DNA Interstrand Crosslinks: Natural and Drug-Induced DNA Adducts that Induce Unique Cellular Responses

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Introduction

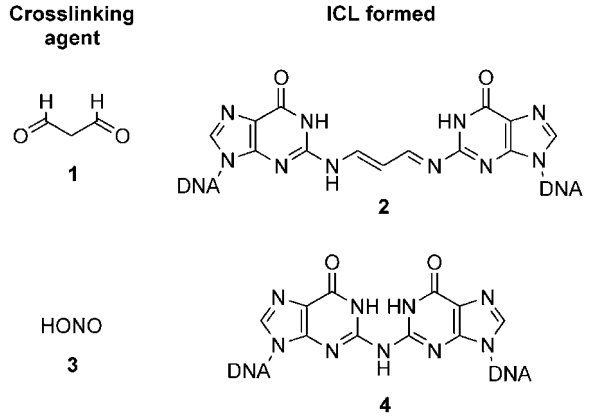
It is a testimony to the complexity of cancer that cytotoxic drugs are still a mainstay of therapeutic approaches to treat that disease. Perhaps even more paradoxical is the fact that cytotoxic therapy has its origin in the highly toxic mustard gas, which was developed for chemical warfare in the First World War. As with other crosslinking agents, such as mitomycin C, the chloroethylnitroso ureas and cisplatin, mustard gas and nitrogen mustards derived from it exert their cytotoxic action by forming DNA interstrand crosslinks (ICLs). ICLs covalently link two strands of DNA, thereby blocking vital aspects of DNA metabolism. Evidence that ICLs can also be formed endogenously, for example by the reaction of DNA with bifunctional lipid peroxidation products, has been obtained much more recently. ICLs are therefore something the cell has to deal with naturally, although these lesions are formed infrequently compared to adducts involving only one DNA strand.

The importance of being able to process ICLs in healthy cells is underscored by the existence of the rare inherited human disorder Fanconi Anemia (FA), which is characterized by extreme sensitivity to ICL forming agents, but not other DNA damaging agents. Here I will review the formation, biological consequences and clinical importance of ICLs. Finally, I will discuss how progress in the chemical synthesis of ICLs will provide opportunities for studying the cellular responses to ICL forming agents.

Formation and Occurrence of ICLs

ICLs are formed by endogenous compounds

The formation of ICLs is readily observed by treating oligonucleotides with various agents. It is however much more difficult to obtain direct evidence for the formation of ICLs in living cells, since they are only formed infrequently. This is especially true for the naturally occurring crosslinks, which, unlike more abundant DNA adducts, such as 8-oxoguanine, have not yet been directly detected in biological samples. The formation of endogenous ICLs has therefore been inferred from treatment of oligonucleotides with the relevant agents or by analysis of the mutations introduced in reporter genes in cells treated with crosslinking agents. Based on such observations, bifunctional electrophiles, such as malonic dialdehyde (**1**), a product of lipid peroxidation, have been linked to endogenous ICL formation (Scheme 1).[1] Similar types of adducts are also formed



Scheme 1. *Examples of ICLs formed by endogenous agents. Malondialdehyde (**1**) can react with exocyclic amines on guanine residues to form the ICL **2**. Nitrous acid (**3**), the hydrated from of nitric oxide can form ICLs between adjacent guanine residues on opposing strands (**4**) by diazotiziation of one of the exocyclic amine groups.*

by unsaturated aldehydes of environmental origin, such as acrolein and crotonaldehyde, as well as from condensation reactions of DNA with formaldehyde or acetaldehyde.[2] It is noteworthy that the condensation reactions between various aldehydes and the aromatic amines of the DNA bases are reversible. This inherent instability is another factor that renders the direct detection of these ICLs difficult.

Another agent of endogenous origin that has been shown to introduce ICLs is nitric oxide. As a gas or in its hydrated form, nitrous acid (**3**), it can induce diazotiziation of the exocyclic amine groups of the bases; this can lead to ICL formation if the N(2) of a guanine on a complementary strand in a (CpG) sequence can react with the diazonium ion before it gets hydrolyzed.[3]

ICLs are formed by antitumor agents by a variety of mechanisms

While these endogenous or environmental ICLs probably contribute to mutagenesis and carcinogenesis and are responsible for the evolution of cellular responses to ICLs, the main driving force in studying ICLs has been their importance as adducts

