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Preparation of Stable Nitrogen Mustard DNA Inter Strand Crosslink Analogs for Biochemical and Cell Biological Studies

Alejandra Castaño^a, Upasana Roy^a, and Orlando D. Schärer^{a,b,c,d,*}

^aDepartment of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400, USA

^bDepartment of Pharmacological Sciences, Stony Brook University, Stony Brook, NY 11794-3400, USA

^cCenter for Genomic Integrity, Institute for Basic Science, Ulsan, Korea

^dDepartment of Biological Sciences, School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan, Korea

Abstract

Nitrogen mustards (NMs) react with two bases on opposite strands of a DNA duplex to form a covalent linkage, yielding adducts called DNA interstrand crosslinks (ICLs). This prevents helix unwinding, blocking essential processes such as replication and transcription. Accumulation of ICLs causes cell death in rapidly dividing cells, especially cancer cells and ICL-forming agents like nitrogen mustards are valuable in chemotherapy. However, the repair of ICLs can contribute to chemoresistance through a number of pathways that remain poorly understood. One of the impediments in studying NM ICL repair mechanisms has been the difficulty of generating site-specific and stable NM ICLs. Here we describe two methods to synthesize stable NM ICL analogs that make it possible to study DNA ICL repair. As a proof of principle of the suitability of these NM ICLs for biochemical and cell biological studies, we use them in primer extension assays with Klenow polymerase. We show that the NM ICL analogs block the polymerase activity and remain intact under our experimental conditions.

Keywords

DNA interstrand crosslink; DNA repair; nitrogen mustard; oligonucleotides; DNA synthesis

1. INTRODUCTION

Bis-(2-chloroethyl)-amine derivatives or nitrogen mustards (NM) are bifunctional alkylating agents widely used in the clinic to treat a variety of cancers (Chabner, Amrein, Druker, Michaelson, Mistsides, Goss et al., 2005). The bifunctional nature of NMs allows for the formation of DNA intra- and interstrand crosslinks. It has been shown that although DNA interstrand crosslinks (ICLs) make up only 1–5% of the total adducts, they are responsible for the cytotoxicity as they provide a complete block to essential processes such as DNA

*Corresponding author: orlando.scharer@ibs.re.kr.

replication and transcription (Noll, Mason, & Miller, 2006; Schärer, 2005). The ability of ICLs to induce apoptosis particularly in replicating cells provides some degree of selective cytotoxicity towards rapidly dividing cancer cells in a therapeutic setting (Deans & West, 2011). However, cellular resistance is often observed in patients treated with nitrogen mustards, in large part due to ICL repair pathways that remove NM ICLs from the genome. Several ICL repair pathways exist, and the best understood of these is coupled to replication and has been biochemically reconstituted in *Xenopus* egg extracts (Knipscheer, Räschle, Schärer, & Walter, 2012; Räschle, Knipscheer, Enoiu, Angelov, Sun, Griffith et al., 2008). Replication-coupled ICL repair makes use of genes involved in a number of pathways, including Fanconi anemia (FA), translesion DNA synthesis (TLS), homologous recombination (HR), and nucleotide excision repair (NER). The coordinated activity of this pathway comprises factors that recognize ICLs at stalled replication forks, remove the ICL and eventually reestablish the replication fork. In another, less understood replication-coupled ICL repair pathway, the replication fork initially bypasses the intact ICL in a process known as replication traverse, presumably followed by ICL removal at a later stage (Huang, Liu, Bellani, Thazhathveetil, Ling, de Winter et al., 2013). In addition, ICL repair is also known to occur outside of replication in an NER- and TLS-dependent manner, but the mechanistic details remain poorly understood as well (Clauson, Scharer, & Niedernhofer, 2013).

