crosslinks during his freshman summer on campus. Cole continued his work in the Rice Lab throughout nearly all ensuing semesters on campus, but also pursued an off-campus research opportunity his sophomore summer as a SURPH Fellow at Duke University studying bone fracture repair in the Alman Lab and curated an independent research opportunity at Geisel School of Medicine at Dartmouth in the Mabaera Lab investigating a negative checkpoint in cancer immunology his junior summer. Outside research, Cole was passionately involved in the Colby Club Hockey team as a long-standing member of the leadership team, worked in Colby Sports Medicine as an Athletic Training Student Worker, and volunteered as a Colby Cares About Kids mentor at Benton Elementary School. Cole will graduate with a Bachelor of Arts in Chemistry-Biochemistry in May 2024. After graduating, Cole will be pursuing post-baccalaureate research in the biomedical field as he prepares to apply for graduate school.

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Abstract

Bifunctional alkylating agents have demonstrated high clinical utility as a chemotherapeutic strategy against cancer. These compounds have been well-characterized for their capacity to exert cytotoxicity via interstrand DNA crosslinking. However, the same electrophilic chemistry can also form DNA-protein crosslinks (DPCs) whose contributions to these drugs' antitumor effects have been less well-defined. Recently, the metalloprotease SPRTN has been implicated as a predominant mediator of DPC repair in mammalian cells. Previous work has demonstrated that SPRTN deficiency increases the sensitivity of cancer cells to DPC-inducing agents such as formaldehyde and cisplatin, but these findings have yet to be translated more broadly across chemotherapies capable of inducing these lesions. Herein, we investigated the effects of impairing SPRTN repair on cancer cells' sensitivity to bifunctional alkylating agents. First, we employed RNA interference to transiently knock down SPRTN expression in HeLa cells by over ten-fold relative to non-targeting controls. Using this SPRTN knockdown model, we then demonstrated a 2.4-fold decrease in the LD_{50} of mechlorethamine relative to control experiments. However, we observed no difference in DPC burden between SPRTN knockdown and control HeLa cells after treatment with a highly lethal dose of mechlorethamine and recovery over an extended period. Our preliminary data suggest a role for SPRTN in HeLa cells' response to bifunctional alkylating agents, but more work is necessary to validate these propositions. Nonetheless, this study may support that inhibition of SPRTN in combination with crosslinking chemotherapies could warrant further investigation as a therapeutic strategy for the treatment of certain cancers.

Introduction

Chemotherapeutic strategies have evolved greatly over the past few decades for clinical intervention against cancer. Today, more than 600 chemotherapy drugs are recognized by the National Cancer Institute for treatment of cancer.[1] Shared across all drug profiles is the aim of reducing tumor growth and survival, which is accomplished by interfering with one or more of the classical hallmarks that define cancer: uncontrolled proliferation, evasion of apoptosis, production of internal growth signals, blockage of external anti-growth signals, invasion of new tissue, and sustained angiogenesis.[2] Activities that counteract these processes are critical to the anticancer action of these compounds; however, the same mechanisms can also contribute to adverse effects in healthy tissues associated with the higher doses required of these drugs for antitumor efficacy.[3]

To reduce adverse effects as well as overcome challenges of chemotherapeutic resistance, strategies of pairing drug treatments together as combination therapies have taken great precedence in the clinic. Since 2007, the proportion of monotherapies in active clinical trials has dropped from 70% to less than 30% in 2021, highlighting a universal shift towards combination therapy development.[4] These strategies are designed to pair drugs whose mechanisms of action may add or synergize to inflict more significant toxicity to cancer cells while requiring less concentrated doses of individual drug, thus reducing patient side effects.[3,5] For instance, gemcitabine, an antimetabolite drug that impedes DNA polymerization, combined with cisplatin, a bifunctional alkylating-like agent that damages DNA, has become a standard treatment regimen for advanced-stage lung, bladder, cervical, pancreatic, and ovarian cancers.[6] These compounds have demonstrated synergy likely through a mechanism of impaired DNA polymerization from gemcitabine impacting the repair of cisplatin-induced DNA damage, which effectively reduces the drug doses required for cancer cell death.[7]

Bifunctional alkylating agents are one class of chemotherapies that have been commonly employed in combination therapy regiments.[8] These compounds function by damaging DNA to disrupt the uncontrolled proliferation and apoptotic evasion of cancer cells. There are several subclasses of bifunctional alkylating agents based on varying structural motifs and corresponding decomposition schemes, which include the nitrogen mustards such as mechlorethamine and bendamustine, alkyl sulfonates such as busulfan, nitrosoureas such as carmustine and lomustine, and hydrazines such as laromustine.[9] Note that platinum-based chemotherapy agents such as cisplatin and carboplatin also exhibit similar DNA-damaging function to the alkylating agents, but contain a platinum rather than organic backbone central to their reactivity.[9–11] Collectively, these compounds are characterized by two electrophilic moieties that can each react with a nucleophilic site of a biomolecule to form a crosslinked product.[9] These covalent attachments occur via either an SN1 intermediate-forming process, which are characteristic to nitrogen mustards and nitrosoureas, or an SN2 direct displacement of the leaving group, which are observed for some alkyl sulfonates and hydrazines.[9,12]

The high clinical utility of bifunctional alkylating agents in inducing cancer cell death has largely been attributed to their well-established reactivity with nucleophilic positions on nitrogenous bases of DNA to form covalent interstrand crosslinks (ICLs). ICLs exert their cytotoxicity by interfering with DNA strand separation necessary for gene transcription and DNA replication[13], which are especially critical to cancer proliferation and thus contribute significantly to observed antineoplastic activity. Bifunctional alkylators from different subclasses exhibit preferences for the nitrogenous base they target and nucleophilic position they react with.[14,15] Several works have demonstrated that nitrogen mustards[15–17] and platinum-based agents[10,15] preferentially alkylate the N7 position of guanine to form guanine-guanine crosslinks. In contrast, nitrosoureas such as carmustine and the sulfonylhydrazine laromustine display an interstrand crosslinking mechanism

through the O6 chloroethylation of guanine proceeded by alkyl cyclization to N1 and crosslinking to the N3 position of its base pair cytosine (Figure 1).[15,18]

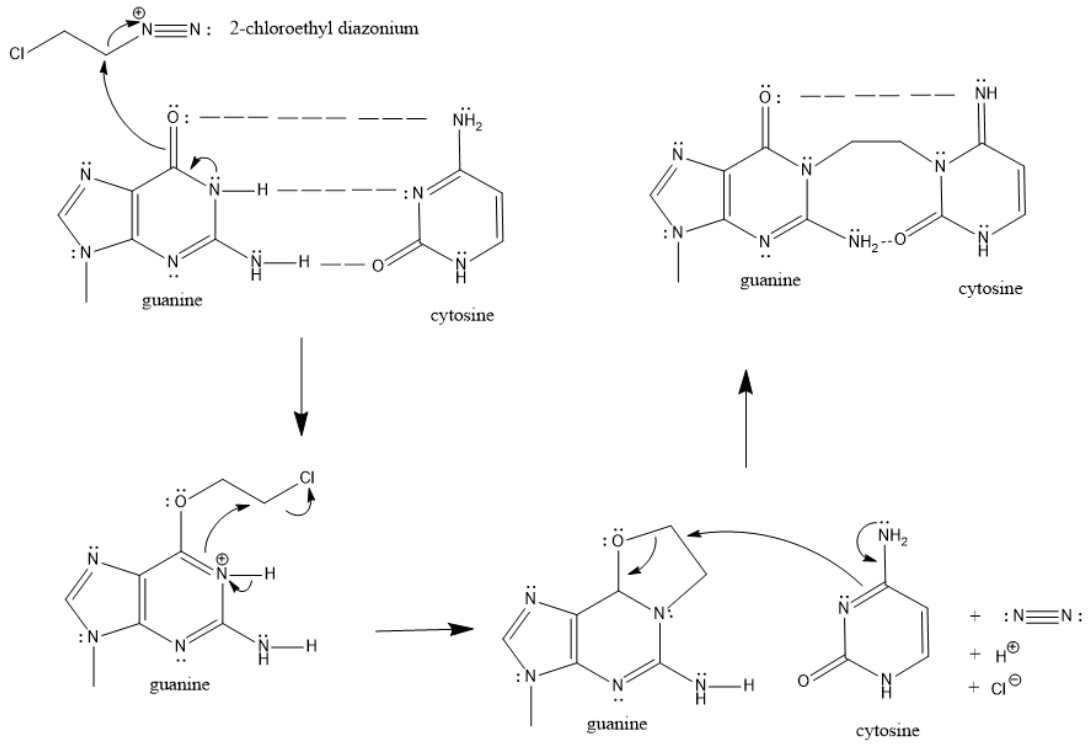

Figure 1. 2-chloroethyl diazonium, the reactive subspecies generated upon the *in situ* decomposition of the sulfonylhydrazine prodrug laromustine, undergoes an SN2 nucleophilic attack process at the O6 position of guanine, which cyclizes to the N1 position upon leaving group ejection and forms an N1 guanine – N3 cytosine interstrand crosslink.

