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PREPARATION OF INTERSTRAND CROSS-LINKED DNA OLIGONUCLEOTIDE DUPLEXES

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1. ABSTRACT

Reaction of cellular DNA with environmental and chemotherapeutic agents can give rise to a variety of lesions including interstrand cross-links. Because interstrand cross-links can prevent DNA strand separation and thus DNA transcription and replication, they represent a serious impediment to cell survival. Cells have developed mechanisms to repair interstrand cross-links in their DNA and in the case of tumor cells, this can lead to resistance to chemotherapeutic agents. Efforts to investigate the mechanisms by which interstrand cross-links are repaired have been hampered by the difficulty of preparing sufficient quantities of well characterized substrates for physical and biochemical studies. This review will describe synthetic strategies that have been developed to synthesize short DNA oligonucleotide duplexes that contain interstrand cross-links. These short duplexes can be used to study the effects of the cross-link on DNA structure or they can be ligated with larger DNA molecules to produce substrates for repair studies. This review will focus on examples of cross-linked duplexes that have been designed specifically to further our understanding of interstrand cross-link structure and repair.

2. INTRODUCTION

Cellular DNA is subject to a wide variety of endogenous and environmentally induced modifications, including base oxidation, deamination, depurination and formation of interstrand cross-links (1). The latter lesion covalently links the two complementary strands of DNA (Figure 1). Interstrand cross-links present a particularly serious problem to the cell because they prevent DNA strand separation and thus interfere with two critical cellular events, DNA transcription and DNA replication.

Interstrand cross-links can arise as a result of normal cellular processes such as the oxidation of lipids, which produces unsaturated aldehydes, such as acrolein or crotonaldehyde (2,3). These compounds act as bifunctional alkylating agents and cross-link guanine residues in DNA (4). Unsaturated aldehydes are also prevalent in the environment and occur as products of combustion and industrial processes (5).

Irradiation of DNA in the presence of intercalating drugs such as trimethylpsoralen leads to the formation of interstrand cross-links (6). The photoactivation of DNA/psoralen complexes with long wavelength ultraviolet light results in photoadduct formation between psoralen and pyrimidine bases in DNA. When psoralen intercalates at 5'-d(TA) sites, interstrand cross-links can form between the two thymines on opposite strands of the DNA.

Cross-links can also be introduced as a consequence of chemotherapy. Many clinically important cancer chemotherapeutic agents are bifunctional alkylating agents (7-10). These compounds include the nitrogen mustards, chloroethylnitrosoureas, cyclophosphamides, mitomycin C and platinum compounds. They react with

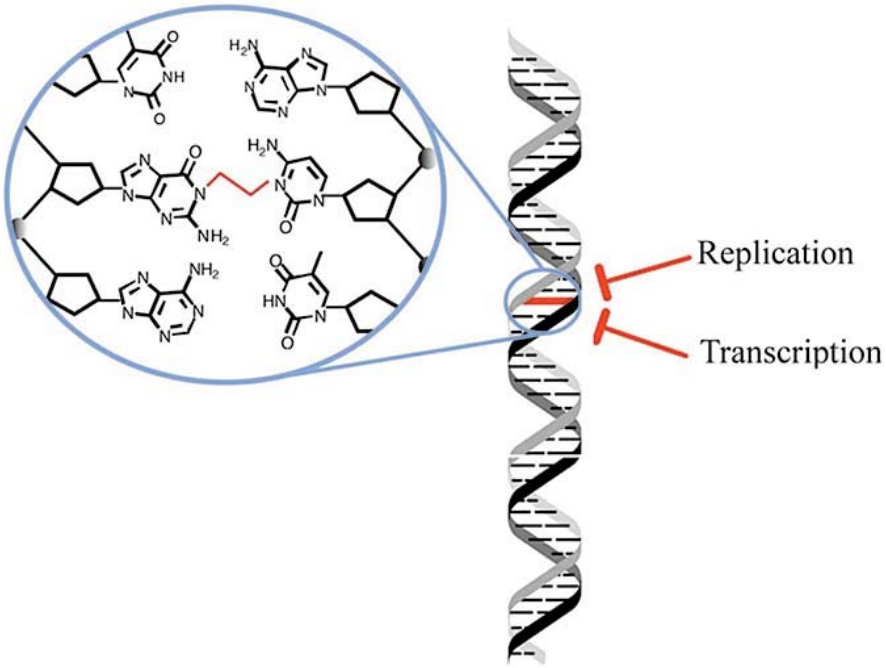

Figure 1. DNA with an Interstrand Cross-link.

DNA to produce, among other lesions, interstrand cross-links, which are considered to be the therapeutically effective lesions in cancer chemotherapy (11-15). There is considerable evidence that indicates interstrand cross-links are recognized by cells and that this recognition can mediate either repair of damage and survival, or accelerated death of the cell (14,16-19). Development of tumors resistant to nitrogen mustards is often an important factor in the lack of response by some patients to therapy with these agents and removal of interstrand cross-links can play a significant role in this resistance (20). Thus, for example, specific resistance of a medulloblastoma cell line to cyclophosphamide and other nitrogen mustards was shown to be associated with interstrand cross-link removal (21,22).

Relatively little is known about the mechanism(s) by which interstrand cross-links are recognized and repaired (1,19,23). Unlike repair of damaged or modified bases, removal of interstrand cross-links is complicated by the presence of an absolute block to replication, the interstrand cross-link itself, and by the absence of a lesion-free strand that can serve as a template for DNA replication. Thus even if the cross-link lesion is removed from one strand of the duplex, the other strand still retains the lesion.

Studies to investigate repair mechanisms rely on the availability of DNA substrates with defined interstrand cross-links. Such substrates can be difficult to prepare and characterize in quantities sufficient for physical and biochemical studies. This review will describe strategies that have been developed to synthesize short DNA oligonucleotide duplexes that contain interstrand cross-links. Such duplexes can be used alone to study the effects of the cross-link on DNA structure or the duplexes can be ligated with larger DNA molecules to produce substrates for repair studies. The present discussion will focus on examples of cross-linked duplexes that have been specifically designed to further our understanding of cross-link structure and repair.

3. SYNTHETIC STRATEGIES

Perhaps the most straightforward strategy for producing interstrand cross-linked DNA involves postsynthetic modification (Figure 2). A short DNA duplex, 10 - 12 base pairs in length, is prepared by annealing two chemically synthesized complementary strands. The duplex is then treated with the cross-linking agent, which in most cases is a bifunctional alkylating reagent. This process usually generates a mixture of products that include: (1) monoadducts, which are formed on either the Watson or Crick strand of the duplex; (2) intrastrand cross-links, which result from reaction of two base residues on the same strand of the duplex; and (3) the desired interstrand cross-link. The interstrand cross-linked duplex is then separated from unreacted duplex and other products of the reaction typically by polyacrylamide gel electrophoresis. Under denaturing conditions, the electrophoretic mobility of the interstrand cross-linked duplex is considerably lower than that of the individual strands of the duplex or their mono- or intrastrand adducts. The overall yield of the interstrand cross-linked duplex depends upon the nature of the cross-linking agent and the stabilities of the products formed. For reactions involving bifunctional alkylating agents, whose reaction efficiency is low and which mainly form monoadducts, the yield is generally less than 10%.