The major limitation in studying repair of NM ICLs has been the difficulty in generating substrates suitable for biochemical and cell biological studies. Two main challenges exist: first, treatment of a DNA duplex with NMs yields the desired ICLs only as a small fraction of products, with monoadducts and intrastrand crosslinks making up the majority of adducts. This makes the isolation of sufficient amounts of ICLs difficult (Millard, Raucher, & Hopkins, 1990; Povirk & Shuker, 1994). Second, the alkylation of guanosine at N7 yields a positive charge on the purine ring (**1**, Fig. 1A), rendering the base prone to spontaneous depurination, leading to loss of the ICL, formation of abasic sites and eventually strand breaks (Fig. 1B) (Gates, 2009).

To overcome these limitations, we have developed two different methods to generate stable analogs of NM ICLs that closely mimic the native NM ICL substrates (Fig. 1A). Our first approach is based on the previously synthesized 7-deaza-guanine phosphoramidite (7CdG, **2**, Fig. 1A) precursors bearing masked diols that are easily incorporated into DNA oligomers by solid DNA synthesis (Angelov, Guainazzi, & Schärer, 2009; Guainazzi, Campbell, Angelov, Simmerling, & Schärer, 2010; Mukherjee, Guainazzi, & Schärer, 2014). After incorporation into 5'-GNC-3' sequences, two complementary oligonucleotides containing 7CdG-residues with alkyl diol side chains at the 7 position are annealed. The diols are oxidized to aldehydes with sodium periodate and coupled with hydrazine resulting in a high yield of the NM ICL mimic **2** (Fig. 1A and 2B). By using alkylaldehyde chains and diamines of different lengths, structurally diverse NM-like ICLs can be generated, for example the 5 atom ICL (7CdG-5a, **2**), a close structural mimic of the native NM ICL, or the 8 atom ICL (7CdG-8a, **4**), that has a longer linkage and no distortion in the DNA (Fig. 1A).

In our second approach, NM ICLs are rendered resistant to glycosidic bond cleavage by incorporation of 2'-deoxy-2'- β -fluoroarabino guanosine (2'FdG, **3**, Fig. 1A) in the 5'-

GNC-3' sequences via solid phase DNA synthesis. The electronegative fluorine atom at the 2' carbon of the sugar base is strongly electron withdrawing and dramatically destabilizes the transient positive charge formed during the glycosidic bond cleavage reaction (6, Fig. 1B), thus de facto eliminating spontaneous depurination (Lee, Bowman, Ueno, Wang, & Verdine, 2008; Watts, Katolik, Viladoms, & Damha, 2009).

The generation of various synthetic stable NM ICL analogs and the relative advantages and disadvantages of our approaches are described. We used these ICLs in primer extension assays with Klenow polymerase to assess the stability of the ICLs. Additionally, we describe a method to generate substrates in which the duplex around the ICL can be resected by incorporation of uracil residues in the oligonucleotide followed by enzymatic cleavage with UDG/EndoVIII (USER, Fig. 2C). These methods allow the synthesis of a panel of NM-like ICLs for the study of ICL repair mechanisms.

2. MATERIALS

2.1. Reagents and Buffers

- 1 mM DNA oligonucleotides in 1x TE: 5'-CCCTCTUCTG*TCCUTCTTTC-3' (20mer), and 5'-GAAAGAAGG*ACAGAAGAGGGTACCATCATAGAGTCAGTG-3' (where G* represents dG, 2'FdG, or 7CdG) (Notes 2 and 4)
- Use ultrapure water 18 MΩ.cm for the preparation of solutions and in all reactions. All the reagents are of analytical grade purity.
- 1x TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA)
- 40 mM sodium cacodylate (pH 7): mix 0.86 g sodium cacodylate trihydrate (Sigma-Aldrich) in 70 mL water. Adjust pH to 7 by addition of 0.2 M HCl, and bring final volume to 100 mL with MilliQ water.
- 5 mM mechlorethamine hydrochloride 98% (5 mM NM): use fresh solution prior reaction with DNA by mixing 1 mg of mechlorethamine (Sigma-Aldrich) in 1 mL 40 mM sodium cacodylate pH 7 (Note 1).
- 50 mM sodium periodate solution: dissolve 0.1 g sodium metaperiodate in 10 mL ultrapure water. Store in the dark at 4°C.
- 0.5 M sodium cyanoborohydride solution: dissolve 31.0 mg sodium cyanoborohydride in 1 mL ultrapure water. Store in the dark at 4°C.
- 5 mM hydrazine solution: Add 2.5 µL of 64–65% hydrazine monohydrate solution (Sigma-Aldrich) to 10 mL ultrapure water. Store in the dark at 4°C.

