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Characterization of Aziridinybenzoquinone DNA Cross-links by LC-IRMPD-MS

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Abstract

DNA cross-linking was evaluated by LC-MS/MS to determine the relative cross-linking abilities of two aziridinybenzoquinones. Reactivities of RH1 (2,5-diaziridiny-3-[hydroxymethyl]-6-methyl-1,4-benzoquinone), a clinically studied anti-tumor cross-linking agent, and an analog containing a phenyl group (PhRH1) rather than a methyl group was compared. The bulky phenyl substituent was added to determine the impact of steric hindrance on the formation of cross-links within a double helical structure. Cross-links formed by RH1 and PhRH1 (2,5-diaziridiny-3-[hydroxymethyl]-6-phenyl-1,4-benzoquinone) were observed at 5'-dGNC sites as well as 5'-dGAAC/dGTTC sites. RH1 was more effective at forming cross-links than PhRH1 for a variety of duplexes. Infrared multiphoton dissociation (IRMPD) and collision induced dissociation (CID) results confirmed the presence and the location of the cross-links within the duplexes, and IRMPD was used to identify the dissociation pathways of the cross-linked duplexes.

Introduction

DNA interactive anti-tumor agents can function through non-covalent interactions, such as intercalators(1,2) and minor groove binders,(3,4) or through covalent modifications of the nucleobases (5–9). In many cases, the mechanism of action is to alter the structure of DNA sufficiently that replication machinery does not recognize relevant binding sites, preventing proliferation (10,11), or the cellular apoptosis pathway is triggered (12,13). While interstrand cross-links occur relatively infrequently in comparison to monoadducts or intrastrand cross-links, the greatest cytotoxic effects of many anti-tumor agents are thought to be based on the formation of key interstrand cross-links (14–17). While cross-links can vary greatly in size and position, a major mode of cytotoxicity is the prevention of strand separation during replication (16).

As anti-tumor agents are designed to prevent cells from replicating, ideally they should exhibit a high level of specificity for tumor cells to prevent widespread death of healthy cells. Bio-reductive agents use the cellular environment of tumor cells to provide specificity (18–20), entering the cell in an inactive, oxidized form and are reduced by enzymes within the cell to become cytotoxic. This reduction is optimized under the hypoxic conditions which are frequently found in tumor cells (19). The most well studied of these is mitomycin C which covalently binds two guanine bases after chemical or enzymatic reduction (21–23). Formation of monoadducts and both interstrand and intrastrand cross-links are possible for mitomycin C with monoadduction being the dominant type of DNA modification (22,23). Mitomycin C has been studied extensively and used clinically in the treatment of many cancers including bladder (24), colorectal (25,26), and gastrointestinal tract (27). Other

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bio-reductive compounds have also been developed including nitroaromatics, aliphatic N-oxides, and heteroaromatic N-oxides (18). All of these types of compounds are reduced by a variety of cellular reductases before becoming biologically active. The types of interactions between the activated compounds and DNA can range from non-covalent intercalation to cross-link formation (18).

Another class of bio-reductive agent is based on the quinone moiety. Benzoquinones with bifunctional mustard moieties have been found to cross-link DNA and the efficiency of the reaction is based upon the other quinone substituents (28–30). Aziridinybenzoquinones contain aziridine rings that react with DNA upon conversion of the quinone moiety to the hydroquinone (31). The reduction to the hydroquinone increases the basicity of the aziridine groups, increasing protonation and improving crosslinking yields at physiological pH. Several of these ligands have been found to react with DNA, and the reactivity has been shown to be extremely sensitive to the other substitutions on the quinone (31–34). One of these agents, RH1, has been studied clinically for its anti-tumor properties (34). Most recently, it was evaluated for possible use as a chemotherapeutic drug for childhood cancers including neuroblastomas, osteosarcomas, and Ewing's sarcoma (35). The two aziridine rings allow cross-linking of guanines via the N7 atoms, and the methyl and hydroxymethyl substituents have been found to support cross-linking specifically at 5'-dGNC sequences (31) (Scheme 1). The presence of the cytosine in this sequence ensures that this guanine will be in close proximity to a guanine on the opposite strand.