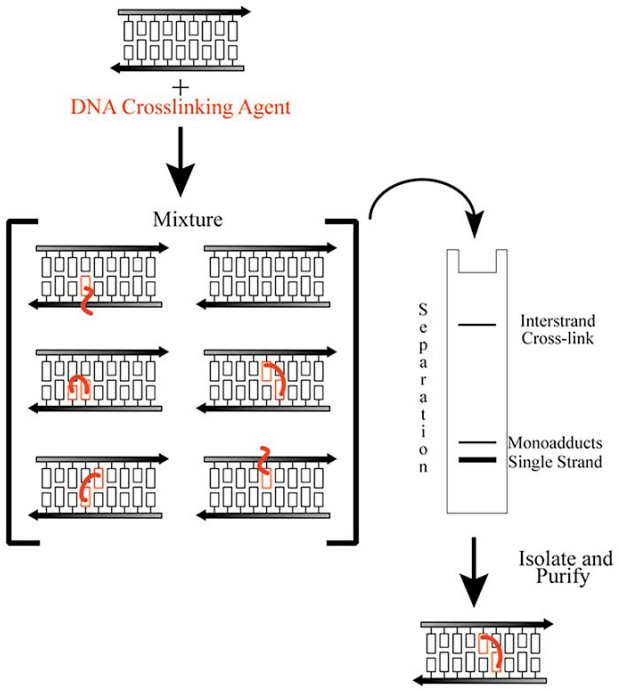

Figure 2. Postsynthetic Cross-link Strategy.

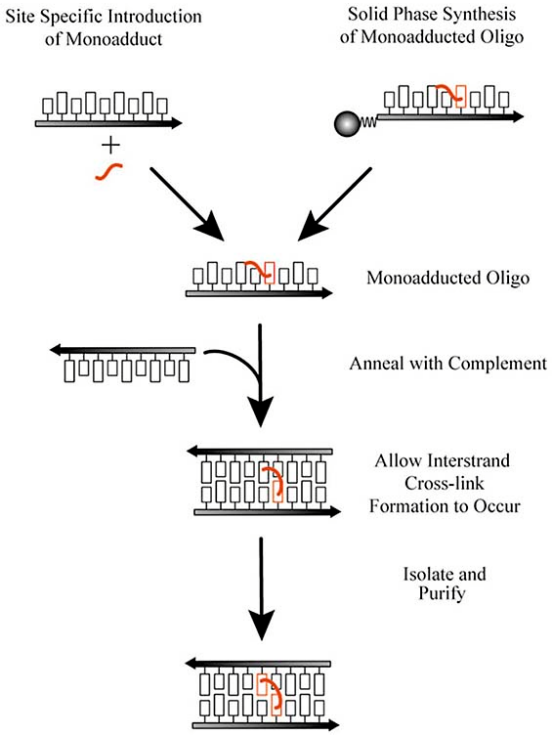

Figure 3. Hybridization Directed Cross-link Strategy.

In some cases it is possible to use hybridization to direct formation of a cross-link between two strands of the duplex (Figure 3). A monoadduct is introduced into one strand of the duplex either postsynthetically or during the synthesis of the CPG-bound strand. In either case the monadducted strand is separated from unmodified strand by HPLC or by polyacrylamide gel electrophoresis. This strand is then annealed with its complementary strand and cross-linking is initiated. The resulting cross-linked duplex is then purified and characterized. This procedure, which

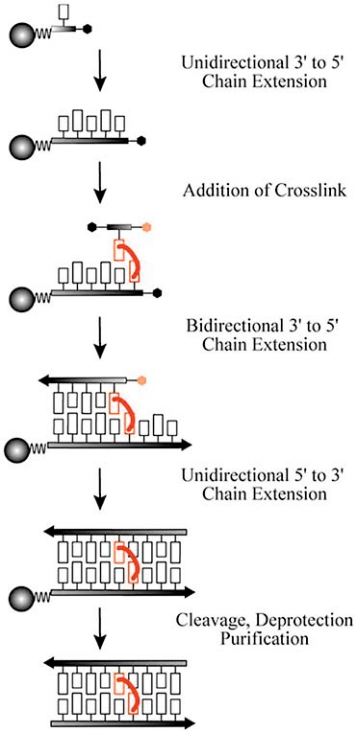

Figure 4. Solid Phase Cross-link Strategy.

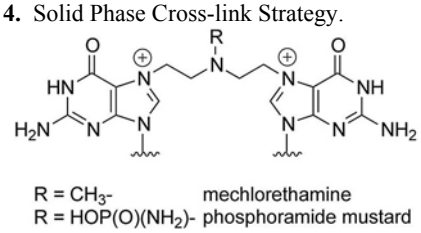

Figure 5. Structure of the Nitrogen Mustard Cross-link.

has been applied to the preparation of platinum and psoralen cross-linked duplexes, potentially provides more control over the site of cross-link placement than does the postsynthetic method.

Even greater control of cross-link placement can be achieved by solid phase automated DNA synthetic techniques (Figure 4). The synthesis begins by first building one arm of the duplex on the solid support, usually standard controlled pore glass beads that have been derivatized with a protected nucleoside. The synthesis proceeds in a stepwise manner from the 3'-end of the duplex arm toward the 5'-end. The cross-link is then introduced at the 5'-end of the arm. This can be done by preparing a phosphoramidite derivative of the chemically synthesized cross-link and coupling this to the 5'-end of the arm. Alternatively, the cross-link can be introduced by reacting a modified nucleoside residue at the 5'-end of the arm, with an incoming modified nucleoside to form the cross-link. Introduction of the cross-link by either procedure results in an oligomer that has two 5'-ends, which are protected with dimethoxytrityl groups, and a single 3'-end, which is protected with a non-acid sensitive protecting group. Synthesis then continues by removing the dimethoxytrityl protecting groups followed by stepwise coupling with protected nucleoside 3'-phosphoramidites. This extends the chains bidirectionally and produces the second and third arms of the duplex. The 5'-dimethoxytrityl groups of the completed arms are removed and resulting 5'-hydroxyl groups are "capped" to prevent further chain extension. The 3'-protecting group of the cross-link is then removed and the fourth arm is created by stepwise additions of protected nucleoside 5'-phophoramidites. The resulting cross-linked duplex is purified by HPLC or polyacrylamide gel electrophoresis after deprotection and cleavage from the support.