²Native and 2'FdG-containing 20mer and 39mer DNA oligos (Integrated DNA Technologies) are ordered HPLC purified and diluted to 1 mM stock solutions in 1x TE.

⁴The 7CdG phosphoramidite building block needs to be synthesized as described or can be requested from the authors. (Angelov et al., 2009; Guainazzi et al., 2010; Mukherjee et al., 2014). It is incorporated into the 20mer and 39mer oligos using Expedite DNA synthesizer. The final 7CdG containing oligos used for NM ICL reactions are deprotected by treatment with concentrated NH₄OH solution at 50°C for 12 hrs and purified by Agilent BondElut C18 columns.

¹CAUTION: Mechlorethamine is a carcinogenic/mutagenic agent! Please refer to its MSDS for proper handling and disposal.

- 5 mM DMEDA solution: Add 5.4 μ L dimethylethylenediamine (Sigma-Aldrich) to 10 mL ultrapure water. Store in the dark at 4°C.
- 1 M sodium phosphate buffer (pH 5.4)
- 5 mM sodium borate buffer (pH 8) for electroelution: mix 1.95 g sodium tetraborate in 800 mL water. Adjust pH to 8 with boric acid and bring up volume to 1 L with milliQ water.
- 9:1 MALDI ToF MS matrix solution: mix 1 mL of 50 mg/mL 3-hydroxypicolinic acid (Protea Bioscience) in 50% acetonitrile/Milli-Q water and 1 mL of 50 mg/mL ammonium citrate (Sigma-Aldrich) in Milli-Q water. Store at 4°C in the dark.
- 50% v/v acetonitrile:water
- 5x Tris–Borate-EDTA buffer (TBE): dissolve 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA in 800 mL ultrapure water. Make up volume to 1 L with ultrapure water (pH 8.0).
- 0.5x TBE DPAGE running buffer: dilute 100 mL 5x TBE stock solution with 900 mL H₂O.
- 20% denaturing polyacrylamide gel electrophoresis (20% DPAGE) casting solution: stir vigorously 210.2 g ultrapure urea, 250 mL Acrylamide/Bis 19:1, 40% (w/v) solution, 50 mL 5x TBE in 400 mL ultrapure water. Once all components have dissolved bring volume up to 500 mL with ultra pure water.
- 0% denaturing polyacrylamide gel electrophoresis (0% DPAGE) casting solution: mix 210.2 g Urea, 50 mL 5x TBE in 400 mL ultrapure water. Once all components have dissolved bring volume up to 500 mL with ultra pure water.
- TEMED
- 10% ammonium persulfate (APS) solution (mix 1 g APS in 10 mL H₂O, store at 4°C)
- 80% formamide/Orange G loading buffer: mix 800 μ L formamide, 0.5 mg orange G and 200 μ L H₂O). Loading buffer can be stored at room temperature.
- 80% formamide/xylene cyanol/bromophenol blue tracking buffer: mix 800 μ L formamide, 0.5 mg xylene cyanol, 0.5 mg bromophenol blue and 200 μ L H₂O. Tracking buffer can be stored at room temperature.
- USER enzyme mix (NEB, M5505S)
- 1x SYBR Gold solution (Life Technologies): dissolve 50 μ L 10000x SYBR gold into 500 mL 1x TBE in amber plastic bottle. Store at 4°C.