Mass spectrometry has proven to be a versatile tool for characterization of both covalent and non-covalent interactions of ligands with DNA and cross-links in particular. Traditional techniques for analyzing DNA adducts, such as NMR (36,37) or gel electrophoresis methods (38,39), are time consuming and typically have lower sensitivity than mass spectrometry. A variety of DNA binding characteristics can be determined using mass spectrometry. For example, the specificity of binding as well as the binding stoichiometries and sequence affinities of DNA interactive ligands can be determined by using ESI-MS (40–48). In some cases, the binding mode of non-covalent interactions can be determined through tandem mass spectrometry (49). In addition, chemical probes in conjunction with dissociation techniques can be used to map the sites of DNA/ligand interactions (50). Ion activation/dissociation techniques can be used to identify the site of reaction for covalently bound ligands (51) and can also be used to characterize the actual atomic linkages between the ligand and the DNA (52–54). Complex samples containing low levels of adducts can be effectively studied by coupling tandem mass spectrometry with HPLC as a front-end separation technique (55–57). These techniques have been used both with small oligodeoxynucleotides (51,58) as well as with larger, biological DNA samples (59).

In this work, the cross-linking abilities of two aziridinybenzoquinone ligands (Figure 1) with duplex oligodeoxynucleotides are characterized. The cross-linking efficacy of the phenyl analog, PhRH1, is compared to the cross-linking ability of RH1 to determine the effect of the phenyl substituent on binding and cross-linking. The bulky phenyl group is expected to cause greater steric hindrance to cross-linking. Comparing the abilities of the two ligands to cross-link various duplexes affords insight into how the quinone substituents modulate the reactivities of the ligands. Further structural characterization by IRMPD-MS provides information on the location of the cross-links within the oligodeoxynucleotides.

Experimental Procedures

Materials

Caution—RH1 and PhRH1 should be considered toxic and handled accordingly. Oligodeoxynucleotides, as listed in Table 1, were purchased from IDT DNA, Inc.

(Coralville, IA) and resuspended in HPLC grade water. RH1 was synthesized as previously described (34,60). The phenyl analog, PhRH1, was custom synthesized (see Supplemental Information for synthesis details). Ammonium acetate, HPLC grade water, and HPLC grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Sodium acetate was purchased from EM Science (Gibbstown, NJ).

Methods

Annealing—Duplexes were created from two complementary oligodeoxynucleotides that were annealed in 150 mM ammonium acetate by placing the oligodeoxynucleotides in a 90°C water bath and allowing the two strands to cool overnight to room temperature, over approximately 12 to 14 hours. The annealed duplexes were stored in the freezer.

Reduction/cross-linking reaction—Approximately 2 μ mol RH1 or PhRH1 was placed in a conical vial. For RH1, 100 μ l of 100 mM sodium acetate (pH 6) was added along with 6.5 nmol of annealed duplex DNA. The same conditions were used for PhRH1, with the exception that PhRH1 was dissolved in 20 μ l of DMSO before the addition of the sodium acetate. The solution containing the quinone ligand and the duplex oligonucleotide was placed in an ice bath with argon bubbled through it. In a separate flask, an approximately 0.5 M solution of sodium hydrosulfite in 100 mM sodium acetate (pH 6) was also kept on ice and under argon. Two 20 μ l aliquots of the sodium hydrosulfite solution were added to the quinone/duplex solution 25 minutes apart. Overall, the reaction took fifty minutes. The solution was then exposed to air. Following the reaction, each quinone/duplex solution was desalted using a 3 kDa molecular weight cutoff filter (Amicon Ultra, Millipore, Billerica, MA) followed by a 0.22 μ m Durapore filter (Amicon Ultrafree-MC, Millipore, Billerica, MA).