This solid phase procedure can be used to synthesize cross-linked duplexes that have symmetrical sequences around the site of the cross-link. Because the chemistry used to introduce the cross-link is highly controlled, the generation of unwanted side products is low and consequently the overall yield of product is quite good. In addition the method is well suited to generate quantities of material sufficient for both biological and physical studies.

4. INTERSTRAND CROSS-LINKED DUPLEXES

The three synthetic strategies outlined above have been used to prepare short DNA duplexes that contain interstrand cross-links. Two types of DNA interstrand cross-links have been prepared: those that result from reaction with therapeutic or environmental agents and those that mimic these types of cross-links.

4.1. Therapeutic and Environmental Interstrand Cross-Links

4.1.1. Nitrogen Mustard Cross-Links

Nitrogen mustards produce interstrand DNA cross-links by alkylation of the N7 of the guanine residues at 5'-d(GNC) sites (Figure 5). The nitrogen mustards are bifunctional alkylating agents that remain the drug of choice for treatment of a number of human cancers. The nitrogen mustards include: mechlorethamine, the simplest of the mustards; phosphoramide mustard, the pharmacologically active metabolite of cyclophosphamide; isophosphoramide mustard, the pharmacologically active metabolite of ifosfamide; and the aromatic nitrogen mustards melphalan and chlorambucil. The first step in DNA interstrand cross-link formation involves the intramolecular cyclization of one of the two chloroethyl moieties of the nitrogen mustard to produce an aziridinium ion that can react with the N7 of guanine. Cyclization of the remaining chloroethyl moiety produces a second aziridinium ion that in turn reacts with a second guanine located on the opposite strand of DNA to give rise to the N7G-alkyl-N7G cross-link characteristic of the nitrogen mustards.

Post-synthetic derivatization strategies have been used to prepare interstrand DNA cross-linked duplexes for each of the nitrogen mustards listed above. Indeed, controversy regarding the exact nature of the cross-linked sequence remained until defined mechlorethamine derived cross-link containing DNA duplexes were prepared and characterized (24,25). The specificity of the 5'-d(GNC)

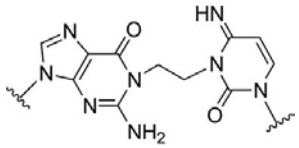

Figure 6. Structure of the Chloroethylnitrosourea Cross-link.

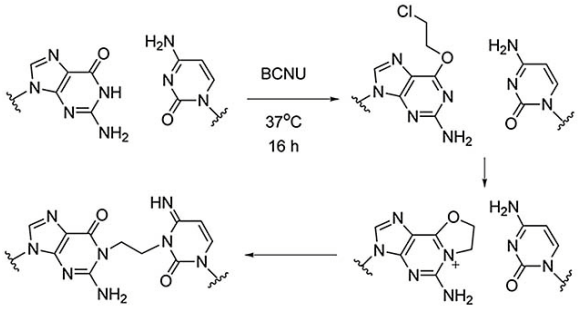

Figure 7. Mechanism of Chloroethylnitrosourea Cross-link Formation.

target sequence of mechlorethamine was clearly demonstrated by Hopkins and coworkers who showed that the substitution of N7-deazaguanine into the target sequence abolished interstrand cross-linking (26). Using analogous methods, cross-linking at 5'-d(GNC) sites was demonstrated for phosphoramide mustard (27), melphalan (28) and chlorambucil (29). It is not surprising that the target sequence of these mustards is identical given that each produces the same 5-atom cross-link, N7G-CH2-CH2-N(R)-CH2-CH2-N7G. Interestingly, post-synthetic derivatization techniques also demonstrated that isophosphoramide mustard displays the same preference for 5'-d(GNC) sites despite the fact that the resulting cross-link is 7 atoms in length N7G-CH2-CH2-NH-P(O_2H)-NH-CH2-CH2-N7G (30).

Site-specific preparation of mechlorethamine cross-linked DNA has been used to study DNA bending induced by cross-link formation (31). This work is of particular interest because the 5-atom tether is not long enough to bridge the distance between the N7 atoms of guanines in the 5'-d(GNC) site without inducing some structural distortion. Gel retardation assays indicate an apparent bend angle between 12.4-16.8° per lesion. Structural studies of nitrogen mustard interstrand cross-links have not been reported, possibly due to the inherent instability of the mustard interstrand cross-links (24).

Interstrand DNA cross-links prepared from mechlorethamine cross-linked duplexes were used to study the repair of these lesions in *E. coli*. Loechler and coworkers treated synthetic DNA duplexes, which contained mechlorethamine interstrand cross-links, with sodium hydroxide to generate the more stable formamidopyrimidine (FAPY) or imidazole ring opened form of N7-alkyl-guanine (24). This treatment resulted in cross-linked DNA species that could be purified and inserted into a plasmid (32). Plasmids prepared in this manner were shown to have an interstrand cross-link at a defined position and were used to study the repair efficiencies in several strains of *E. coli*. The ability of wild-type and repair deficient *E. coli* strains to replicate cross-linked plasmid or a non-cross-link containing plasmid were determined (33,34). These studies demonstrated that nucleotide excision repair plays a role in repair of these lesions and that RecA-mediated homologous recombination is not involved in the repair of these interstrand DNA cross-links. Furthermore, *E. coli* DNA polymerase II (*pol beta*) was shown to be involved in the repair of these mechlorethamine derived interstrand DNA cross-links.

4.1.2. *Bis*-Chloroethylnitrosourea Cross-Link

Chloroethylnitrosourea interstrand DNA cross-links consist of an ethyl cross-link between the N1 atom of guanosine and N3 atom of cytosine in G-C Watson-Crick base pair (Figure 6). The chloroethylnitrosoureas (CENUs) encompass an important class of chemotherapeutic agents that are known to modify both DNA and proteins (35). These agents exhibit anticancer properties against a variety of human tumors including lymphomas, malignant melanoma and cancers of the gastrointestinal tract (36,37). *In vivo* these agents decompose to generate both carbamoylating species and alkylating intermediates. The latter are responsible for the alkylation of DNA with the cytotoxic activity of this class of therapeutics attributed to the formation of interstrand DNA cross-links. Under aqueous conditions *bis*-chloroethylnitrosourea (BCNU) decomposes to generate a highly reactive carbonium ion intermediate that transfers a chloroethyl group to the O6 of deoxyguanosine (Figure 7) (38,39).. An intramolecular cyclization with the N1 atom of deoxyguanosine results in displacement of chloride to afford the highly reactive tricyclic intermediate that is then attacked by N3 of a cytosine on the opposite strand resulting in the formation of the ethyl cross-link.