2.2. Equipment and Consumables

- NanoDrop or UV spectrophotometer
- Ziptip c18 pipette tips (Millipore)

- MALDI plate (MTP Anchorchip, Bruker Daltonics)
- AutoFlex II MALDI-TOF mass spectrometer (Bruker Daltonics)
- FlexAnalysis 3.0 software
- Thermomixer compact (Eppendorf)
- Heat Block
- Elutrap™ device (Schleicher & Schuell)
- Bio-Trap membranes (BT1 glycerinated and BT2 dry, Whatman)
- Sub-Cell GT Horizontal Electrophoresis system, 15 × 25 cm tray (BioRad) to hold the Elutrap device
- Bench-top centrifuge (Sorvall Legend Mach 1.6R with swing bucket rotor)
- 0.5 mL and 1.5 mL microcentrifuge tubes
- 15 mL Falcon tubes
- Amicon Ultra-0.5 3 KDa MWCO (centrifugal filter device, Millipore)
- Amicon Ultra-4 3 KDa MWCO (centrifugal filter device, Millipore)
- Bio Rad PowerPac (400W) power supply
- Electrophoresis tank (model V15.17 Whatman)
- V-series electrophoresis glass sandwich plates (Apogee): long (19.7 cm wide × 19.1 cm long) and short (19.7 cm wide × 16.0 cm long)
- 1.5 mm semi-prep comb (with a single and a long well, and 1.5 mm spacers and 0.75 mm spacers and analytical combs)
- TLC Glass Plates with fluorescence indicator for UV shadowing (EMD/Millipore)
- Scalpel, and tweezers
- Hand held UV Lamp (254nm)
- Typhoon 9400 Fluoroimager (GE Healthcare)
- ImageQuant Software to analyze fluorescent images.

3. METHODS

3.1. Preparation of NM ICLs

3.1.1. Native and 2'FdG-containing NM ICL analogs

1. In a 1.5 mL microcentrifuge tube, mix 100 µL 1 mM 20mer 5'-CCCTCTUCTG*TCCUTCTTTC-3' and 100 µL 1 mM 39mer 5'-GAAAGAAGG*ACAGAAGAGGGTACCATCATAGAGTCAGTG-3' (where G* represents dG or 2'FdG) in 200 µL of 40mM sodium cacodylate pH 7 (250 µM final duplex concentration) (Note 2).

2. Heat this 250 μ M DNA solution at 95°C for 5 min in a preheated heat block. Switch off heating block and let it cool slowly to allow annealing of the oligos.
3. NM ICL reaction: Add 3 equivalents of freshly made 5 mM NM solution (pH 7) to the annealed DNA solution (Note 1). Incubate the reaction mixture at 37°C for 3 hours in a thermomixer in the dark.
4. The resulting NM ICLs (~7% yield for 2'FdG and ~2% for canonical G) can be purified by denaturing polyacrylamide gel electrophoresis (DPAGE) as described in section 3.3 (Note 3).

3.1.2. 7CdG-containing NM ICL analogs

1. In a 1.5 mL microcentrifuge tube, mix 25 nmol each of the 7CdG modified 20mer (5'-CCCTCTUCTG*TCCUTCTTTC-3') and 39mer (5'-GAAAGAAGG*ACAGAAGAGGGTACCATCATAGAGTCAGTG-3') where G* denotes the modified 7CdG, in 125 μ L of 100 mM NaCl. Heat this mixture to 95°C for 5 minutes and cool slowly to allow the oligos to anneal. (Note 4)
2. Add 10 μ L of 50 mM sodium periodate solution and 15 μ L 1 M sodium phosphate buffer (pH 5.4). Incubate this mixture at 4°C overnight in the dark, and allow oxidation to occur.
3. Transfer the mixture to a Millipore Amicon column (3K MWCO) and add 0.1 M sodium phosphate buffer (pH 5.4) up to a volume of 500 μ L. Centrifuge at 11,000 rpm for 30 minutes.
4. Discard flow through, and repeat twice.
5. Collect the final solution by inverting the Amicon column into a new collection tube. Centrifuge at 8,000 rpm for 2 minutes.
6. Transfer oxidized oligos from the collection tube to a new 1.5 mL microcentrifuge tube.
7. Add 10 μ L 5 mM aqueous solution of the amine (hydrazine or DMEDA) and 10 μ L 0.5 M sodium cyanoborohydride. Incubate overnight in the dark at room temperature to allow the crosslinking reaction to take place.
8. The ICL formation can be analyzed by loading 5 pmol of the reaction on a 15% denaturing PAGE gel. The DNA can be visualized by SYBR Gold staining.
9. The ICL can be isolated and purified by denaturing PAGE (Section 3.3).