The reactivities of bifunctional alkylating agents through these mechanisms are not exclusive only to nucleophilic positions of DNA. A large body of evidence demonstrates that many of these compounds are also capable of crosslinking DNA to chromatin-associated proteins.[11,19–23] Although these lesions are less well-understood than ICLs, it is proposed that they occur through a similar DNA alkylation mechanism described above at one electrophilic moiety of the compound while interacting with a nucleophilic protein residue in close proximity at the other electrophilic position, forming a covalent DNA-protein crosslink (DPC).[15] The entrapment of a bulky protein

crosslinked to DNA can obstruct critical DNA metabolic processes, including DNA transcription and replication similar to ICLs but also DNA repair mechanisms intended to reverse these damages.[21] Thus, DPCs are considered a highly cytotoxic form of DNA damage that may contribute to the anticancer potential of bifunctional alkylating agents.[21]

DNA-protein crosslinks are also greatly relevant outside the context of bifunctional alkylating agents. Several endogenous and environmental effectors have been demonstrated to induce DPCs, including formaldehyde as an essential metabolic byproduct and free oxygen radicals generated from ultraviolet radiation.[20,21] Formaldehydes have been characterized extensively for their DNA-protein crosslinking capacity, which is highlighted by preferential reactivity for linking guanine DNA bases to nucleophilic lysine and cysteine amino acid residues.[24] In addition to being present at concentrations of up to 100 μM in human blood[25], formaldehyde is a notable byproduct of histone demethylation that occurs at the DNA-protein interface.[26] Consequently, it has been demonstrated that that endogenous, formaldehyde-induced DPCs are formed in detectable quantities in human cells.[27]

To mitigate the cytotoxic effects of endogenous DPCs, cells have adapted multiple mechanisms of DPC repair. Given their profound relevance to cell survival, these processes have garnered significant interest in recent years, but a comprehensive understanding of their interplay and regulation still requires further development. Early works first suggested that DPCs can be resolved through canonical DNA repair pathways of nucleotide excision repair (NER) and homologous recombination (HR), which broadly function via nuclease activities to excise DNA regions containing DPCs and replace them with non-crosslinked replicates.[28] However, NER and HR are initiated under different conditions of DNA damage and are mechanistically distinctive. In brief, NER occurs upon the protein factor XPC recognizing a distortion in double stranded DNA (dsDNA) at the site of the DNA lesion and recruiting a repair complex chain, which has

endonuclease activity that cleaves 5' and 3' to the lesion and DNA polymerization/ligation activity that restores the original dsDNA template.[29] NER is characterized by its role in repairing moderately bulky DNA adducts in comparison to the substrates of base excision repair. In the context of DPCs, NER is only capable of excising small crosslinked proteins with a mass of 16 kDa or less, which limits its capacity for contributing to DPC repair.[28] In contrast, HR occurs as a replication-dependent repair process in response to induced double strand breaks.[30] Mechanistically, an enzyme complex containing Mre11 and Rad51 is recruited to the site of damage, which enables nucleolytic resection of the DPC-containing region and homologous DNA strand recognition, respectively, to facilitate DNA restoration.[30,31] Surprising, recent work investigating these canonical DNA repair pathways in DPC repair found that knockdown of key components of NER and HR did not increase DPC formation in HeLa cells compared to wildtype controls.[32] These minimal changes in DPC yield demonstrated that neither NER nor HR are the predominant mechanism for DPC repair, which instead suggests that an alternative pathway may be largely responsible for resolving DPC damage.

A DPC repair pathway that proceeds with proteolytic rather than nucleolytic cleavage, termed DNA-protein crosslink proteolytic repair (DPC-PR), has become widely accepted in recent years as a primary mechanism through which eukaryotes repair these lesions.[20,26,28,32,33] Central to the function and coordination of DPC-PR in higher eukaryotes is SPRTN, a 55 kDa metalloprotease that is responsible for specific recognition and cleavage of DPCs during DNA replication.[26] The gene that encodes this protease, *SPRTN*, is conserved across metazoans and shares 24% identity with the yeast functional homolog Wss1.[33] Structurally, SPRTN consists of an N-terminus SprT protease domain, a single-strand DNA (ssDNA) binding Zinc Binding Domain (ZBD), a dsDNA binding Basic Region (BR) domain, and several protein binding domains including a ubiquitin binding domain (UBZ) located near the C-terminus.[26] These domains are critical to the specific activity and tight regulation of SPRTN, which are discussed below.

Functionally, the catalytic center of the SprT proteolytic active site lacks a sequence-specific binding pocket, which requires that SPRTN is tightly controlled by other modes of regulation to ensure its DPC-cleaving specificity.[20] Recent works have suggested that SPRTN exhibits three main regulatory mechanisms: post-translational modifications controlling recruitment and retention at chromatin, a DNA binding switch responsible for activating the SprT protease domain, and autocleavage activity to deactivate DPC-PR (Figure 2).[26,34] SPRTN is maintained in an inactive, 'closed' conformation by monoubiquitylation of its UBZ domain.[35] Deubiquitylation of SPRTN via the deubiquitylating enzyme VCPIP enables conformational opening, which paired with ensuing acetylation by PCAF or GCN5 acetyltransferases and phosphorylation by the replication stress response kinase CHK1, increases recruitment of SPRTN to chromatin.[35–37] At the chromatin, the SprT protease domain is held conformationally inactive by the DNA switch until the ZBD binds to ssDNA concurrent to the BR domain binding dsDNA, which is characteristic to the ssDNA/dsDNA junction of a replication fork stalled by the presence of a DPC.[26,33] It is proposed that ssDNA binding by the ZBD induces a conformational change that activates the SprT protease domain for DPC-localized, sequence-non-specific cleavage.[35] Note that this proteolysis does not impart any nucleolytic activity to remove the crosslink to DNA itself. Therefore, further processing of the small remaining crosslinked adduct by canonical DNA repair mechanisms such as NER is necessary to complete DPC resolution. Finally, to prevent aberrant degradation of proteins closely associated to the chromatin, SPRTN undergoes a negative feedback loop of autocleavage to render itself inactive.[35]