LC-MS—The desalted solutions were analyzed by capillary LC-MS. A Dionex (Sunnyvale, CA) Ultimate 3000 capillary HPLC system fitted with a 0.3×150 mm Zorbax 300SB-C18 column (5 μ m particle size, Agilent, Santa Clara, CA) was used for the separation. The aqueous mobile phase (A) was 10 mM ammonium acetate and the organic mobile phase (B) was 50% 10 mM ammonium acetate/50% ACN. The gradient elution method varied depending on the sequences of interest, but the general method involved a gradient from 99% A to 85–80% A over 38 minutes. The flow rate was 4 μ l/min. The LC system was directly coupled to a Thermo Scientific (San Jose, CA) LTQ XL linear ion trap mass spectrometer. The mass spectrometer was operated in the negative ESI mode and the capillary temperature was set to 120°C. The system had been modified for IRMPD by the addition of a zinc selenide window at the back of the instrument as described previously (61). IRMPD was achieved using a Synrad (Mukilteo, WA) 50W CO₂ laser at a wavelength of 10.6 μ m. For these experiments, the laser power was set to approximately 10 W, and the laser was triggered during the activation step for 2 to 10 ms. DNA fragments were labeled according to established nomenclature (62).

Relative quantitation—To determine the relative cross-linking efficiencies of the quinones with the duplexes, peak areas were determined from the UV chromatogram. The UV chromatograms were used for relative quantitation to alleviate discrepancies due to variable DNA ionization efficiencies from the results. The peak areas for the cross-linked duplexes were divided by the summed peak areas of all DNA species, including both cross-linked DNA and unmodified single strands, as shown in the following equation:

$$\text{Relative Crosslinked Duplex (\%)} = \frac{\frac{PA_{CL}}{\epsilon_a + \epsilon_b}}{\frac{PA_{CL}}{\epsilon_a + \epsilon_b} + \frac{PA_{ssa}}{\epsilon_a} + \frac{PA_{ssb}}{\epsilon_b}} \quad (1)$$

where PA_{CL} is the peak area of the cross-linked duplex and PA_{ssa} and PA_{ssb} refer to the peak areas of each of the unmodified single strands. Each peak area was normalized with the molar extinction coefficient for the corresponding oligonucleotide sequence, ϵ_a or ϵ_b . For the cross-linked duplex, the two extinction coefficients were summed with the effect of the cross-linker ignored. Due to the low absorptivities of RH1 and PhRH1 at 260 nm, their contributions to the total absorbances were neglected in the calculation of the cross-linking efficiencies. The unmodified intact duplexes did not survive the separation conditions and thus were not observed.

UV/vis thermal denaturation curves—DNA melting studies were performed on a Varian Cary Bio 100 UV/vis spectrophotometer (Palo Alto, CA) outfitted with a Peltier temperature control system. The concentration of the duplex was 5 μ M in 100 mM sodium acetate (pH 6). The duplexes were heated at 1 $^{\circ}$ C/min over a temperature range of 15–80 $^{\circ}$ C. The melting point was determined by finding the maximum of the first derivative curve.

Results and Discussion

Both LC-MS and LC-UV methods were used to characterize the reactions between RH1 or PhRH1 and oligodeoxynucleotide duplexes. The relative abundances of the cross-linked duplexes were compared for different duplexes upon reaction with RH1 and PhRH1, and the fragmentation patterns of the cross-linked duplexes revealed the positions of cross-link formation.

LC-MS detection of cross-links

RH1 was incubated with each selected duplex and reacted under the conditions specified in the experimental section to form the reduced hydroquinone and to encourage the production of interstrand cross-links. Cross-links were formed between RH1 and five of the duplexes including DS1, DS2, DS3, DS4, and DS5, but not with DS6, DS7, and DS8, as described later. For the five positive cases, the species detected by LC-MS included the unmodified single strands, the cross-linked duplexes, and low abundances of single strand monoadducts. Examples of ESI mass spectra arising from the LC-MS experiments are shown in Figure 2 and display the multi-charged molecular ion signatures of the single strands, the monoadducts, and the cross-linked duplexes. Intact unmodified (i.e. non-cross-linked) duplexes were not detected. While G/C rich duplexes may remain intact during HPLC separation and ESI-MS analysis, the organic solvent component of the mobile phase disrupts the weaker hydrogen bonding interactions of A/T rich duplexes, thus preventing their survival and detection. In general, the cross-linked duplexes were typically detected in the 5- and 6- charge states. Under similar reaction conditions, PhRH1 formed cross-links with the same duplexes as formed with RH1. The PhRH1-cross-linked duplexes tended to elute slightly later than the RH1-cross-linked duplexes. Monoadducted single strands were not observed in high abundances for either RH1 or PhRH1. With many cross-linkers, monoadducts are more abundant than cross-links; however, the reaction mechanism of RH1 and other aziridinylbenzoquinones enhances cross-link formation. After the first aziridine group has opened and has alkylated N7 on the guanine base, an electron pair is donated to the aromatic ring system which increases the reactivity of the remaining aziridine ring improving the yield of the cross-link over the monoadduct (31). The low levels of monoadducts could not be accurately quantified due to chromatographic overlap with other