In order to investigate the sequence specificity and chemical structure of DNA treated with BCNU, Hopkins and coworkers studied the cross-linking reaction with synthetic oligonucleotides on both analytical and preparative scales using excess BCNU (40). These duplexes were analyzed using denaturing polyacrylamide gel electrophoresis with yields for the cross-linked duplexes ranging from 0.4-3.7%. The identity of the cross-link was confirmed by enzymatic digestion of the cross-linked duplex to component nucleosides. HPLC analysis of the digest revealed in addition to the parent mononucleosides, a peak that had spectroscopic features identical to that of an authentic sample of chemically synthesized 1-[N3-deoxycytidyl]-2-[N1-deoxyguanosyl] ethane.

To date, there is no report of a high resolution NMR or crystal structure of duplexes containing this adduct. The mechanism for repair of this cross-link is also unknown, although O6-alkylguanine alkyltransferase has been shown to remove the chloroethyl group from the O6 of guanine, thus preventing the formation of the interstrand cross-link (41-43).

4.1.3. Nitrous Acid Cross-Link

Nitrous acid can produce an interstrand DNA cross-link between two deoxyguanosine residues at 5'-

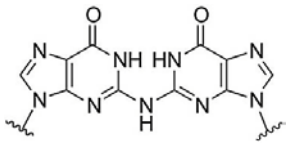

Figure 8. Structure of the Nitrous Acid Cross-link.

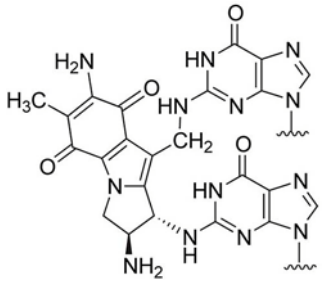

Figure 9. Structure of the Mitomycin C Cross-link.

(CG) sites with the two guanines linked though a common exocyclic amine (N2 atom) functionality (Figure 8). Although nitrous acid is not related to a therapeutic regimen, there is interest in the initiation of this cross-link due to various dietary and environmental exposures to this agent (44,45). The proposed reaction mechanism begins with diazotization of an exocyclic amino group on one of the guanines, followed by nucleophilic attack of the second exocyclic amine of the guanine located on the opposite strand.

Earlier reports on the preparation of DNA duplexes containing this lesion described treatment of chemically synthesized DNA with sodium nitrite under acidic conditions followed by purification via denaturing polyacrylamide gel to isolate the cross-linked duplex (46,47). However, this direct method may not be the most desirable route to obtain the cross-link due to various deamination reactions. More recent reports describe the synthesis of the cross-linked duplex using a combination of solution and solid-phase synthesis methodologies (48,49). In solution, the key reaction to produce the deoxyguanosine dimer involved a palladium-catalyzed coupling between 2-bromo-deoxyinosine and deoxyguanosine. Two different approaches were taken to construct the duplex using either a symmetrical bis-phosphoramidite or 3'-O-allyloxycarbonyl protected phosphoramidite. Purification of the cross-linked duplex synthesized by either approach was accomplished via polyacrylamide gel electrophoresis and afforded the cross-linked oligomer in approximately 10 % overall yield.

NMR studies of a DNA duplex containing this lesion revealed an absence of peaks diagnostic of imino protons at the site of the cross-link, which indicated a lack of base pairing (48). This is in agreement with molecular modeling studies that suggest that although the lesion should be accommodated in B-form duplex it induces a severe propeller twist at the site of the cross-link (46).

4.1.4. Mitomycin C Cross-Link

Mitomycin C (MC) is a genotoxic cancer chemotherapeutic agent that reacts in the minor groove with the exocyclic amino groups of guanines in the sequence 5'-d(CG) to produce an interstrand cross-link (Figure 9) (10,50). In the absence of chemical or enzymatic reduction of its quinone moiety, MC is relatively stable and unable to alkylate DNA. Reduction of the quinone initiates a series of spontaneous transformations that result in the generation of a highly unstable vinylogous quinone methide. Reaction of this species with the exocyclic amino group of guanine initiates a second round of rearrangements that produce a second reactive species that can react with the amino group of another guanine to give the interstrand cross-link. The exact mechanism(s) of bioactivation of MC and related compounds remains an active area of investigation and excellent reviews of the literature are available (10). Like many of the bifunctional alkylating agents, the interstrand cross-link formed is a small fraction of the total covalently adducted DNA formed. Other adducts include the monoadduct (51) and an intrastrand cross-link arising at the sequence 5'-d(GG) (52).

Hamilton and coworkers utilized a postsynthetic methodology to prepared 23-bp MC cross-linked DNA oligomers (50). A synthetic DNA duplex was prepared that contained a single embedded -CG- site. The incubation of this duplex with MC in the presence of sodium dithionite under anaerobic conditions resulted in the formation of interstrand cross-linked DNA. Purification of the cross-linked oligomer was accomplished using high temperature size exclusion column chromatography.

Tomasz and coworkers developed a hybridization directed method for the preparation of MC interstrand cross-linked oligomers (53). Mitomycin C was incubated with a DNA duplex in the presence of sodium dithionite, however this was done under aerobic conditions which allows only the formation of the initial adduct at C1. The MC monoalkylated oligonucleotides were purified by HPLC. Interstrand cross-linking could then be initiated by annealing the monoalkylated strand to a complementary strand and then incubating under anaerobic conditions in the presence of the sodium dithionite.

Using a combination of strategies described above a series of oligonucleotides were prepared that possessed a site-specific MC monoadduct, an interstrand 5'-d(CG) MC cross-link or an intrastrand 5'-d(GG) MC cross-link (54). Analysis of the alkylated DNA for anomalous electrophoretic mobility by nondenaturing gel electrophoresis revealed that neither the monoalkylated nor the interstrand cross-link produced appreciable bending while the intrastrand MC cross-link induced a 14° bend.

NMR studies on monoalkylated MC DNA (55) and interstrand cross-linked MC DNA (56) are largely consistent with molecular models and the available biophysical data. Mitomycin C cross-linking does not result in the disruption of the Watson-Crick base pairing, however the orientation of the MC complex within the minor groove induces a widening of the groove as evident from distortions in the phosphorus resonances and the appearance of unique NOE connectivities.

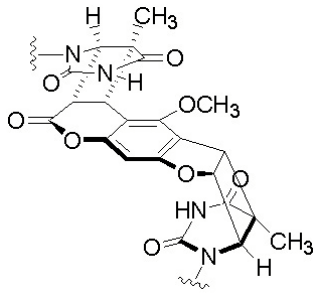

Figure 10. Structure of the 8-Methoxypsoralen Cross-link.