3.2. Resection of NM ICLs by USER

1. In a 1.5 mL microcentrifuge tube, dilute 500 pmol of purified ICL in 80 μ L ultra pure water. Add 10 μ L 0.1 M Tris-Cl (pH 7.6) and 10 μ L USER enzyme mix. Incubate at 37°C for 6 hours.

³DNA mixture can be buffer exchanged with 1x TE utilizing an Amicon Ultra-0.5 column (3K MWCO) at 12K rpm and stored at -20°C if the newly formed NM ICL is not purified immediately.

2. The completion of digestion can be checked by loading an aliquot (~5 pmol) on an analytical denaturing PAGE, and visualizing the DNA by SYBR Gold staining (section 3.3.). Once the digestion is complete, the resected ICL is ready to be purified by denaturing PAGE.

3.3. Purification and Characterization of NM ICLs

1. Assemble the DPAGE sandwich using the long and short glass plates with 2 × 1.5 mm spacers and rest horizontally on a solid support (Note 5).
2. In a 100 mL beaker, prepare 60 mL 15% DPAGE by mixing 45 mL 20% DPAGE gel buffer, 14.4 mL 0% DPAGE gel buffer, 0.6 mL 10% APS, and 20 µL TEMED.
3. Using a serological pipette, cast the gel solution into glass sandwich without introducing bubbles. Place the 1.5 mm semi-prep comb into the sandwich preventing the formation of bubbles, and allow the gel solution to polymerize for at least 40 min.
4. Suspend the NM ICL to be purified in an equal volume of 80% formamide/orange G buffer. Denature the NM ICL sample by heating the solution to 95°C for 5 min, and place sample vial on ice immediately for 5 min or until ready to load.
5. Fix the polymerized 15 % DPAGE sandwich gel vertically in electrophoresis tank (longer plate facing outwards), and fill the buffer reservoirs with 0.5x TBE running buffer to completely cover the wells. Carefully remove the comb, and rinse the formed wells with running buffer. Attach the temperature probe to the outer gel glass, and connect the tank to the PowerPac, setting it to 20 W and 50°C. Pre-run the gel for 40 min or until the temperature reaches 50°C. Rinse the wells with 0.5x TBE to remove excess urea.
6. Load the denatured DNA solution into the wide well, and load tracking dye buffer to the single well.
7. Run the PowerPac at 20 W and 50°C until the bromophenol blue dye reaches the bottom of the glass plate and orange G dye exits the gel (Note 6).
8. Disconnect the power supply and the temperature probe, and dislodge the glass sandwich. With a plastic wedge, carefully separate the plates without breaking the gel.
9. Place the gel on top of a TLC plate covered with saran wrap.
10. In a dark room, hold the UV lamp directly above the gel (254 nm wavelength), and slice the resolved bands: NM ICL 59mer (top band), uncrosslinked 39mer

⁵The sizes of the spacers and combs can vary depending on the amount of DNA to be purified.

⁶For analytical gels, (0.75 mm spacers) 30 mL DPAGE solution is sufficient. Running times can be adjusted depending the DNA sizes to be resolved. Here, we are resolving a 20mer, 39 mer and 59mer, thus the bromophenol blue migrates to about 42 bp in a 15% DPAGE. We use orange G in the loading buffer to visualize the loading. Orange G migrates faster than bromophenol blue thus it completely exits the gel.

(middle band), and uncrosslinked 20mer (bottom band) with a clean scalpel (Fig. 3A, C) (Note 7). Turn off UV lamp.