more abundant DNA base loss products. As a control experiment, RH1 was incubated with DS5 under the same conditions usually employed albeit without the reducing agent. No crosslinks were observed, thus indicating that the reduction of RH1 from the quinone to the hydroquinone is crucial to crosslink formation.

To compare the reaction efficiencies of RH1 and PhRH1, the relative percentages of cross-linked duplexes were calculated as described above in Equation 1 (Figure 3). Cross-linking of the duplexes by RH1 appeared to be sensitive to the overall sequence of the duplex. The relative cross-linking efficiencies of RH1 with DS1, DS3, and DS4 were all similar (approximately 4%). DS1 and DS4 both have target sequences of 5'-dGNC while DS3 has a target sequence of 5'-dGAAC/5'-dGTTC. DS4 has two cross-linking sites each with 5'-dGAC/5'-dGTC sequences. At first glance, a comparison of the cross-linking yields for DS2, with one 5'-dGAC/5'-dGTC target site, and DS4, with two 5'-dGAC/5'-dGTC target sites, would appear to indicate a linear increase in cross-linking with the number of target sequences. However, MS/MS characterization of the cross-linked DS4, as discussed below, indicates that only one of the two target sites actually undergoes cross-linking. This would suggest that the increase in cross-linking efficiency of DS4 is not due to the increase in the number of target sequences but could be related to the change in duplex conformation and stability that occurs when G/C rich sequences are located at the termini of oligodeoxynucleotides. These differences were further explored by determining the melting temperatures of the five duplexes which are discussed in more detail below. The cross-linking efficiency of DS5 was significantly greater than observed for the other four duplexes. This duplex contains two target sites with 5'-dGAC/5'-dGTC and 5'-dGAAC/5'-dGTTC sequences. The increase in cross-linking, in combination with dissociation results discussed below, indicates that cross-linking may occur at either target site which therefore increases the probability of cross-linking.

For reactions of PhRH1, the lowest cross-linking efficiency was observed for DS1, indicating that this target site is the least accessible for reaction with the bulky phenyl analog. Cross-linking of DS2, DS3, and DS4 were generally similar, suggesting that the longer target sequence of DS3 did not provide an increase in flexibility that might enhance PhRH1 cross-linking. The increase in binding to DS4 over DS1, similar to the increase observed with RH1 as the crosslinker, could not be attributed to the presence of a second crosslinking site as the fragmentation pattern indicated no crosslinking occurs at the terminal crosslinking site. The increase in crosslinking, as was observed with RH1, is attributed to the change in duplex structure due to higher G/C content. As with RH1, the cross-linking of DS5 increased significantly over that of the other four duplexes, and cross-links at both target sites were confirmed by tandem MS.

The cross-linking efficiencies of PhRH1 were lower than RH1 for all five duplexes. The difference in cross-linking efficiency was especially notable for DS1, suggesting that the target site of DS1 is fairly specific for the smaller RH1 and intolerant of the bulkier PhRH1. The difference in cross-linking efficiencies was also significant for DS3, DS4, and DS5, but the difference was diminished for reactions of RH1 and PhRH1 with DS2. The similarity in cross-linking for DS2 might reflect greater flexibility in the target site which would permit PhRH1 cross-linking to a similar extent as RH1. These results were probed further by examining the melting temperatures of the five duplexes, as follows.