Mitomycin C cross-linked oligomers and cross-link containing plasmid substrates have been prepared and used to examine the mechanisms of recognition and repair of these lesions (57,58). Electrophoretic mobility shift assays were used to demonstrate the specific recognition of MC cross-linked oligonucleotide duplexes by xeroderma pigmentosum group A protein (XPA) and a minimal DNA binding domain of XPA (58). MC cross-link containing plasmid substrates have been used in an *in vivo* reactivation assay that suggests the presence of a cross-link repair pathway independent of homologous recombination (59).

4.1.5. Psoralen Cross-Links

Psoralens are linear furocoumarins that are able to generate interstrand cross-links by photoalkylating thymines at 5'-d(TA) sites in DNA (Figure 10) (6,60). The photoactivation of psoralen has been used to treat skin diseases, cutaneous T-cell lymphoma and to sterilize blood products. Intercalation of psoralen at 5'-d(TA) sites is the initial step in interstrand cross-link formation. The subsequent absorption of a photon upon long wavelength (320-410 nm) ultraviolet-irradiation results in [2 + 2] photocycloaddition at the 3,4 or 4',5' double bonds of psoralen and the 5,6 double bond of thymidine. Interstrand cross-linking can arise only from the sequential formation of a furan-side adduct followed by a pyrone-side adduct because [2 + 2] photocycloaddition with the pyrone ring destroys the ability of the adducted psoralen to absorb an additional photon. The resulting cyclobutane rings possess *cis-syn* stereochemistry.

In general, hybridization directed methodologies have been used to prepare psoralen interstrand cross-linked oligonucleotide duplexes. Hearst and coworkers exploited the use of high intensity lasers to produce large quantities of furan-side monoalkylated oligonucleotides (61), whereas Essigmann and coworkers chemically synthesized oligonucleotides containing a site-specific furan-side monoadduct (62-64). These mono furan-side containing oligonucleotides can then be annealed with a complementary oligonucleotide and interstrand cross-link formation accomplished by ultraviolet irradiation.

Using self-complementary DNA oligomers containing a single 5'-d(TA) step and 4,5',8-trimethylpsoralen, Hearst and coworkers prepared furan-side mono adducts by irradiating samples with a Krypton ion laser emitting light at 406.7 nm and 413 nm (61). Irradiation at these wavelengths is sufficient to induce furan-side photocycloaddition but is unable to cause pyrone-side photocycloaddition, and as such allows the furan-side adduct to accumulate in the reaction. The mono-adducted oligonucleotide was then purified by HPLC. This methodology allowed preparation of micromole quantities of furan-side psoralen adducted DNA. Oligonucleotides prepared in this manner were annealed to complementary strands and interstrand cross-link formation achieved by irradiation with an Argon laser emitting a 366 nm.

A second strategy utilized by Essigmann and coworkers to generate furan-side monoalkylated oligonucleotides involved the preparation of a furan-side psoralen-thymidine phosphoramidite suitable for use in the chemical synthesis of oligonucleotides (62-64). 2'-Carboxypsoralen was prepared and coupled to the 5'-hydroxyl of thymidine. Irradiation at 300 nm in the presence of the photosensitizer acetone resulted in a single photoproduct that was the desired furan-side psoralen-thymidine adduct. This nucleoside was converted to the protected nucleoside phosphoramidite using standard procedures. The psoralen-thymidine phosphoramidite was used in an automated DNA synthesizer using standard methods. Deprotection of the furan-side containing oligonucleotide was accomplished using 10% DBU in ethanol at room temperature for 24 hours. Following treatment with sodium carbonate to convert the 2'-methyl-ester of psoralen to the carboxylic acid, the resulting oligonucleotide was purified by HPLC. Oligonucleotides prepared in this manner were annealed to complementary strands and interstrand cross-link formation achieved by irradiation with 366 nm light (9.0 J/m^2).

Structural and biochemical characterization of psoralen cross-linked DNA has revealed surprisingly small structural deformations. Early models suggested psoralen cross-links would introduce considerable deformation of the helix. These models were based in large part on incorrect assumptions that the helix would propagate normal to the plane of the adducted thymine rather than as a modified surface formed by the buckled thymine. The availability of specific psoralen cross-linked DNA oligomers has allowed detailed biophysical and structural studies to be carried out. Bending studies using phased psoralen cross-links were consistent with little deformation of the helix (i.e. bend angle < 10°) and a 1-bp unwinding of the helix, changes that are not significantly different from those produced by classical intercalators. NMR solution structures of 4'-amino-4,5',8-trimethylpsoralen (65) and 4,5',8-trimethylpsoralen (66,67) cross-linked 8-mers both clearly demonstrated that psoralen interstrand cross-links are well accommodated by the DNA helix. The presence of the cross-linked psoralen results in a helical unwinding of ~25° and gives rise to an overall helical repeat of 11-bp, both consistent with intercalation of a planar compound. Furthermore, both structures demonstrated that the local deformations that occur upon photocycloaddition do not distort the overall helical geometry and are largely confined to the base pairs immediately flanking the cross-link. In both structures guanines adjacent to the 5'-d(TA) site stack onto the surface formed by the 5-methyl, 6-H, O2 and N3H

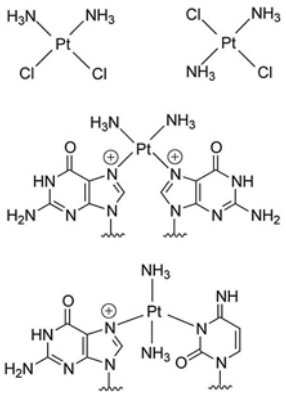

Figure 11. Structure of Platinum Cross-links.

of the adducted thymines and hydrogen bonding is maintained by the adducted thymines. More recently, X-ray crystallography was used to determine the structure of psoralen containing Holliday junctions (68). Models of B-form DNA containing psoralen interstrand cross-links derived from these structures are consistent with the solution structures described above.

Efforts to understand the repair of interstrand DNA cross-links in general have been greatly influenced by psoralen interstrand cross-links. The incision/recombination model originally proposed by Cole was proposed to account for the requirement of the uvrA, uvrB, uvrC, uvrD, recA, and polA gene products for the *in vivo* survival of *E. coli* in the presence of the psoralen (69,70). The key steps of this repair pathway include: (a) initiation of repair by NER excinuclease nicking of the DNA on both sides of the cross-link thereby generating an 11-nt DNA fragment that remains covalently linked to the non-incised strand; (b) displacement of the nicked strand via RecA-mediated homologous recombination with a lesion free homologous chromosome; (c) resolution of this intermediate to produce an error-free template strand followed by the recognition and repair of the remaining three-way junction via classical NER.

The availability of robust methodologies for the preparation of site-specific psoralen interstrand cross-links has facilitated efforts to examine the *in vivo* and *in vitro* repair of these cross-links. Several of the steps postulated in the incision/recombination repair pathway described above have been demonstrated using psoralen cross-link oligonucleotide substrates, including the initial recognition and dual incisions by the NER excinuclease (71) and the processing of the three-way junction by NER have been demonstrated *in vitro* (72,73). Additionally, *in vitro* studies found that while the dual nicks depicted in the model were not sufficient to stimulate RecA-mediated homologous recombination, the processing of the nick into a gap could stimulate RecA-mediated exchange (74).