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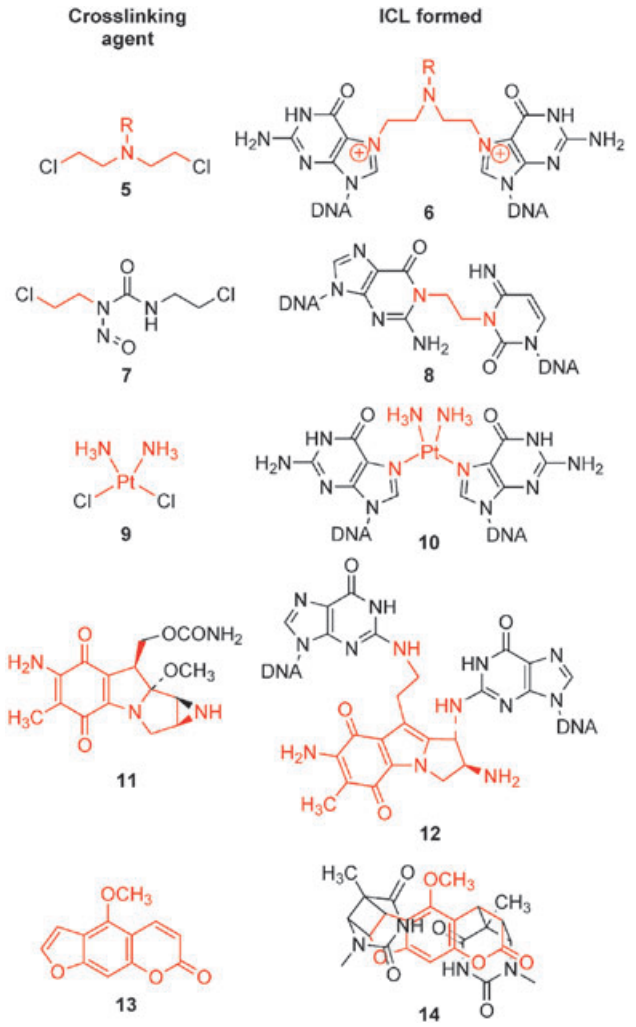
formed by a number of antitumor agents. The first ICLs to be identified were the ones formed by the nitrogen mustards (NMs, **5**; Scheme 2; reviewed in ref. [4]). Studies in the 1960s revealed that the N(7) positions of guanine were the most reactive positions toward alkylation by the NMs and that separation of dsDNA was inhibited by NM adducts.[5] The final proof of the structure of these ICLs and the determination that they are preferentially formed in complementary GNC sequences only followed much later, after the advent of solid-phase DNA synthesis.[6,7] NMs with aromatic substituents (chlorambucil, melphalan) or a bioactivatable phosphoramide group (cyclophosphoamide) are still frequently used in the clinic.



Scheme 2. *Examples of ICLs formed by agents used in antitumor therapy. The crosslinking agents chlorambucil (**5**, R = C_4H_6-C_2H_4-CO_2H, a nitrogen mustard), carmustine (**7**, a chloroethylnitroso urea), cisplatin (**9**), mitomycin C (**11**), and psoralen (**13**) and the main ICLs formed by them are shown. The parts of the crosslinking agents that are transferred to the DNA and the modified residues in the DNA bases are indicated in red.*

A second prototypical class of alkylating agents is the chloroethylnitroso ureas (CENUs), represented by BCNU/carmustine (**7**) in Scheme 2. While the CENUs have also been shown to form crosslinks between two N(7) atoms of guanines, the most frequently formed ICL is one between N(3) of dC and N(1) of dG.[8,9] This ICL is unusual because it is formed in a multistep process involving initial alkylation of O(6) of dG, rapid conversion to form 1,O^6-ethanoguanine followed by slow rearrangement to the ICL between dG and dC (**8**). For CENUs it has been clearly demonstrated that not only the removal of the ICLs themselves, but also of the precursor mono adduct (O^6-(2-chloroethyl)guanine) by DNA-repair enzyme AGT (alkylguanine transferase) contributes to the resistance of tumor cells to treatment by CENUs.[10] Inhibitors of AGT are therefore being developed as drugs for combination therapy with the CENUs.

ICLs can also be formed in the major groove of DNA by metal complexes. The most important metal complex used in cancer chemotherapy is cisplatin (**9**) and related compounds derived from it.[11]

Certain natural products, for example mitomycin C (MMC, **11**), also have the ability to form ICLs. MMC reacts with N(2) groups of two dG residues at adjacent positions on opposing strands to form an ICL.[12] A reductive activation cascade that transforms the quinone moiety of MMC to the phenol form precedes adduct formation. This activation step is important for the selectivity of its antitumor activity, since many solid tumors are hypoxic compared to normal tissues and therefore more efficiently activate MMC.