To further investigate the underlying reasons for variations in cross-linking by RH1 as a function of the DNA sequences, melting temperatures were measured for the first five duplexes in Table 1. The melting points spanned the range of 36.1 to 47.9 °C (Table 2), and the order of melting temperatures was DS2 < DS1 < DS3 < DS5 < DS4. The sequences with A/T rich terminal sequences, DS1, DS2, and DS3, all have lower melting temperatures than

the sequences with G/C bases near the ends which is consistent with the well-known impact of the weaker hydrogen-bonding interactions of A/T versus G/C base pairs. DS2 is the least thermally stable duplex and had the lowest cross-linking efficiency by RH1. In addition, the cross-linking efficiencies obtained for DS2 with PhRH1 or RH1 were very similar- a result not observed for the other duplexes. It is possible that the helix was destabilized and partially distorted, thus preventing RH1 from forming a stable interstrand cross-link. The higher melting temperatures of the other sequences suggest greater stability which would better allow the duplex to remain in a conformation more suitable for cross-linking. DS4 and DS5, which contain the same target sequence as DS2, had melting temperatures approximately 12 °C and 8 °C higher than DS2, respectively. The additional stability of these two duplexes would allow the duplex to remain in a helical structure most conducive to cross-linking which explains the increased difference in cross-linking between RH1 and PhRH1 in comparison to DS2.

In addition to the duplexes described above, the reactions of RH1 with three other duplexes (DS6, DS7, DS8) resulted in no cross-linking although monoadducts were observed for the individual single strands of both DS6 and DS7 which contain guanine residues. The lack of cross-linking to either DS6, a self-complementary 12-mer with a 5'-dCG binding site, or DS7, a 14-mer with a 5'-dGC binding site, suggests that a minimum distance between guanine bases is required to facilitate cross-linking. With the 5'-dCG or 5'-dGC sequences, the guanine bases are on adjacent base pairs- a distance too constricted for optimal bridging by the RH1 ligand. Collisional activation of the monoadducted single strands of DS6 or DS7 resulted in loss of the modified guanines and subsequent backbone fragmentation, pathways consistent with covalent modification of the DNA solely at the guanine bases. DS8 was chosen to determine if reactions with adenine or thymine were possible in the absence of guanine. No monoadduction or cross-linking was observed for this duplex.

Fragmentation Patterns of Cross-linked Duplexes

The sites of the cross-linking reactions were probed based on the fragmentation patterns generated upon CID or IRMPD of the cross-linked DNA/RH1 ions. Examples of the resulting CID and IRMPD mass spectra are shown in Figure 4 for (DS3 + RH1)⁶⁻, and similar patterns were observed for all the cross-linked duplexes in the 6-charge state. CID spectra of the cross-linked duplexes in the 6- charge state generally resulted in fragmentation occurring at the site of cross-linking. For instance, cleavage of the N-glycosidic bond between the guanine and deoxyribose sugar resulted in the formation of complementary ions: single strand ions with one guanine lost and single strand ions containing both the cross-linker and an extra guanine (the one lost from the companion strand). A summary of the general fragmentation pathways of the cross-linked duplexes is shown in Scheme 2. The retention of the cross-linker and one guanine from the opposite strand confirms that cross-linking is occurring between the two strands. While these complementary fragment ions are helpful for confirming that interstrand cross-linking has occurred, they do not reveal the specific site of cross-linking. Backbone fragmentation occurs as well, resulting in the production of a – B and w ions. The 5-charge states of the cross-linked duplexes were also examined by CID. As has been seen with duplexes in low charge states in the past (63), the major dissociation pathway was loss of unmodified bases. Backbone fragmentation, similar to what was observed upon dissociation of the 6- charge state, was also observed.