Repair studies in higher organisms including *Saccharomyces cerevisiae* and mammalian cells have revealed a significantly more complicated repair process. Repair of psoralen cross-link containing plasmid substrates in yeast suggests the involvement of two pathways, an error-free pathway that involves the formation of double stranded break intermediates, which is followed by recombination and a second error-prone pathway that results in errors at the site of the cross-link (75). Processing of interstrand psoralen cross-links in mammalian cell-free extracts appears to involve the formation of dual incisions, both of which are 5' of the cross-link and result in a gap (76). This gap induces a futile process of DNA synthesis, which may be a signal for other cellular responses (77).

4.1.6. Platinum Cross-Links

Cis-Diamminedichloroplatinum II (*cis*-DDP or cisplatin) is one of the most widely used anticancer agents. It is particularly effective in the treatment of testicular tumors where it can afford cure rates greater than 95% (78). Like the therapeutic nitrogen mustards, cisplatin forms intra- and interstrand DNA cross-links with reaction predominantly at the N7 positions of purine bases. This damage to DNA may be responsible for the therapeutic efficacy of cisplatin. Studies performed *in vitro* demonstrate that cisplatin treated DNA contains approximately 2% of interstrand adducts (79), whereas the *trans*-isomer forms approximately double that quantity (80).

Cisplatin is a neutral, square planar coordination complex of platinum (II). It is coordinated to two chloride and two ammonia groups, where the chloride ligands are in the *cis*-geometry (Figure 11). While the ammonia groups are strongly coordinated to platinum (II), the chloride ligands are easily displaced by nucleophiles. These cross-links are formed preferentially at the 5'-d(GC) sites between guanine residues (Figure 11) (81,82). *Trans*-DDP forms interstrand cross-links between N7 and N3 atoms of complementary guanine and cytosine residues respectively of a GC base pair, although the kinetics is markedly slower (12%) (80,83,84).

Duplexes containing platinum interstrand cross-links can be prepared by hybridization directed cross-linking. Both *cis* and *trans* $[Pt(NH_3)_2Cl_2]$ bind preferentially to guanine residues at the N7 position, producing a monofunctional adduct that reacts again to form the bifunctional lesion. Thus an oligonucleotide containing a unique G residue is first reacted with the appropriate *cis* or *trans*- platinum complex, $[Pt(NH_3)_2(N7$-N-methyl-2,7-diazapyrenium$)Cl]^{2+}$, generated from *cis* or *trans*-$[Pt(NH_3)_2Cl_2]$ (85). After purification by ion-exchange FPLC, the platinated oligonucleotide is mixed with its complementary strand and incubated in 1M sodium chloride for 20 h in the dark at 37°C. Duplexes containing the interstrand cross-link are then repurified by FPLC.

The NMR solution structure (86,87) as well as the crystal structure (88) of duplex DNA containing a single cisplatin interstrand cross-link shows that the *cis*-diammineplatinum(II) bridge lies in the minor rather than the major groove of the helix. The helix is bent by 45° towards the minor groove (89) causing this groove to be slightly enlarged. A net unwinding of 79° is observed, by both techniques. The two cytosines complementary to the

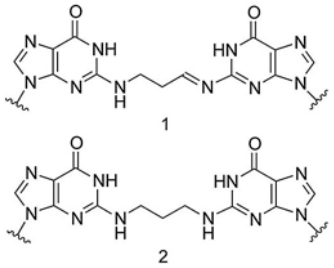

Figure 12. Structures of the Acrolein and Trimethylene Cross-links.

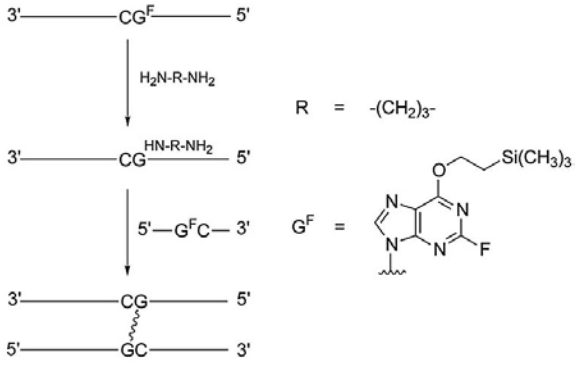

Figure 13. Synthesis of Trimethylene Cross-link.

platinated guanines are extrahelical, and exposed to solvent. Consequently they are hyperreactive to chemical probing. However the adduct does not induce a local denaturation within the flanking sequences since the adenine and thymidine residues do not react with probing reagents (90). The interstrand cross-link of *trans*-DDP bends the DNA helix towards the major groove by 26° and unwinds it by 12° (80). The 2D NMR structure (91) of a duplex DNA containing a single interstrand cross-link of trans-DDP shows distortion over 2 base pairs on either side of the adduct and bending towards the major groove. The platinated guanine adopts a *syn*-conformation. The rotation, mediated by the platinum moiety, results in a Hoogsteen-type pairing between the complementary platinated guanine and cytosine.

Homologous recombination may be involved in the repair of cisplatin interstrand cross-links in mammalian systems (92). Interstrand cross-links are not by themselves the critical DNA lesion induced by cisplatin because an inability to repair this lesion only leads to a very modest increase in cellular sensitivity. In *S. cerevisiae*, cells deficient in photolysase are more resistant to cisplatin than wild type cells, suggesting that the enzyme binds to cisplatin adducts and shields them from repair. The cells are not differentially sensitive to trans-DDP, consistent with the lack of affinity for trans-DDP adducts (93). A DNA substrate containing a single cisplatin interstrand cross-link is poorly recognized by replication protein A (RPA) (94). T4 endonuclease VII, an enzyme that resolves branched DNA structures, can cleave interstrand cross-links of both cisplatin and trans-DDP, although the efficiency of cleavage of the cisplatin cross-link is higher (95).

4.2. Interstrand Cross-Link Mimics

4.2.1. Trimethylene Cross-Links

Acrolein and crotonaldehyde are mutagens and tumor initiators formed in cells via lipid peroxidation and are also present in tobacco smoke (96,97). In addition, they are produced in large quantities by industry for use as synthetic intermediates. These unsaturated aldehydes and malondialdehyde are bis-electrophiles that are capable of reacting with nucleosides either through their carbonyl group(s) or double bond. Simple acyclic nucleoside adducts are generally not observed because they react a second time to produce fused ring adducts of the nucleobase (98-101). Thus, for example, conjugate addition of the exocyclic amino group of deoxyguanosine to acrolein followed by cyclization of the initially formed N2-(3-oxopropyl) intermediate gives the 8-hydroxypropano adduct which is the major product of the reaction of acrolein with DNA (96,100,102-104). The 8-hydroxypropano adduct can revert back to the N2-(3-oxopropyl) intermediate, which can react with the exocyclic amino group of a second guanine to form a cross-linked species, 12.1 (Figure 12). Crotonaldehyde (103-107) and malondialdehyde (108) react in a similar manner.