11. Cut the gel bands further into ~ 1cm gel pieces and save in labeled falcon tubes until next step.
12. DNA isolation from the gel is done via electroelution utilizing an EluTrap system. Rinse two BT1 glycerinized membranes with ultrapure water and mount tightly at the ends of the Elutrap device. Mount one dry BT2 membrane ~2.5 cm away from positive end of the trap (collection chamber) and another dry BT2 membrane ~ 0.5 cm from positive end (gel piece chamber).
13. Load the purified NM ICL gel pieces into the gel piece chamber, and place the Elutrap device horizontally into the Sub-Cell GT electrophoresis tray. Fill all the reservoirs with 5 mM sodium borate buffer (pH 8) covering the gel pieces, the collection chamber and the outer electrophoresis tank.
14. Cover the electrophoresis tank and connect it to the power supply. Run at 200 V for 15 min. Pipette out the buffer in the collection chamber into a clean-labeled vial and place on ice.
15. Measure DNA content via Nanodrop and record the absorbance. Calculate the ng/uL NM ICL recovered.
16. Refill the collecting chamber with 5 mM sodium borate buffer and repeat steps 14–15 at least 2 more times until no DNA is detected in the collected fraction.
17. Pool all the saved fractions into a Amicon Ultra-3K 4 mL filter device and concentrate the DNA using the bench-top centrifuge at 3.5 K rpm, 4°C for 45 min. Buffer exchange with 1x TE at least twice.
18. Pipette the NM-ICL solution out of the Amicon column and save into a new labeled vial.
19. Take 2 µL of NM-ICL solution and dilute with 48 µL water. Briefly vortex and measure UV absorbance via Nanodrop. Calculate ng/uL DNA in both diluted sample and stock solution.
20. MALDI ToF MS is used to verify the mass of the crosslink. Spot 1 µL of MALDI matrix onto a MALDI plate and let dry at room temperature.
21. ZipTip the diluted NM ICL sample (at least 10 pmol) following the manufacturer's protocol (Millipore).
22. Load 1 µL zip-tipped NM ICL on spot containing MALDI matrix and allow to dry at room temperature.
23. Mount the MALDI plate to MALDI carrier and insert it into the AutoFlex II MALDI-TOF mass spectrometer (previously calibrated with known high molecular weight oligo calibrants).

⁷Make sure to wear appropriate personal protection equipment (UV resistant goggles, long sleeve lab coat and nitrile gloves) when shining UV light to the gel. Clean the scalpel every time you slice the different types of gel bands to reduce contamination.

24. Measure the mass/charge ratio (m/z) in a linear negative mode using ion source acceleration voltage of 20.00 kV at a frequency of 50Hz across a m/z of 7000 to 20000 Da. To achieve a high signal-to-noise ratio (SNR), represent each spectrum integrating at least 600 individual laser shots.
25. The collected spectra is analyzed and visualized using the FlexAnalysis 3.0 software (Table 1).
26. To verify the purity of the NM ICL and to quantify any degraded products arising during purification steps, run a 15% analytical DPAGE gel. Use 0.75 mm spacers and a 14 well comb in the DPAGE sandwich. Always run the appropriate controls (e.g. the 20mer and 39mer used for original reaction) along with the NM ICL. Load 2 pmol in 10 μ L 80% formamide/orange G loading dye (Note 6). Run the gel as described above.
27. To visualize the gel via fluorescence detection, suspend the gel in a solution containing 1x SYBR gold for 30 min in the dark.
28. Place gel onto Typhoon fluoroimager plate, and scan gel using the appropriate filter (Fig. 3B, D, Fig. 4).
29. Analyze bands with ImageQuant software, and calculate percentage of degradation (if any).