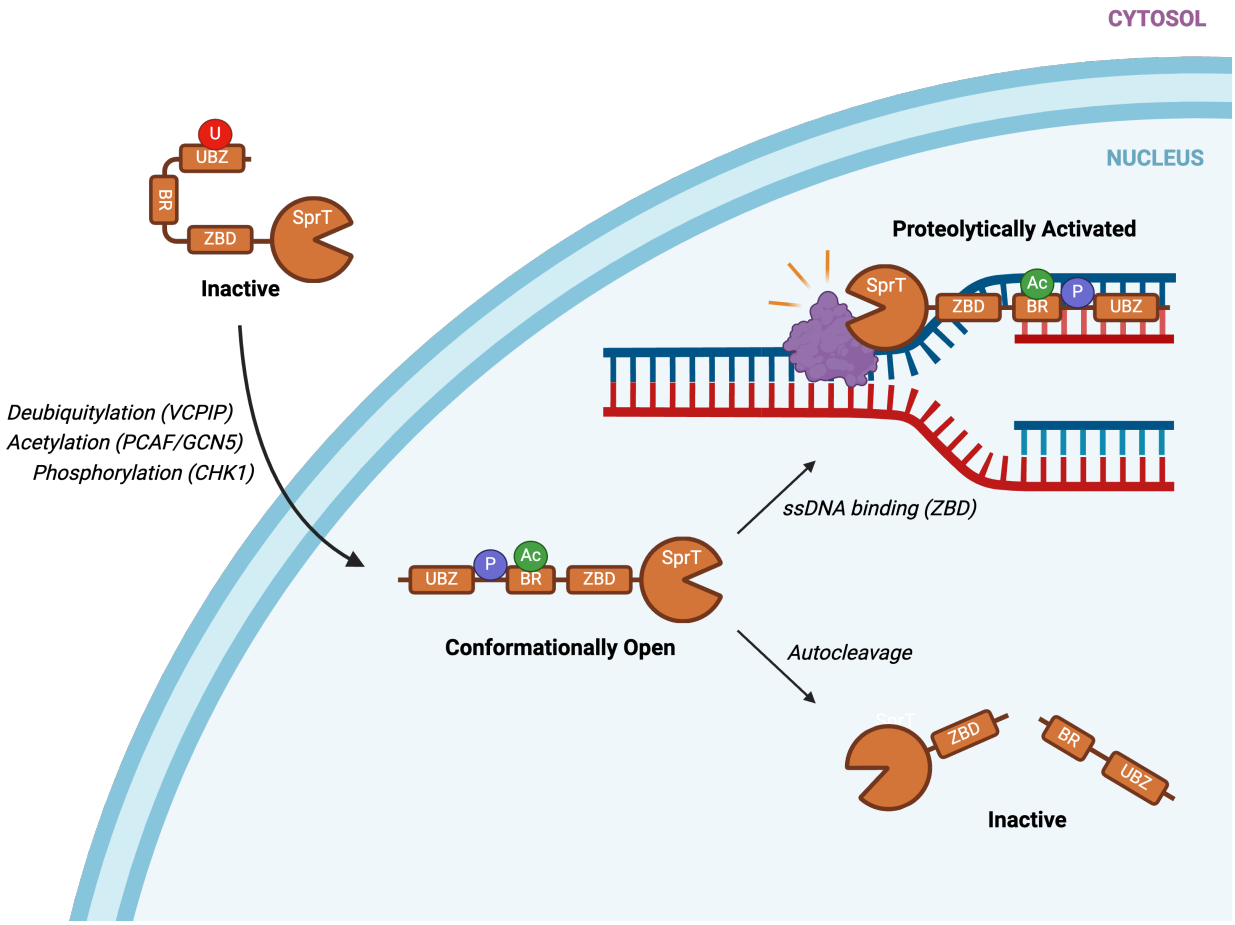

Figure 2. A series of regulatory events control the activation and specificity of SPRTN for removing DPCs. *Adapted from Ruggiano and Ramadan*[34] *and created with BioRender*.

A compelling body of evidence supports that SPRTN function is critical to DPC removal and thus cellular homeostasis. Several studies have indicated that decreases in cellular SPRTN increased the prevalence of DPCs formed both under endogenous conditions and in response to crosslinking agents such as formaldehyde.[32,35,38] Accordingly, cell survival is markedly diminished in the same SPRTN-knockdown models treated with formaldehyde in comparison to SPRTN-proficient controls receiving the same treatments.[35,38] Moreover, genetic ablation of *Sprtn* was found to be embryonically lethal in mouse models, and inducible *Sprtn* knockout in murine fibroblast cells significantly reduced cell proliferation and increased cell death.[39] These findings collectively indicate that SPRTN plays an essential role in overcoming the cytotoxicity of DPCs.

Based on its key activity in DPC resolution and prominent regulation scheme, SPRTN is of particular interest in the context of cancer. As a repair mechanism highly relevant during DNA replication, SPRTN-mediated DPC-PR may be critical for supporting proliferation and survival of cancer cells constitutively exposed to mutagenic stress. Interestingly, an increase in SPRTN expression was observed in HeLa cells in response to treatment with formaldehyde.[38] This finding suggests that SPRTN may be employed by cancer cells as a mechanism of defense against DNA-protein crosslinking stress.

Given the capacity of bifunctional alkylating agents to induce DPCs as a potential mode of anticancer activity, we suspect that SPRTN may function in cancer as a mechanism of resistance to reduce the cytotoxicity of these crosslinking chemotherapies. Herein, we demonstrate that knockdown of SPRTN sensitized cancer cells to treatment with bifunctional alkylating agents. A transient SPRTN siRNA knockdown (siSPRTN) was employed to reduce SPRTN expression, which was confirmed using RT-qPCR. Next, cell survival and proliferation in siSPRTN HeLa cells treated with mechlorethamine (HN2) were evaluated in comparison to drug-treated, non-targeting siRNA-transfected control cells using clonogenic assays. We found that siSPRTN decreased the LD_{50} concentration of HN2 by 2.4-fold in comparison to controls, which supported a model of SPRTN knockdown mediating sensitization to crosslinking chemotherapy. Finally, we quantified the accumulation of DPCs by HeLa cells transfected with siSPRTN after treatment with high doses of mechlorethamine using the ARK assay.[40] More comprehensive measurements of DPC burden paired with exhaustive cell survival characterization in these models should enable us to confirm that SPRTN knockdown synergizes with the DPC inducing activity of bifunctional alkylating agents to decrease cancer growth. At large, the implications of our findings could suggest that SPRTN-targeted therapy paired with DPC induction via crosslinking agents could be a novel combination therapy strategy to improve the effectiveness of these treatments for cancer.

Materials and Methods

Reagents

Gibco Roswell Park Memorial Institute 1640 (RPMI-1640) medium, Gibco Dulbecco's Modified Eagle Medium (DMEM) growth medium containing high glucose (4.5 g/L) and sodium pyruvate (110 mg/L), Gibco Opti-MEM reduced serum medium, Gibco DPBS without calcium or magnesium, RMBio FetalGro bovine growth serum (FBS), Gibco Penicillin-Streptomycin (10,000 U/mL), Invitrogen Lipofectamine RNAiMAX, Quant-iT PicoGreen dsDNA assay kit, and fluorescein isothiocyanate (FITC) were obtained from Thermo-Fisher (Rockport, IL). BioWhittaker L-Glutamine (200 mM) was obtained from Lonza (Walkersville, MD). Mechlorethamine hydrochloride, crystal violet, and KiCqStart Probe Assays for SPRTN and GAPDH conjugated to 6FAM and HEX fluorophores, respectively, were obtained from Sigma-Aldrich (St. Louis, MO). SPRTN TriFECTa RNAi Kit and PrimeTime One-Step RT-qPCR Master Mix were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Zymo Direct-zol RNA MiniPrep Kit with Tri Reagent was obtained from Zymo Research (Irvine, CA). Buffer RLTplus was obtained from Qiagen (Germantown, MD).

Cell Culture

Henrietta Lacks (HeLa) cervical cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5g/L) and sodium pyruvate (110mg/L) supplemented with 10% fetal bovine serum (FBS), L-Glutamine (2mM), and Penicillin-Streptomycin (100U/mL) at 37°C with 5% CO_2 and 100% relative humidity. HeLa cultures were passaged regularly prior to reaching full confluence, in which cells were washed three times with DPBS and treated with 0.25% trypsin-EDTA in Hank's Salts for 3-5 min at 37°C before neutralization in five volumes of complete DMEM and resuspension in fresh DMEM for plating to appropriate density.

RNAi knockdown of SPRTN in HeLa cells

HeLa cells were transfected with siRNA via lipofection using Lipofectamine RNAiMAX reagent following manufacturer's protocols. In brief, HeLa cells were seeded to reach 60-80% confluence overnight and treated with 1 nM of hs.SPRTN.13.2 or non-targeting dsiRNA duplexes complexed with Lipofectamine RNAiMAX in Opti-MEM reduced serum growth medium for 48 hr prior to gene expression analyses or reseeding for downstream applications.

RT-qPCR validation of SPRTN-KD

RNA was extracted from untreated and siRNA-transfected HeLa cells 48 hr post-treatment using Zymo Direct-zol RNA MiniPrep Kit with Tri Reagent following manufacturer's protocols. In brief, HeLa cells were lysed in-well with Zymo Tri Reagent and RNA was isolated from lysates via sequential spin-column separations with proprietary RNA washes flanking DNase I treatment. The yield and purity of RNA eluants in nuclease-free water were assessed on a Thermo Scientific Nanodrop One Microvolume UV-Vis Spectrophotometer. For RT-qPCR, RNAs from biological triplicates of each treatment group were diluted to concentrations consistent with 100ng for each technical triplicate. Reaction mixtures were prepared in 2X IDT PrimeTime One-Step RT-qPCR Master Mix with KiCqStart dual-label probe and primer oligos at 200 nM and 300 nM, respectively, for SPRTN and GAPDH (Sigma-Aldrich). RT-qPCR was performed using a Bio-Rad CFX Opus 96 Real-Time PCR System programmed according to manufacturer's protocol for the one-step reaction: 50°C for 15 min, 95°C for 3 min, and 40 cycles of 95°C for 5 s followed immediately by 60°C for 30 s. SPRTN gene expression was normalized to GAPDH expression per sample and presented relative to SPRTN expression of non-transfected HeLa cells.