As an alternative to conventional CID, IRMPD was also used to characterize the cross-linked duplexes. In IRMPD, ions are energized by absorption of IR photons rather than by a collisional process, typically affording very efficient dissociation with the extent of fragmentation dependent on the irradiation time. For very low irradiation times, the most abundant product occurred due to the loss of water. Using slightly longer irradiation times (3 ms), CID-like spectra were produced. The dominant fragmentation process involved

separation of the strands via cleavage adjacent to a cross-linked guanine site, resulting in formation of complementary single strands, either with a guanine loss or with retention of the RH1 cross-linker plus an extra guanine from the other strand (see Scheme 2). Low abundances of backbone fragment ions, such as w_8^{2-} ions from both strands, were also observed. Increasing the irradiation time to 5 ms promoted secondary dissociation of the single strand species described above, yielding backbone fragment ions as shown in Figure 4. For example, both the pair of w_8^{2-} ions and the complementary $a_6 - G$ ions are observed, in this case confirming the site of cross-linking at those guanines as illustrated in Scheme 2.

CID and IRMPD spectra were also obtained for the cross-linked DS1 and DS2 products (spectra not shown). As with DS3, fragmentation was observed at the site of cross-linking with the primary cleavage occurring at the N-glycosidic bond between the modified guanine and the sugar. Backbone fragments indicating fragmentation 3' to the cross-linked nucleosides were observed for both cross-linked duplexes.

IRMPD was especially useful for characterizing the cross-linked DS4 duplex (Figure 5a). There are two potential cross-linking sites, and it was unclear whether a terminal target sequence would have the correct secondary structure to permit cross-linking. The IRMPD mass spectrum of the RH1 cross-linked duplex in the 6- charge state after 5 ms of irradiation time shows the $(a_4 - G)^-$ ion from ssb as the most abundant fragment. The corresponding w_{10}^{2-} ion is also observed. Fragments from the other strand include the $(a_9 - G)^{2-}$ ion as well as the complementary w_5^- ion. The significant abundance of these fragment ions, as well as the presence of both sets of complementary fragment ions indicates that the majority of the cross-linking occurred at the internal target sequence. There is a low abundance fragment ion corresponding to the w_2^- fragment from strand b. This could indicate cross-linking at the terminal guanine, but there are no complementary fragment ions observed such as the $(a_{12} - G)^{2-}$ - a fragment ion which would be within the m/z range of the instrument. This suggests that the fragmentation at that base is not related to cross-linking.

As with DS4, there were two possible sites of cross-linking for duplex DS5: a 5'-dGAC/5'-dGTC sequence and a 5'-dGAAC/5'-dGTTC sequence. A duplex with two different binding sites that vary in the distance between the guanines was chosen to determine if a cross-linking preference would be seen between the two target sequences. As shown in Figure 5b, the IRMPD spectrum acquired after 3 ms irradiation displays the single strand-type fragment ions described earlier (both single strands with guanine loss and single strands with the additions of the cross-linker and the guanine from the opposite strand). This indicates that cross-links are present but does not indicate to which guanine the cross-linkers were attached. Backbone fragments from strand a present in the dissociation spectrum consist of the $a_3 - G$ and w_{11}^{2-} ions and the $(a_9 - G)^{2-}$ and w_5^- ions. Strand b fragment ions are $a_3 - G$ and w_{11}^{2-} ions and the $(a_{10} - G)^{2-}$, $(a_{10} - G)^{3-}$ and w_4^- ions. These sets of complementary fragment ions correspond to fragmentation at both possible cross-linking sites, indicating that both cross-linking sites are active. In addition to the complementary fragments, two backbone fragments containing the cross-link were observed, including w_{11} ions from each strand with each retaining the cross-linker RH1 and an extra guanine. The w_{11} ion usually does not retain the neighboring base (from the same strand) after fragmentation, but the ions are relatively low in abundance. The addition of the cross-linked guanines to the w_{11} ions indicate that, for these particular ions, the cross-link occurred at the 5' guanine on each strand. The presence of this ion from each strand is a further indication that both sites, the 5'-GNC and the 5'-GNNC sites, participate in interstrand cross-linking.