3.4. Primer Extension Assays with NM ICL analogs

1. Dilute the purified NM ICL analogs and FAM labeled primer P15 (5'-FAM-CACTGACTCTATGATG) to 1 μ M in 1x TE.
2. Mix 7.5 μ L 1 μ M ICL, 2.5 μ L 1 μ M primer P15, 5 μ L 10x annealing buffer (100 mM Tris-Cl pH 8, 500 mM NaCl) and 35 μ L of ultrapure water. Incubate overnight at room temperature to allow annealing (Note 8).
3. For primer extension assays, mix 1 μ L of the annealed mixture, 1 μ L NEB2 buffer, 1 μ L 1 mM dNTPs and 6 μ L ultrapure water.
4. Incubate at 37°C, and add 1 μ L 10 nM Klenow (exo-).
5. Incubate for 5 mins at 37°C, then add 10 μ L formamide loading buffer.
6. Heat to 95°C for 5 mins and then snap chill on ice.
7. Load on a 10% DPAGE gel and visualize bands by scanning the gel using Typhoon fluoroimager (Fig. 5).

5. SUMMARY AND CONCLUSIONS

This chapter outlines methods to synthesize stable NM ICL analogs for biochemical and cell biological studies. Formation of native NM ICLs by reaction of duplex oligonucleotide with nitrogen mustards typically gives rise to a mixture of products with a very low yield of the

⁸Heating of ICLs to 95°C should be avoided to preserve the stability of the ICL. All annealing reactions should be done overnight at room temperature.

desired ICL. Additionally, NM ICLs are hydrolytically unstable, as depurination of the positively charged base in a native NM ICL leads to loss of the ICL and strand breaks (Fig. 1B), thereby severely limiting their use for functional studies. We compared the stability of our modified analogs to that of native NM ICLs (Fig. 4 and 5) and showed that the modified ICLs are stable under the experimental conditions used, whereas the native NM ICLs are not. We describe two approaches to generate analogs that mitigate the limitations of native NM ICLs. In one, the glycosidic bond is stabilized by introduction of a fluorine substituent at the 2' position (2'FdG) (**3**, Fig. 1A), which eliminates depurination, yielding a stable ICL during purification and polymerase assays (Fig. 4 and 5). Although the yield of ICL formation is low, the 2'FdG NM ICL can be synthesized from commercially available DNA oligonucleotides and nitrogen mustards without the need to conduct organic synthesis. Importantly, it contains a positively charged purine ring and only differs from the native ICL by addition of a fluorine atom at the 2' position in the β -orientation, which has been shown to only minimally affect DNA structure (Martin-Pintado, Yahyaee-Anzahae, Campos-Olivas, Noronha, Wilds, Damha et al., 2012). The 7CdG NM ICL requires the synthesis of a specific phosphoramidite precursor (Angelov et al., 2009; Guainazzi et al., 2010), but can be used in any sequence context to generate a site-specific, stable NM ICL analog in a high yielding crosslinking reaction. This approach can furthermore be modified to produce structural variants such as NM ICLs that contain crosslinks of various lengths, allowing for the synthesis of ICLs of different structures for functional studies (Angelov et al., 2009; Mukherjee et al., 2014; Roy, Mukherjee, Sharma, Frank, & Schärer, 2016). 7CdG NM analogs have also been successfully incorporated into substrates for biochemical and cell based DNA repair studies (Ho, Guainazzi, Derkunt, Enoiu, & Schärer, 2011; Hodskinson, Silhan, Crossan, Garaycochea, Mukherjee, Johnson et al., 2014; Pizzolato, Mukherjee, Schärer, & Jiricny, 2015; Räsche et al., 2008; Roy et al., 2016). A possible drawback of the 7CdG NM ICLs is that there are three substitutions in the crosslink compared to the native NM ICL and the crosslinked purines are not positively charged. Although our modeling studies have shown that this induces only minor effects on the DNA structure (Guainazzi et al., 2010), the main stalling point of Klenow at the 2'FdG ICL and 7CdG ICL differs by one nucleotide (compare Fig. 5B, lanes 4 and 5), indicating that the charge may influence interaction with proteins. Thus, we anticipate that the two types of modified NM ICLs will each be useful for specific applications.

In summary, we describe the generation of two types of stable NM ICL analogs that have been and will continue to be of use to the scientific community to study biological pathways triggered by ICLs.