Cell survival assays

Clonogenic cell proliferation assays were used to assess HeLa cell survival for dose response curves against mechlorethamine treatments and in combination with SPRTN knockdown. In brief, HeLa cells were seeded overnight at a density of 2,000 cells/well and treated with 0 μM, 0.5 μM, 1 μM, 2 μM, 10 μM, 50 μM, or 100 μM of mechlorethamine hydrochloride. Aliquots of 20 mM, 0.5 mM, and 0.25 mM aqueous stock solutions of mechlorethamine were used for treatments, which were prepared several months prior from an aqueous 200 mM mechlorethamine stock solution and stored at -20°C until use. The range of mechlorethamine doses was selected based on previous clonogenic assays performed by our group and validated using wild type HeLa cell survival experiments. Media were replaced after 3 hr drug treatments on day 1 and again on day 4. On day 7, cells were washed with cold PBS prior to fixation with methanol/acetic acid (7:1) and remaining colonies were stained with 0.5% crystal violet for 2 hr. Colonies were imaged using an Azure Biosystems 600 Imaging System and clonogenicity was quantified as the blank-adjusted average absorbance at 590 nm across a 37 pt WellScan for each sample, representative of crystal violet staining distribution and intensity, using a Molecular Devices SpectraMax M5 Microplate Reader. LD_{50} concentrations for SPRTN knockdown and non-targeting control experimental groups treated with mechlorethamine were calculated by fitting the two-parameter regression, $y = \frac{1}{1+(\frac{x}{LD_{50}})^a}$, to the experimental data using optimizations for the LD_{50} and Hill coefficient (a).

ARK assay for DPC extraction

HeLa cells were transfected for RNAi knockdown of SPRTN as described above, and 48 hr post-transfection, were treated with 0 μM or 100 μM of 20 mM mechlorethamine (detailed above) for 3 hr before incubation at 37°C for 16 hr. For isolation of DPCs, the ARK assay was performed as described previously.[40] In brief, cells were lysed in Buffer RLTplus pre-warmed to 55°C for 10 min,

lysates were sheared with an 18-gauge needle six times, and DNA was precipitated in 50% chilled ethanol prior to centrifugation at 21,000 x g for 20 min at 4°C to recover free DNA and DPCs. Then, pellets were washed in 20 mM Tris–HCl (pH 6.5), 150 mM NaCl, and 50% ethanol before being dissolved in pre-warmed 1% SDS, 20 mM Tris-HCl (pH 7.5) and incubated at 42°C for 6 min. Samples were sheared with a 26-gauge needle five times and an equal volume of 200 mM KCl, 20 mM Tris-HCl (pH 7.5) was added to precipitate SDS-bound proteins and DPCs. After chilling on ice for 6 min, samples were centrifuged at 21,000 x g for 5 min at 4°C to pellet the protein precipitate, and supernatants were collected as containing free DNA. Twice over, protein pellets were washed with 100 mM KCl and 20 mM Tris-HCl (pH 7.5) buffer, incubated at 55°C for 10 min, chilled on ice for 6 min, centrifuged at 20,000 x g for 5 min at 4°C, and supernatants were collected as containing free DNA. Washed pellets were dissolved in proteinase K buffer (100 mM KCl, 20 mM Tris-HCl, pH 7.5, and 10 mM EDTA) and incubated with 0.2 mg/ml proteinase K for 45 min at 55°C. Digestions were chilled on ice for 6 min before undergoing centrifugation at 20,000 x g for 10 min at 4°C to pellet debris. The supernatant containing DPC-associated DNA was collected separately, and 8 μL from the 4mL of recovered free DNA and 50 μL from the 1 mL supernatant of DPC-associated DNA were diluted to 100 μL for analysis using Quant-iT PicoGreen dsDNA assay following manufacturer's protocols. In brief, 100 μL of sample were incubated at room temperature with 100 μL of 1x PicoGreen working solution for 5 min and fluorescence emission at 520 nm was measured from excitation at 480 nm. Percent DNA bound to protein as a measure of DPC content was calculated for each sample by scaling blank-adjusted fluorescence signals with their corresponding dilution factor, and averages from technical triplicates for each biological triplicate were presented.

Results

Transfection with SPRTN-targeting siRNA significantly reduced SPRTN expression in HeLa cells

To develop a model for impairing the capacity of cancer cells to perform DPC proteolytic repair, we sought to target SPRTN as the key mediator of this process. Given that genetic knockout of *SPRTN* is embryonically lethal[39], we employed RNA interference (RNAi) to transiently knock down SPRTN expression as a strategy consistent with that of several previous reports.[32,38,41] Henrietta Lacks (HeLa) human cervical cancer cells were selected as the model cell line for investigation based on their precedent as being easily transfected with SPRTN siRNA by lipofection.[32,38,41] To confirm that siRNA is delivered into HeLa cells sufficiently via this route, we assessed transfection efficiency 24 hr post-treatment using Lipofectamine RNAiMAX complexes containing a fluorescent TYE 563-labeled, non-targeting control dsiRNA (Figure 3A). By overlaying Cy3 fluorescence indicative of TYE 563 with brightfield images of HeLa cell morphology, we qualitatively demonstrated TYE 563 internalization across a vast majority of cells transfected with Lipofectamine-TYE 563, which is consistent with a high transfection efficacy and thus suitable for RNAi-mediated knockdown (Figure 3A).

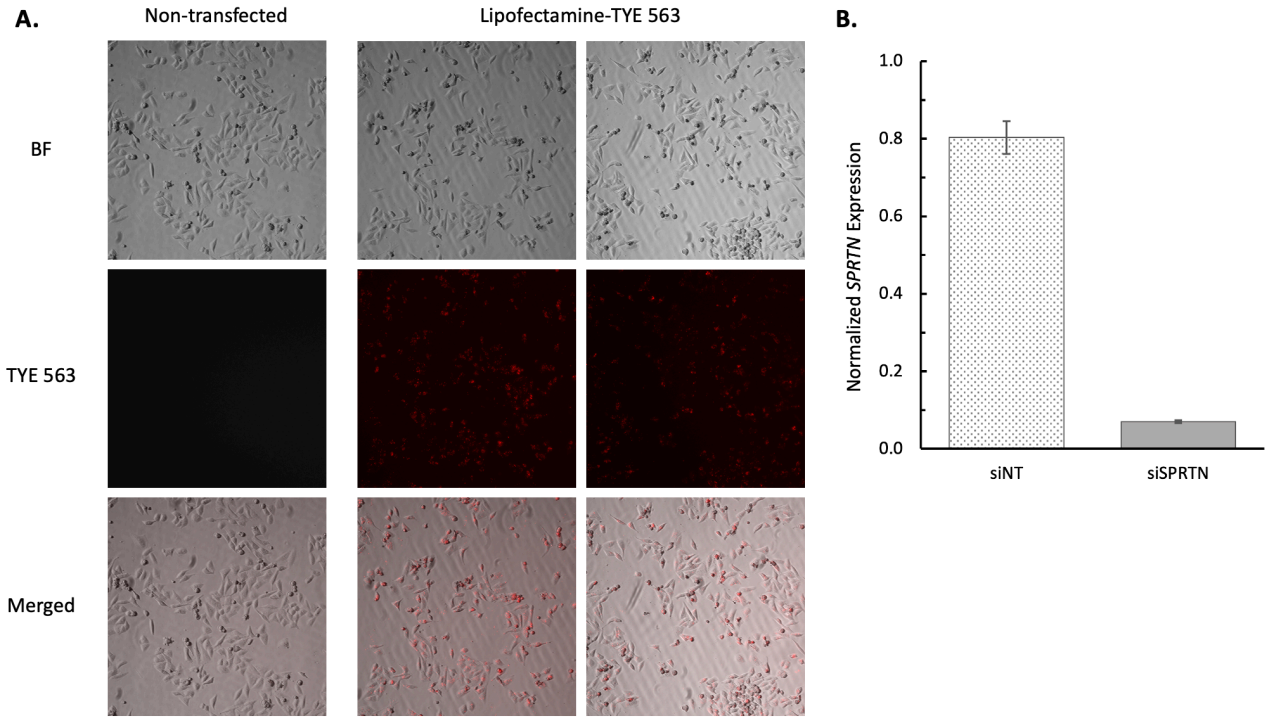

Figure 3. Transfection with SPRTN-targeting siRNA induced successful knockdown of *SPRTN* expression. **A.** Representative brightfield and fluorescence images of HeLa cells 24 hr after lipofection using Lipofectamine RNAiMAX complexed with 10 nM of fluorescent TYE 563-labeled control dsiRNA (n=2 per group). **B.** Gene expression measured via RT-qPCR of *SPRTN* normalized to *GAPDH* expression in HeLa cells transfected 48 hr prior with 1 nM of non-targeting (siNT) or SPRTN-targeting (siSPRTN) siRNA. Error bars represent SEM for biological triplicates for siNT and biological duplicates for siSPRTN.