These cross-links are inherently unstable. Therefore Harris and coworkers have synthesized duplexes that contain a trimethylene interstrand cross-link mimic, 12.2 (Figure 12). Their synthetic methodology (Figure 13) was similar to the hybridization directed cross-link strategy discussed above. An oligonucleotide containing the 6-(2-(trimethylsilyl)ethoxy)-2-fluoropurine-9-(2'-deoxyribonucleoside) was reacted with an excess of 1,3-diaminopropane to give the 3-aminopropyl adduct. Further reaction of this adduct, after desilylation, with an oligonucleotide containing the O6-TMSE-2-fluoropurine residue yielded several products, one of which was the desired cross-linked duplex. Similarly modified oligonucleotides containing the 6R and 6S methyl analogues of crotonaldehyde were synthesized using a parallel strategy (109,110).

The trimethylene linkage proved to be relatively stable under conditions that maintain duplex structure, undergoing no more than 20% reversion to the starting oligonucleotides after 16 h at room temperature in 0.05M phosphate buffer, pH = 7.0. Duplexes were prepared in which the trimethylene linker was inserted into either a -CG- or -GC- sequence in the center of an 8 base pair DNA duplex(108, 111). Thermal denaturation experiments showed that the cross-link stabilized the -CG- cross-linked duplex to a remarkable extent (108). Thus the denaturation temperature of the -CG- cross-linked duplex is greater than 85ºC and was estimated to be more than 60ºC higher than the melting temperature of the non-cross-linked control duplex. Examination of the cross-linked -CG- duplex by high resolution proton NMR and molecular dynamics suggested that the cross-link causes little distortion of the duplex (108). In addition essentially no bending was observed when multimers generated by ligation of a 21 base pair duplex that contained a single cross-link were examined by gel electrophoresis.

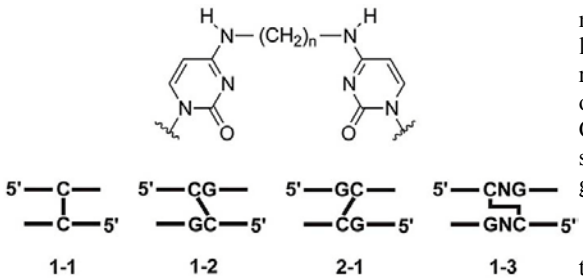

Figure 14. Structure and Orientation of the N4C-Alkyl-N4C Cross-link.

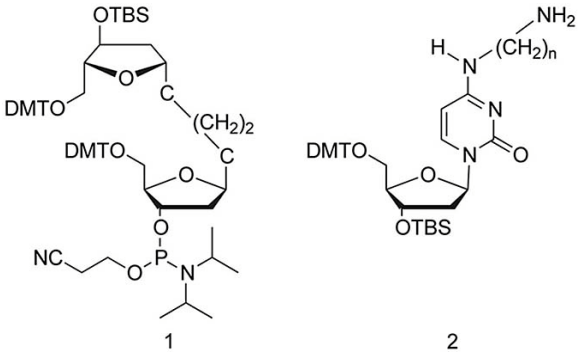

Figure 15. Reagents for the Preparation of N4C-Alkyl-N4C Cross-links.

In contrast to this behavior, the trimethylene cross-link destabilized the -GC- duplex (111). This duplex denatures in a cooperative manner and has a denaturation temperature of 52ºC, which is 10ºC less than that of the non-cross-linked duplex. Analysis by high resolution proton NMR showed that the trimethylene group induces a bend and twist in the helical axis at the site of the cross-link (111). This distortion may account for the destabilizing effect of the cross-link, although bending deformations were not detected by gel electrophoretic mobility assays.

Little is known about the repair of the trimethylene cross-link. Studies by Mu and coworkers (112) on a duplex containing the trimethylene -CG- cross-link showed that XPF-ERCC1, an endonuclease involved in nucleotide excision repair, in combination with RPA, could make incisions at phosphodiester linkages 5 and 9 nucleotides 5' of the cross-link.

4.2.2. N^4C-Alkyl-N^4C Cross-Links

We have used the solid phase synthetic strategy (Figure 4) to prepare DNA duplexes that contain N^4C-alkyl-N^4C interstrand cross-links (Figure 14) (113-116). This cross-link connects the exocyclic amino groups of two cytosine residues on opposite strands of DNA via an alkyl linker that contains two, four or seven methylene groups. As far as we know, N^4C-alkyl-N^4C type interstrand cross-links are not produced when DNA reacts with bifunctional alkylating agents. The N^4C-alkyl-N^4C interstrand cross-link has been incorporated into DNA duplexes in a number of different orientations (Figure 14). The 1-1 type cross-link connects two mismatched cytosine residues via a two methylene linker whereas the 1-2 and 2-1 staggered cross-links link the cytosines of a -CG- and -GC- sequence respectively. The 1-3 series of duplexes contain a two, four or seven methylene linker connecting the cytosines of a -CNG- sequence. This latter cross-link is in some respects similar to the nitrogen mustard cross-links that connect guanine residues in a -GNC- sequence.

Two different approaches were used to introduce these cross-links into the DNA duplex. In the first approach, the protected N^4C-alkyl-N^4C cross-link phosphoramidite, 15.1 (Figure 15), was coupled to the 5'-end of the first arm of the duplex. This phosphoramidite contains two orthogonal protecting groups, the 5'-O-dimethoxytrityl groups and the 3'-O-*t*-butyldimethylsilyl group, which can be removed selectively by treatment with dichloroacetic acid and tetra-*n*-butylammonium fluoride respectively. In the second approach, an O^4-triazole-2'-deoxyuridine residue at the 5'-end of the first arm was reacted with protected N^4-(aminoalkyl)-2'-deoxycytidine, 15.2. Synthesis of the remaining arms of the duplex was then completed as described above. The duplexes were readily purified by strong anion exchange HPLC. Cross-linked duplexes containing up to 11 base pairs and having blunt or overhanging ends have been prepared by this method in quantities sufficient for analysis by high resolution proton NMR.

The physical properties of the N^4C-alkyl-N^4C cross-linked duplexes have been characterized by a variety of methods including circular dichroism (CD), ultraviolet thermal denaturation studies, gel electrophoretic mobility studies and high resolution NMR spectroscopy. The CD spectra of the cross-linked duplexes are similar to those of the non-cross-linked duplexes. These results suggest that introduction of the cross-links does not create large distortions of the helix and that overall the cross-linked duplexes adopt a geometry similar to that of B-form DNA.