To map the fragmentation trends and pathways in more detail, time-variable IRMPD experiments were undertaken. For this strategy, the irradiation time was varied to alter the total energy deposition as well as the secondary activation of primary product ions. The

resulting graph obtained for $[DS4 + RH1]^{6-}$ ion is shown in Figure 6. The time-variable curves for the other cross-linked duplexes were similar. As the irradiation time was increased, the proportion of informative backbone fragments increased. Even longer irradiation times resulted in both an increase in backbone fragmentation at the site of cross-linking and additional backbone fragments remote from the cross-linking sites with the latter remaining relatively minor. The graph in Figure 6 indicates that the first dissociation event is cleavage between the cross-linked guanine and the sugar, leading to strand separation. These primary product ions then dissociate further to produce backbone fragments at the cross-linked nucleobase. These major backbone fragmentation patterns are summarized in Figure 7 for all five duplexes. Further irradiation leads to high energy fragments along the backbone away from the cross-linked guanines to form. Not only can IRMPD be used to identify cross-links and where they occur, but it can also be used to determine fragmentation pathways.

Conclusions

RH1 was found to form cross-links between oligonucleotide duplexes with several target binding sequences. In addition to two 5'-dGNC sequences, RH1 formed cross-links at a binding site with a 5'-dGAAC sequence indicating that the cross-link is capable of spanning an additional distance. However, no cross-links were produced for a duplex containing a 5'-GC sequence, suggesting that a minimum distance between the guanine bases is necessary for cross-linking. In addition, the terminal binding site did not result in formation of cross-links indicating that a local helical structure is necessary for cross-linking. The replacement of the methyl group of RH1 by a phenyl group (PhRH1) resulted in lower cross-linking efficacies for all of the duplexes studied. For the cross-linked duplexes, IRMPD provided key information for determination of the sites of cross-linking. IRMPD of the crosslinked duplexes resulted almost entirely in fragmentation at the site of the crosslink. This greatly simplified the identification of the site of crosslinking.

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Figure 1.
Aziridinyl benzoquinones.

Figure 3.
Relative percentages of crosslink formation for RH1 and PhRH1.

Figure 4.

CID (a) vs. IRMPD (b) spectra for $[\text{DS3}+\text{RH1}]^{6-}$. Ssa and ssb refer to the single strands as described in Table 1. The irradiation time for IRMPD was 5 ms. Fragments from ssa are in bold italics.

Figure 5. IRMPD spectra for selected crosslinked duplexes. Dissociation spectrum from [DS4+RH1]⁶⁻ after 5 ms irradiation time and dissociation spectrum from [DS5+RH1]⁶⁻ after 3 ms irradiation time. Fragments from ssa are in bold italics. Fragments containing the crosslink are noted by ■.

Figure 6.
Time-variable IRMPD of $[DS4+RH1]^{6-}$.

Figure 7.
Major fragmentation pathways for duplexes crosslinked with RH1 or PhRH1.

Scheme 1.

Reduction mechanism and possible crosslink structure for RH1. Adapted from ref. 36.

Scheme 2.

Fragmentation process of a duplex with an interstrand crosslink at two guanine bases after IR irradiation.

Table 1

Oligonucleotide sequences used in this work

Name	Sequence
DS1	a: 5'-ATATAGCCTATAT-3' b: 3'-TATATCGGATATA-5'
DS2	a: 5'-AAATATGACTATAT-3' b: 3'-TTTATACTGATATA-5'
DS3	a: 5'-AAATTGAACAAATT-3' b: 3'-TTTAACTTGTTTAA-5'
DS4	a: 5'-GACAAATTGACATA-3' b: 3'-CTGTTTAACTGTAT-5'
DS5	a: 5'-TAGACATAGAACAA-3' b: 3'-ATCTGTATCTTGTT-5'
DS6	a: 5'-ATATACGTATAT-3' b: 3'-TATATGCATATA-5'
DS7	a: 5'-AAATATGCATATAT-3' b: 3'-TTTATACGTATATA-5'
DS8	a: 5'-AATTAATTAATTAA-3' b: 3'-TTAATTAATTAATT-5'

Table 2

Melting temperatures of DS1-DS5

Double Strand	Melting Temperature (°C) ^a
DS1	38.2 ± 0.2
DS2	36.1 ± 0.4
DS3	41.6 ± 0.3
DS4	47.9 ± 0.3
DS5	44.1 ± 0.4