Using our validated transfection method, we next aimed to confirm that SPRTN-targeting siRNA, henceforth referred to as siSPRTN, can successfully knock down *SPRTN* expression in HeLa cells. For gene expression analysis via RT-qPCR, we elected to use *GAPDH* as a reference gene to normalize *SPRTN* expression across samples in accordance with previous work.[35] The *GAPDH* gene encodes for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, which is ubiquitously expressed across identical cell types and therefore commonly used for gene expression normalization.[42] Additionally, to ensure that changes in normalized *SPRTN* expression were attributable to the specificity of siSPRTN for SPRTN mRNA and not the activity of the transfection reagents themselves, we compared the expression of HeLa cells transfected with

siSPRTN against HeLa cells transfected with a control, non-targeting siRNA (siNT). Indeed, we observed an eleven-fold knockdown in normalized *SPRTN* expression of HeLa cells transfected with 1 nM of siSPRTN in comparison to those treated with 1 nM of siNT after 48 hr of exposure (Figure 3B). These findings supported that our transient SPRTN knockdown model in HeLa cells should be sufficient for assessing cancer cell survival and DPC formation in response to bifunctional alkylating agents.

SPRTN knockdown increased sensitivity of HeLa cells to mechlorethamine-mediated cytotoxicity

Previous groups have demonstrated that SPRTN knockdown can sensitize cancer cells to cytotoxicity induced by the crosslinking agent formaldehyde.[35,38] Therefore, we sought to consider whether our model of SPRTN knockdown in HeLa cells could confer sensitivity to bifunctional alkylating agents of therapeutic relevance. We elected to treat siNT- and siSPRTN-transfected HeLa cells with mechlorethamine for our preliminary study given both this compound's previous history in the clinic and strong capacity to induce DPCs that has been well-characterized previously.[17,23,43] Concentrations for mechlorethamine doses were determined based on preliminary clonogenic survival assays performed with non-transfected HeLa cells (not pictured). Upon pairing SPRTN knockdown with mechlorethamine (HN2) treatment, we observed a pronounced decrease in clonogenicity for siSPRTN HeLa cells at 0.5 μM relative to 0 μM compared to that of siNT as made visible by crystal violet (CV) staining 7 days post-treatment (Figure 4A).

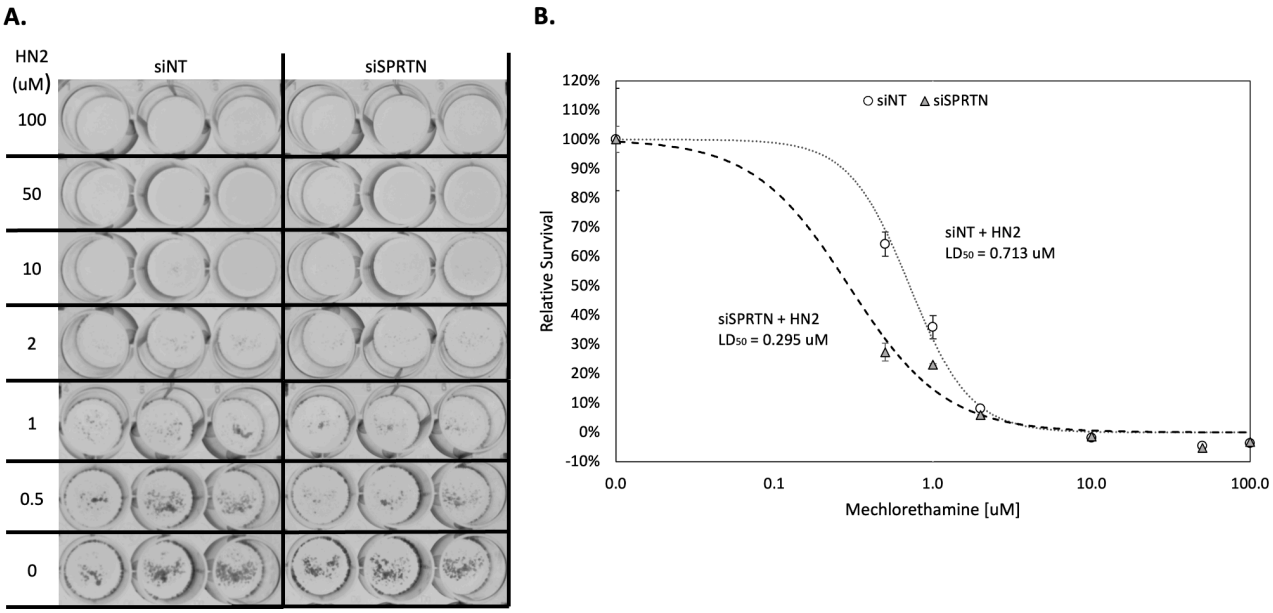

Figure 4. SPRTN knockdown increased sensitivity of HeLa cells to mechlorethamine-induced cytotoxicity. HeLa cells were transfected with 1 nM of siNT or siSPRTN for 48 hr and reseeded at 2,000 cells per well prior to 3 hr treatment with varying doses of mechlorethamine (HN2). The clonogenicity of siNT and siSPRTN triplicates were **A.)** visualized after 7 days using 0.5% crystal violet staining and **B.)** quantified as relative survival compared to their respective untreated groups based on blank-adjusted mean absorbances at 590 nm for a 37-point well scan averaged across each treatment group. Error bars represent SEM for biological triplicates.

To quantify these findings, we implemented an unbiased strategy to standardize the detection of CV-stained colonies (Figure 4B). Since the peak absorbance of CV occurs at 590 nm, we averaged the absorbance at 590 nm measured across 37 uniform points of each well to yield a numerical representation of the intensity and distribution of CV-stained colonies in each well, which we have demonstrated to maintain a linear relationship with the number of live cells present. Thus, we used this strategy to quantify the relative survival of each HN2 treatment group normalized against its respective HN2-untreated transfection group for siNT and siSPRTN HeLa cells from Figure 4A (Figure 4B). Considering that SPRTN knockdown may alter cell viability independent of HN2 treatment, this approach enabled unbiased comparison of changes in clonogenicity attributable exclusively to drug treatment across siSPRTN and siNT groups.

Consistent with the visibly apparent trend in Figure 4A, we observed a relative survival of 27 ± 3% for siSPRTN compared to 64 ± 4% for siNT when treated with 0.5 μM HN2 (Figure 4B). Note that this trend was also maintained for 1 μM HN2 treatments with relative survivals of 23.1 ± 0.3% and 36 ± 4% for siSPRTN and siNT, respectively (Figure 4B). We also calculated the LD_{50} concentrations of mechlorethamine for siSPRTN and siNT groups by fitting a two parameter LD_{50} regression to these data, which gave concentrations of 0.295 μM HN2 for siSPRTN HeLa cells and 0.713 μM HN2 for siNT cells, respectively (Figure 4B).