The thermal denaturation curves of the cross-linked duplexes are sigmoidal in shape and are qualitatively similar to those of the UV melting curves of the corresponding non-cross-linked duplexes. These results suggest that the duplexes denature in a cooperative manner, although the strands do not physically separate as they do in normal DNA. In most cases, the denaturation transition temperature is significantly higher than the melting temperature of the corresponding non-cross-linked duplex. This increase most likely occurs because unlike melting of a non-cross-linked duplex, which is a bimolecular process, denaturation of a cross-linked duplex is a unimolecular process and consequently the decreased entropy results in an increased thermal stability.

A remarkably high transition temperature, 81ºC, was observed for a 10 base pair duplex containing a 1-2 type cross-link (115). In this case the transition temperature is 49ºC higher than the melting temperature of the corresponding non-cross-linked duplex. Examination of molecular models and preliminary NMR analysis suggest that this cross-link is accommodated well in the major groove of the helix with essentially no disruption of

hydrogen bonding interactions of either the cross-linked bases or surrounding base pairs. In contrast to this behavior, the transition temperature of a duplex containing a 2-1 type cross-link was observed to be 10ºC lower than the melting temperature of the non-cross linked version of the duplex. In this case the orientation of the cross-link is such that the distance between the N^4C exocyclic amino groups of the -GC- sequence is greater than the length of the two methylene cross-link. Molecular models suggest that as a consequence, the base pairing by the cross-linked cytosines is disrupted as well as base pairing at the sites adjacent to the cross-link.

The length of the cross-link can also affect duplex stability as shown by thermal denaturation experiments with 11 base pair duplexes that contain a 1-3 type interstrand cross-link (116). Thus the transition temperature of a duplex with a two methylene cross-link, 42ºC, is 16ºC higher than the melting temperature of the non-cross-linked duplex, whereas the transition temperatures of duplexes having the four and seven methylene cross-links are 47ºC and 46ºC higher respectively than the melting temperatures of the control duplex. Molecular models show the distance between the two N^4C exocyclic amino groups in the -CNG- sequence is 7.2 Å in a B-form helix. The two methylene cross-link, which could maximally span 3.8 Å is too short to cover this distance. Geometry optimization of B-form DNA that contains this cross-link shows considerable perturbation of the cross-linked base pairs resulting in severe propeller twisting and displacement of the cytosines into the major groove of the helix. Geometry optimization shows the four methylene cross-link, which can span a distance of 6.23 Å, causes less perturbation of helix geometry with modest propeller twisting or rolling of the cross-linked C-G base pairs, whereas the seven methylene cross-link, which is 10.0 Å long, fits comfortably into the major groove without perturbing base pairing or base stacking at or adjacent to the site of the cross-link.

Bending deformations caused by the presence of distortions in helix geometry can be measured by non-denaturing gel electrophoresis (117-119) and are signaled by the anomalous mobilities of multimers of the oligomer when the source of the deformation is in phase with the helical turn. Cross-linked duplexes were constructed with complementary overhanging ends, which upon T4 DNA ligase-mediated self-ligation produce multimers in which the cross-links are phased every 10 base pairs or approximately one helical turn. Multimers derived from self-ligation of duplexes with the 1-1 or 1-2 type cross-links had mobilities essentially identical to those of the non-cross-linked controls, indicating these cross-links do not induce bending deformations (115). The duplex with the 2-1 type cross-link did not undergo self-ligation indicating this duplex is too distorted to serve as a substrate for the T4 DNA ligase.

All three duplexes with the 1-3 cross-link underwent self-ligation (116). In contrast to the duplexes with 1-1 and 1-2 type cross-links, anomalous electrophoretic mobilities were observed for multimers generated from the 1-3 cross-linked duplexes that contained two and four methylene linkers, whereas multimers generated from the seven methylene cross-linked duplex migrated with the non-cross-linked control. These results suggest that the two and four methylene cross-links produce an overall bending deformation whereas the seven methylene linker does not bend the duplex. The deformations most likely result from static bending or anisotropic flexibility (120) and not from formation of a hinge joint (118) at the sites of the cross-links. Thus multimers produced from self-ligation of duplexes in which the cross-link is phased every 14 base pairs did not show anomalous electrophoretic mobilities. The bending angles of the two methylene and four methylene cross-links were 20º and 14º respectively as determined from the electrophoretic mobilities (117). The degree of bending in these duplexes is similar to bending caused by A6 tracts in DNA (20º) and by a interstrand transplatin G-C cross-link (20º-26º) (80,91) or by an interstrand mechlorethamine G-G cross-link (~14º) (31). These results show that it is possible to incrementally deform the helix by changing the length of the interstrand N4C-alkyl-N4C cross-link when it is in the 1-3 orientation.

The solution structure of an 11 base pair duplex containing the 1-1 type cross-link has been determined by high resolution proton NMR and restrained molecular dynamics (121). The structure shows a widening of the major groove at the site of the cross-link to accommodate the two methylene linker, which projects into the major groove. The mismatched cross-linked cytosines deviate somewhat from planarity and this distortion perturbs the stacking of the base pairs on either side of the cross-link. The backbone is also distorted at the site of the cross-link which produces a kink that bends the backbone approximately 32º towards the major groove.

Cross-linked duplexes with 1-1, 1-2 and 2-1 type cross-links have been inserted into plasmid DNA and the resulting cross-linked plasmids have been used as substrates to study repair in *E. coli* and in human cell extracts. Preliminary results show that each of the cross-links in repaired in wild type *E. coli*. The pathways that are involved in this repair are currently under investigation.

5. PERSPECTIVE

Although tremendous progress has been made in studying DNA repair, the mechanism(s) involved in the repair of interstrand cross-links is only poorly understood. This has been due in part to the difficulty of preparing well characterized, stable interstrand cross-linked duplexes that can serve as substrates for repair studies. The advent of solid phase DNA methodology and the development of novel synthetic strategies provides a means to prepare duplexes with interstrand cross-links and cross-link mimics. Because these methods allow synthesis of relatively large amounts of material, it will be possible to study the structure of duplexes by methods such as high resolution NMR spectroscopy and X-ray crystallography. Such studies can provide valuable information on the effect(s) of interstrand cross-links on helix structure and

geometry. This information can in turn be used to understand how interstrand cross-links are recognized and repaired in the cell. Defining the chemical, structural and dynamic properties of DNA that contain interstrand cross-links and understanding the mechanism(s) by which this damage is repaired will ultimately contribute to our basic understanding of tumor cell resistance to therapeutic agents. Such information could eventually lead to the development of more effective therapeutic agents and treatment modalities.

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