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Figure 1. Native and stable NM ICLs

A. Nitrogen mustard (NM) interstrand crosslinks (ICLs) formed by reaction of DNA with NM (**1**) and stable analogs in which the glycosidic bond is stabilized by using 7-deazaguanine bases (**2**, 7CdG-5a), to remove the positive charge in the purine ring) or 2'-fluoro-deoxyribose sugars (**3**, 2'FdG), to prevent depurination by destabilizing the positive charge formed during glycosidic bond cleavage). The approach to generate 7CdG ICL additionally allows for the synthesis of structurally diverse ICLs, such as **4** (7CdG-8a). **B.** The positive charge in the native NM ICL destabilizes the glycosidic bond (**5**) and leads to depurination (**6**) resulting in the formation of an abasic site (**7**) and strand cleavage (**8**).

Figure 2. Scheme for the formation of NM ICLs

A. Native or 2'FdG NM ICLs are formed upon reaction of a duplex DNA containing a site specific 5'-GNC-3' sequence with excess mechlorethamine. **B.** 5a or 8a 7CdG NM ICLs are formed by oxidation of the diol-bearing 7-deaza-guanosines (incorporated within a 5'-GNC-3' sequence in a duplex) with sodium periodate to the aldehydes followed by reductive amination using hydrazine or DMEDA in the presence of sodium cyanoborohydride. **C.** Resected NM ICLs are produced by cleavage of uracil residues flanking the ICL with USER mix (UDG and ENDOVIII).

Figure 3. Purification of stable NM ICL analogs

A. Separation of a 25 nmol 2'FdG NM ICL reaction by 15% DPAGE and visualization by UV shadowing. **B.** 3 pmol of the annealed duplex (lane 1), crude reaction mixture (lane 2) and the purified 2'FdG ICL (lane 3) were resolved by 15% denaturing PAGE and visualized by SYBR gold staining. **C.** Separation of 30 nmol of a 5a 7CdG NM ICL formation reaction by 10% denaturing PAGE and visualization by UV shadowing. **D.** 5 pmol of the 39mer (lane 1), crude ICL reaction (lane 2) and the purified 5a ICL (lane 3) were resolved by 10% denaturing PAGE and visualized by SYBR gold staining. The positions of the ICL, 39mer and 20mer single-stranded oligonucleotides are indicated.

Figure 4. Analysis of purified NM ICLs by 15% PAGE

3 pmol 39mer (lane 1), NM ICL analogs (lanes 2–7) and Native NM ICL (lane 8) were resolved by 15% denaturing PAGE and DNA visualized by SYBR Gold staining. The NM ICL analogs were either untreated (lanes 2,4,6) or treated with 0.1 U/ μ L USER for 6 hours at 37°C to generate the resected forms of the ICL (lanes 3,5,7). Note that the native NM ICL decomposed during purification, isolation and analysis, while the modified NM ICLs show no significant decomposition.

Figure 5. NM ICL analogs block the polymerase reaction by Klenow (exo-) fragment

A. Substrates used for primer extension assay. The modified base and crosslink are highlighted in red. **B.** Primer extension assay of full length NM ICL templates with Klenow using non-crosslinked controls (lanes 2 and 3), and full NM ICL-containing templates (lanes 4–7). Note that decomposition of the ICL in the native NM ICL allow for bypass, while the stable analogs block the polymerase. **C.** Primer extension with resected NM ICLs. Primer (lane 1), non-crosslinked control (lane 2) and resected NM ICLs (lanes 3–5). All substrates were annealed to the FAM labeled primer P15 and incubated with 1 nM Klenow for 5 min at 37°C. Products were resolved by 10% denaturing PAGE.

TABLE 1

MALDI-ToF MS of synthesized NM ICLs

Name	Calculated mass [M-H] ⁻ , Da	Experimental mass [M-H] ⁻ , Da	Error, %
Full 5a deaza NM ICL	18179.9	18136.4	0.2
Full 8a deaza NM ICL	18194	18226.9	0.2
Full FANA NM ICL	18200.95	18251	0.3