SPRTN knockout may not impact DPC abundance induced by lethal doses of mechlorethamine after extended time

Based on our preliminary findings of sensitivity to bifunctional alkylating agents conferred by SPRTN knockdown, we sought to characterize whether deficiency in DPC-PR may mediate this phenotype by impairing the capacity of HeLa cells to repair DPCs in response to these drugs and therefore result in increased DPC burden. We employed a modified K-SDS protein precipitation assay, termed the "ARK" assay by Hu *et al.*, to quantify DPCs induced by mechlorethamine in siNT and siSPRTN HeLa cells with increased sensitivity and reduced background compared to the traditional assay format.[40] This improved recovery was achieved primarily through the addition of a chaotropic and detergent-based lysis that enables more stringent separation of non-covalent DNA-protein interactions than traditional lysis via sonication.[40] Then, consistent with conventional K-SDS assays, DPC-associated DNA was separated from free DNA via protein precipitation and DPC abundance was measured indirectly using PicoGreen dsDNA quantification as the percent of DNA proteolytically released from DPCs compared to total free and DPC-associated DNA.[40,44]

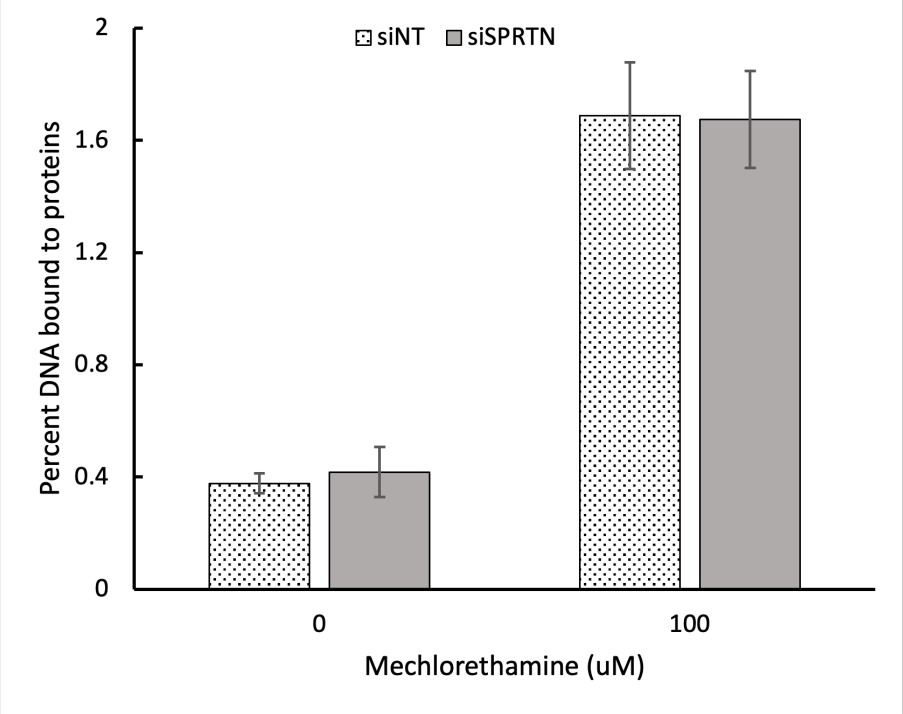

Figure 5. SPRTN knockdown may not alter the DPC burden maintained in HeLa cells well after treatment with lethal doses of mechlorethamine. HeLa cells were transfected with siNT or siSPRTN for 48 hr and treated with 100 μM HN2 for 3 hr. After 16 hr recovery at 37°C, DPC-associated DNA was separated from free DNA for each sample via a modified ARK assay and DPCs were measured as the percent DNA bound to proteins using PicoGreen dsDNA quantification. Error bars represent SEM of biological triplicates from averaged technical triplicates.

For our initial assessment of DPCs induced by mechlorethamine, we selected a dose of 100 μM HN2 for 3 hr followed by 16 hr recovery at 37°C based on previous work by our group that demonstrated strong signal-to-noise ratios for this regiment using the conventional K-SDS assay. Thus, employing the ARK assay, we aimed to validate this approach for quantifying differences in DPC formation and maintenance among HeLa cells transfected with siNT and siSPRTN in response to 100 μM HN2 treatment. We observed significant increases in DPC accumulation upon drug treatment for both groups, which yielded changes from 0.38 ± 0.04% to 1.7 ± 0.2% for siNT and 0.42 ± 0.09% to 1.7 ± 0.2% for siSPRTN (Figure 5). However, note that no significant differences between DPC abundance measured for siNT and siSPRTN cells were evident even after 100 μM HN2 treatment (Figure 5).

Discussion

Several groups have demonstrated that SPRTN knockdown can increase the burden of DPCs induced by endogenous and exogenous crosslinking agents, which is likely responsible for the increased cytotoxicity observed in response to these treatments.[32,35,36,38,40] Of particular interest for these studies has been the endogenous metabolic byproduct formaldehyde, the platinum crosslinking chemotherapy cisplatin, and the topoisomerase inhibiting chemotherapies etoposide and camptothecin. However, very little work has investigated the potential for synergistic effects between SPRTN deficiency and DNA alkylating agents as a therapeutic avenue against cancer, especially in the context of nitrogen mustards well-characterized for their capacity to induce DPCs.

Herein, we reported preliminary findings for SPRTN knockdown to sensitize HeLa cells to treatment with mechlorethamine, a nitrogen mustard that has a rich clinical history and is an analog to the more clinically relevant compound bendamustine. Using clonogenic survival assays, we observed significant decreases in relative survival of HeLa cells with SPRTN knockdown when treated with 0.5 μM and 1 μM of HN2, which correlated with a 2.4-fold decrease in calculated LD_{50} concentration. Although these results were demonstrative of knockdown-associated sensitization, it must be considered that our clonogenic survival curves lacked mechlorethamine treatments at doses below 0.5 μM in the range that our calculated LD_{50} for siSPRTN in concert with HN2 treatment fell. Therefore, we rationalized that a more exhaustive scale of mechlorethamine doses below the bottom threshold of 0.5 μM will be required to validate these calculated LD_{50} values.

Suspecting that an increased DPC burden may be the cause of SPRTN knockdown-associated sensitization to mechlorethamine, we were initially surprised to find no measurable increase in percent DNA bound to protein in siSPRTN-transfected HeLa cells compared to siNT-transfected cells for treatment with 100 μM. However, pairing these findings with our clonogenic survival data presented above, we reasoned that the highly lethal dose of 100 μM HN2 may

overwhelm any DPC-PR efforts afforded by SPRTN-proficient cells during the 3 hr treatment and therefore result in no distinguishable differences in DPC burden accumulated even after a 16 hr window for recovery. Note that previous work has indicated that high doses of mechlorethamine can induce crosslinking damage that persists with minimal change in abundance even 24 hr after treatment in human leukemia HL-60 cells[45], which was modestly consistent with our finding for DPCs independent of SPRTN deficiency. Nonetheless, our DPC quantification study did demonstrate over four-fold increases in DPC burden upon mechlorethamine treatment when comparing within siNT and siSPRTN groups respectively. These findings were not only consistent in scale with previous studies by our group, but also demonstrated lower background signals in accordance with a more stringent DPC quantification afforded by the ARK assay. Taken together, we propose that the ARK assay is suitable for detecting DPCs induced at concentrations lower than 100 μM HN2, which will be required in combination with shorter recovery time periods to more comprehensively evaluate whether SPRTN knockdown may increase DPC burden in response to bifunctional alkylating chemotherapies.

Collectively, this work provides preliminary evidence to support that interfering with SPRTN-mediated DPC-PR to sensitize cancer cells to crosslinking chemotherapy may be a promising potential therapeutic avenue to fight cancer.[35] However, several considerations must be made before a SPRTN inhibition strategy may gain traction for further therapeutic development. Transient knockdown of SPRTN expression by over ten-fold as reported here represents an effective model for preliminary investigation into synergism with crosslinking agents, but this magnitude is likely much higher than that feasible for therapeutically relevant inhibition strategies *in vivo*. Therefore, we propose that further investigations into SPRTN knockdown-mediated sensitization to crosslinking drugs should also be performed with siSPRTN transfections titrated to produce less potent knockdowns.

Additionally, although previous reports have suggested that SPRTN expression increased in HeLa cells challenged by formaldehyde crosslinking stress[38], these responses have not been characterized for treatment with crosslinking chemotherapeutics nor comprehensively across different cancer and healthy tissue types. Recently, Ruggiano *et al.* demonstrated that knockdown of BRCA2, a key factor in HR, sensitized SPRTN-deficient HeLa cells to formaldehyde-induced cytotoxicity, and conversely that SPRTN knockdown in BRCA2-deficient HeLa cells also decreased cell viability.[41] *BRCA1/2* mutations are characteristic to several cancer types including ovarian and breast cancers, which can also exhibit resistance to traditional PARP inhibitor and platinum crosslinking drug-based combination therapies.[46] Therefore, we propose that screening for SPRTN expression across different cancer types –especially in the context of HR deficiency– may enable the identification of tumors that could be particularly susceptible to potential SPRTN inhibitors alone or in combination with bifunctional alkylating agent strategies. Should there be tumors that demonstrate strong sensitization to crosslinking chemotherapies upon knockdown of SPRTN, this may represent a novel targeted therapy strategy that could overcome other acquired resistance and/or decrease the dose of toxic chemotherapies required for patient remission, thus potentially reducing adverse effects and improving patient experiences in their fight against cancer.

References

(1) *Cancer Drugs - NCI*. https://www.cancer.gov/about-cancer/treatment/drugs (accessed 2023-12-10).
(2) Hanahan, D.; Weinberg, R. A. The Hallmarks of Cancer. *Cell* **2000**, *100* (1), 57–70. https://doi.org/10.1016/S0092-8674(00)81683-9.
(3) Nygren, P. What Is Cancer Chemotherapy? *Acta Oncol*. **2001**, *40* (2–3), 166–174. https://doi.org/10.1080/02841860151116204.
(4) Yang, J.; Kang, H.; Lyu, L.; Xiong, W.; Hu, Y. A Target Map of Clinical Combination Therapies in Oncology: An Analysis of Clinicaltrials.Gov. *Discov. Oncol*. **2023**, *14* (1), 151. https://doi.org/10.1007/s12672-023-00758-4.
(5) Mokhtari, R. B.; Homayouni, T. S.; Baluch, N.; Morgatskaya, E.; Kumar, S.; Das, B.; Yeger, H. Combination Therapy in Combating Cancer. *Oncotarget* **2017**, *8* (23), 38022–38043. https://doi.org/10.18632/oncotarget.16723.
(6) *Definition of gemcitabine-cisplatin regimen - NCI Drug Dictionary - NCI*. https://www.cancer.gov/publications/dictionaries/cancer-drug/def/cisplatin-gemcitabine-regimen (accessed 2024-03-28).
(7) Moorsel, C. J. A. V.; Pinedo, H. M.; Veerman, G.; Bergman, A. M.; Kuiper, C. M.; Vermorken, J. B.; Van Der Vijgh, W. J. F.; Peters, G. J. Mechanisms of Synergism between Cisplatin and Gemcitabine in Ovarian and Non-Small-Cell Lung Cancer Cell Lines. *Br. J. Cancer* **1999**, *80* (7), 981–990. https://doi.org/10.1038/sj.bjc.6690452.
(8) Bharti, A. C.; Vishnoi, K.; Singh, S. M.; Aggarwal, B. B. Chapter 1 - Pathways Linked to Cancer Chemoresistance and Their Targeting by Nutraceuticals. In *Role of Nutraceuticals in Cancer Chemosensitization*; Bharti, A. C., Aggarwal, B. B., Eds.; Cancer Sensitizing Agents for Chemotherapy; Academic Press, 2018; Vol. 2, pp 1–30. https://doi.org/10.1016/B978-0-12-812373-7.00001-2.
(9) Colvin, M. Alkylating Agents. In *Holland-Frei Cancer Medicine*; Kufe, D., Pollock, R., Weichselbaum, R., Eds.; BC Decker Inc.: Hamilton, ON, 2003.
(10) Malinge, J.-M.; Giraud-Panis, M.-J.; Leng, M. Interstrand Cross-Links of Cisplatin Induce Striking Distortions in DNA. *J. Inorg. Biochem*. **1999**, *77* (1), 23–29. https://doi.org/10.1016/S0162-0134(99)00148-8.
(11) Ming, X.; Groehler, A.; Michaelson-Richie, E. D.; Villalta, P. W.; Campbell, C.; Tretyakova, N. Y. Mass Spectrometry Based Proteomics Study of Cisplatin-Induced DNA–Protein Cross-Linking in Human Fibrosarcoma (HT1080) Cells. *Chem. Res. Toxicol*. **2017**, *30* (4), 980–995. https://doi.org/10.1021/acs.chemrestox.6b00389.
(12) Bardos, T. J.; Datta-Gupta, N.; Hebborn, P.; Triggle, D. J. A Study of Comparative Chemical and Biological Activities of Alkylating Agents [1]. *J. Med. Chem*. **1965**, *8* (2), 167–174. https://doi.org/10.1021/jm00326a006.
(13) Rycenga, H. B.; Long, D. T. The Evolving Role of DNA Inter-Strand Crosslinks in Chemotherapy. *Curr. Opin. Pharmacol*. **2018**, *41*, 20–26. https://doi.org/10.1016/j.coph.2018.04.004.
(14) Hopkins, P. B.; Millard, J. T.; Woo, J.; Weidner, M. F.; Kirchner, J. J.; Sigurdsson, S. Th.; Raucher, S. Sequence Preferences of DNA Interstrand Cross-Linking Agents: Importance of Minimal DNA Structural Reorganization in the Cross-Linking Reactions of Mechlorethamine, Cisplatin and Mitomycin C. *Tetrahedron* **1991**, *47* (14), 2475–2489. https://doi.org/10.1016/S0040-4020(01)81782-8.

(15) Rajski, S. R.; Williams, R. M. DNA Cross-Linking Agents as Antitumor Drugs. *Chem. Rev.* **1998**, *98* (8), 2723–2796. https://doi.org/10.1021/cr9800199.
(16) Rink, S. M.; Solomon, M. S.; Taylor, M. J.; Rajur, S. B.; McLaughlin, L. W.; Hopkins, P. B. Covalent Structure of a Nitrogen Mustard-Induced DNA Interstrand Cross-Link: An N7-to-N7 Linkage of Deoxyguanosine Residues at the Duplex Sequence 5'-d(GNC). *J. Am. Chem. Soc.* **1993**, *115* (7), 2551–2557. https://doi.org/10.1021/ja00060a001.
(17) Loeber, R. L.; Michaelson-Richie, E. D.; Codreanu, S. G.; Liebler, D. C.; Campbell, C. R.; Tretyakova, N. Y. Proteomic Analysis of DNA–Protein Cross-Linking by Antitumor Nitrogen Mustards. *Chem. Res. Toxicol.* **2009**, *22* (6), 1151–1162. https://doi.org/10.1021/tx900078y.
(18) Penketh, P. G.; Shyam, K.; Sartorelli, A. C. Comparison of DNA Lesions Produced by Tumor-Inhibitory 1,2-Bis(Sulfonyl)Hydrazines and Chloroethylnitrosoureas. *Biochem. Pharmacol.* **2000**, *59* (3), 283–291. https://doi.org/10.1016/S0006-2952(99)00328-7.
(19) Tretyakova, N. Y.; Groehler, A.; Ji, S. DNA–Protein Cross-Links: Formation, Structural Identities, and Biological Outcomes. *Acc. Chem. Res.* **2015**, *48* (6), 1631–1644. https://doi.org/10.1021/acs.accounts.5b00056.
(20) Perry, M.; Ghosal, G. Mechanisms and Regulation of DNA-Protein Crosslink Repair During DNA Replication by SPRTN Protease. *Front. Mol. Biosci.* **2022**, *9*, 916697. https://doi.org/10.3389/fmolb.2022.916697.
(21) Barker, S.; Weinfeld, M.; Murray, D. DNA-Protein Crosslinks: Their Induction, Repair, and Biological Consequences. *Mutat. Res. Mutat. Res.* **2005**, *589* (2), 111–135. https://doi.org/10.1016/j.mrrev.2004.11.003.
(22) Reardon, J. T.; Cheng, Y.; Sancar, A. Repair of DNA–Protein Cross-Links in Mammalian Cells. *Cell Cycle* **2006**, *5* (13), 1366–1370. https://doi.org/10.4161/cc.5.13.2892.
(23) Michaelson-Richie, E. D.; Ming, X.; Codreanu, S. G.; Loeber, R. L.; Liebler, D. C.; Campbell, C.; Tretyakova, N. Y. Mechlorethamine-Induced DNA–Protein Cross-Linking in Human Fibrosarcoma (HT1080) Cells. *J. Proteome Res.* **2011**, *10* (6), 2785–2796. https://doi.org/10.1021/pr200042u.
(24) Lu, K.; Ye, W.; Zhou, L.; Collins, L. B.; Chen, X.; Gold, A.; Ball, L. M.; Swenberg, J. A. Structural Characterization of Formaldehyde-Induced Cross-Links Between Amino Acids and Deoxynucleosides and Their Oligomers. *J. Am. Chem. Soc.* **2010**, *132* (10), 3388–3399. https://doi.org/10.1021/ja908282f.
(25) Swenberg, J. A.; Lu, K.; Moeller, B. C.; Gao, L.; Upton, P. B.; Nakamura, J.; Starr, T. B. Endogenous versus Exogenous DNA Adducts: Their Role in Carcinogenesis, Epidemiology, and Risk Assessment. *Toxicol. Sci.* **2011**, *120* (Supplement 1), S130–S145. https://doi.org/10.1093/toxsci/kfq371.
(26) Weickert, P.; Stingele, J. DNA–Protein Crosslinks and Their Resolution. *Annu. Rev. Biochem.* **2022**, *91* (1), 157–181. https://doi.org/10.1146/annurev-biochem-032620-105820.
(27) Liu, C.-W.; Tian, X.; Hartwell, H. J.; Leng, J.; Chi, L.; Lu, K.; Swenberg, J. A. Accurate Measurement of Formaldehyde-Induced DNA–Protein Cross-Links by High-Resolution Orbitrap Mass Spectrometry. *Chem. Res. Toxicol.* **2018**, *31* (5), 350–357. https://doi.org/10.1021/acs.chemrestox.8b00040.
(28) Vaz, B.; Popovic, M.; Ramadan, K. DNA–Protein Crosslink Proteolysis Repair. *Trends Biochem. Sci.* **2017**, *42* (6), 483–495. https://doi.org/10.1016/j.tibs.2017.03.005.
(29) Scharer, O. D. Nucleotide Excision Repair in Eukaryotes. *Cold Spring Harb. Perspect. Biol.* **2013**, *5* (10), a012609–a012609. https://doi.org/10.1101/cshperspect.a012609.
(30) Li, X.; Heyer, W.-D. Homologous Recombination in DNA Repair and DNA Damage Tolerance. *Cell Res.* **2008**, *18* (1), 99–113. https://doi.org/10.1038/cr.2008.1.

(31) Zhang, H.; Xiong, Y.; Chen, J. DNA–Protein Cross-Link Repair: What Do We Know Now? *Cell Biosci.* **2020**, *10* (1), 3. https://doi.org/10.1186/s13578-019-0366-z.
(32) Vaz, B.; Popovic, M.; Newman, J. A.; Fielden, J.; Aitkenhead, H.; Halder, S.; Singh, A. N.; Vendrell, I.; Fischer, R.; Torrecilla, I.; Drobnitzky, N.; Freire, R.; Amor, D. J.; Lockhart, P. J.; Kessler, B. M.; McKenna, G. W.; Gileadi, O.; Ramadan, K. Metalloprotease SPRTN/DVC1 Orchestrates Replication-Coupled DNA-Protein Crosslink Repair. *Mol. Cell* **2016**, *64* (4), 704–719. https://doi.org/10.1016/j.molcel.2016.09.032.
(33) Ruggiano, A.; Ramadan, K. DNA–Protein Crosslink Proteases in Genome Stability. *Commun. Biol.* **2021**, *4* (1), 11. https://doi.org/10.1038/s42003-020-01539-3.
(34) Ruggiano, A.; Ramadan, K. The Trinity of SPRTN Protease Regulation. *Trends Biochem. Sci.* **2021**, *46* (1), 2–4. https://doi.org/10.1016/j.tibs.2020.10.007.
(35) Stingele, J.; Bellelli, R.; Alte, F.; Hewitt, G.; Sarek, G.; Maslen, S. L.; Tsutakawa, S. E.; Borg, A.; Kjær, S.; Tainer, J. A.; Skehel, J. M.; Groll, M.; Boulton, S. J. Mechanism and Regulation of DNA-Protein Crosslink Repair by the DNA-Dependent Metalloprotease SPRTN. *Mol. Cell* **2016**, *64* (4), 688–703. https://doi.org/10.1016/j.molcel.2016.09.031.
(36) Huang, J.; Zhou, Q.; Gao, M.; Nowsheen, S.; Zhao, F.; Kim, W.; Zhu, Q.; Kojima, Y.; Yin, P.; Zhang, Y.; Guo, G.; Tu, X.; Deng, M.; Luo, K.; Qin, B.; Machida, Y.; Lou, Z. Tandem Deubiquitination and Acetylation of SPRTN Promotes DNA-Protein Crosslink Repair and Protects against Aging. *Mol. Cell* **2020**, *79* (5), 824-835.e5. https://doi.org/10.1016/j.molcel.2020.06.027.
(37) Halder, S.; Torrecilla, I.; Burkhalter, M. D.; Popović, M.; Fielden, J.; Vaz, B.; Oehler, J.; Pilger, D.; Lessel, D.; Wiseman, K.; Singh, A. N.; Vendrell, I.; Fischer, R.; Philipp, M.; Ramadan, K. SPRTN Protease and Checkpoint Kinase 1 Cross-Activation Loop Safeguards DNA Replication. *Nat. Commun.* **2019**, *10* (1), 3142. https://doi.org/10.1038/s41467-019-11095-y.
(38) Glumac, M.; Polović, M.; Batel, A.; Gelemanović, A.; Maček, B.; Velić, A.; Marinović-Terzić, I. SPRTN-Dependent DPC Degradation Precedes Repair of Damaged DNA: A Proof of Concept Revealed by the STAR Assay. *Nucleic Acids Res.* **2023**, *51* (6), e35–e35. https://doi.org/10.1093/nar/gkad022.
(39) Maskey, R. S.; Kim, M. S.; Baker, D. J.; Childs, B.; Malureanu, L. A.; Jeganathan, K. B.; Machida, Y.; Van Deursen, J. M.; Machida, Y. J. Spartan Deficiency Causes Genomic Instability and Progeroid Phenotypes. *Nat. Commun.* **2014**, *5* (1), 5744. https://doi.org/10.1038/ncomms6744.
(40) Hu, Q.; Klages-Mundt, N.; Wang, R.; Lynn, E.; Kuma Saha, L.; Zhang, H.; Srivastava, M.; Shen, X.; Tian, Y.; Kim, H.; Ye, Y.; Paull, T.; Takeda, S.; Chen, J.; Li, L. The ARK Assay Is a Sensitive and Versatile Method for the Global Detection of DNA-Protein Crosslinks. *Cell Rep.* **2020**, *30* (4), 1235-1245.e4. https://doi.org/10.1016/j.celrep.2019.12.067.
(41) Ruggiano, A.; Vaz, B.; Kilgas, S.; Popović, M.; Rodriguez-Berriguete, G.; Singh, A. N.; Higgins, G. S.; Kiltie, A. E.; Ramadan, K. The Protease SPRTN and SUMOylation Coordinate DNA-Protein Crosslink Repair to Prevent Genome Instability. *Cell Rep.* **2021**, *37* (10), 110080. https://doi.org/10.1016/j.celrep.2021.110080.
(42) Thellin, O.; Zorzi, W.; Lakaye, B.; De Borman, B.; Coumans, B.; Hennen, G.; Grisar, T.; Igout, A.; Heinen, E. Housekeeping Genes as Internal Standards: Use and Limits. *J. Biotechnol.* **1999**, *75* (2–3), 291–295. https://doi.org/10.1016/S0168-1656(99)00163-7.
(43) Loeber, R.; Michaelson, E.; Fang, Q.; Campbell, C.; Pegg, A. E.; Tretyakova, N. Cross-Linking of the DNA Repair Protein O^6-Alkylguanine DNA Alkyltransferase to DNA in the

Presence of Antitumor Nitrogen Mustards. *Chem. Res. Toxicol.* **2008**, *21* (4), 787–795. https://doi.org/10.1021/tx7004508.
(44) Torrecilla, I.; Ruggiano, A.; Kiianitsa, K.; Aljarbou, F.; Lascaux, P.; Hoslett, G.; Song, W.; Maizels, N.; Ramadan, K. Isolation and Detection of DNA–Protein Crosslinks in Mammalian Cells. *Nucleic Acids Res.* **2024**, *52* (2), 525–547. https://doi.org/10.1093/nar/gkad1178.
(45) Le, P. M.; Silvestri, V. L.; Redstone, S. C.; Dunn, J. B.; Millard, J. T. Cross-Linking by Epichlorohydrin and Diepoxybutane Correlates with Cytotoxicity and Leads to Apoptosis in Human Leukemia (HL-60) Cells. *Toxicol. Appl. Pharmacol.* **2018**, *352*, 19–27. https://doi.org/10.1016/j.taap.2018.05.020.
(46) Krais, J. J.; Johnson, N. BRCA1 Mutations in Cancer: Coordinating Deficiencies in Homologous Recombination with Tumorigenesis. *Cancer Res.* **2020**, *80* (21), 4601–4609. https://doi.org/10.1158/0008-5472.CAN-20-1830.