One last crosslinking agent that will be discussed here is psoralen (**13**), a three-ring heterocyclic compound that forms ICLs with two thymine residues at adjacent positions on opposing strands of DNA upon irradiation with long-wave UV light.[13] Since psoralen ICLs can be formed with higher specificity and under somewhat controlled conditions, it has been frequently used as a model ICL for biological studies. Clinically, the psoralens are used to treat a number of dermatological diseases, such as cutaneous T-cell lymphoma and psoriasis.[14] Treatment protocols with psoralen involve first the oral administration of the drug followed by local UV irradiation of the area to be treated with long-wave UV light to form the ICLs. In this way ICLs can be introduced with some selectivity in the skin, which is accessible to UV irradiation.

ICLs are the most relevant adducts formed by crosslinking agents

Two points deserve to be mentioned here when thinking about the biological consequences of ICLs. For all the agents that induce ICLs mentioned above, *inter*strand crosslinks only make up a small fraction (typically 1–5%) of all the adducts formed, while the majority are monoadducts or *intra*strand crosslinks. This is partly due to spatial reasons, as the majority of monoadducts formed initially do not satisfy the geometrical constraints required for crosslink formation. Nonetheless, in the vast majority of cases, it is the interstrand crosslinks that are the physiologically most relevant adducts.[15] One exception to this rule is provided by cisplatin, where the most abundant 1,2d(GpG) intrastrand crosslink has been shown to be the main contributor to cytotoxicity, since it is bound by various cellular proteins and is refractory to repair.[11] These observations demonstrate the potent cytotoxicity of ICLs compared to DNA ad-

ducts involving only one strand of DNA. The low percentage of relative ICL formation gives rise to two main challenges in the study of its consequences. One is that it is not always straightforward to differentiate the effects of various adducts induced by crosslinking agents at the cellular level if a particular endpoint, such as survival or induction of a response pathway, is examined. The other challenge is that synthesis of defined ICL adducts for biological studies has been difficult.

ICLs are structurally diverse DNA adducts

Another important aspect is that although ICLs are generally categorized as one class of lesion, they induce a variety of structural distortions into DNA. ICLs may be formed in the major and minor grooves of DNA, intercalated between two base pairs, or they may connect two opposing bases through atoms involved in base pairing (Schemes 1 and 2). This suggests that they induce a variety of structural alterations into dsDNA, a notion supported by available structural information. Certain ICLs in the minor groove of DNA, such as those formed by MMC or a stable mimic of the malondialdehyde ICL only induce minor distortions in the overall structure of DNA.[16,17] In contrast, certain ICLs formed by cisplatin,[18] psoralen,[19] or nitrous oxide[20] induce higher degrees of distortion in the DNA helix. This structural diversity raises the question of whether there is a significant difference in the biological responses to the various ICLs or whether the common denominator of all ICLs, the blockage of DNA strand separation, is the key feature responsible for the severe consequences induced by ICLs. This question has not yet been explored, although the effects of covalent linkage between the two strands appear to be the common element responsible for the high cytotoxicity of ICLs. Various structural features of ICLs are likely, however, to influence a number of secondary responses or affect the rate with which they are removed from DNA.

Cellular Responses to ICL-Forming Agents

The defining effect of ICLs is that they block essential aspects of cellular metabolism, in particular DNA replication and transcription. It has been estimated that a single unrepaired ICL can kill a bacterial or yeast cell, while about 40 ICLs can kill a repair-deficient mammalian cell.[4,21] Available evidence in mammals suggests that the replication block induced by ICLs is the defining event responsible for their high cytotoxicity and for the triggering of the key cellular responses to ICL formation (Figure 1).[22] ICLs are processed to double-strand breaks (DSBs) after an encounter with a replication fork. Like the formation of DSBs by other mechanisms, this triggers a number of events including cell-cycle arrest, homologous recombination to repair the breaks, or apoptosis, if the damage load is too large for the cell to process. The DSBs induced by crosslinking agents differ from the ones formed by agents that induce direct breakage of the strands such as ionizing radiation. Little is known about whether the events that lead to apoptosis are different in response to frank DSBs or ICL-induced DSBs.[23]

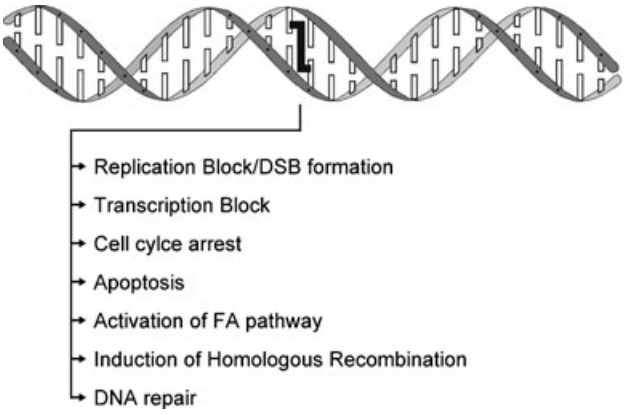
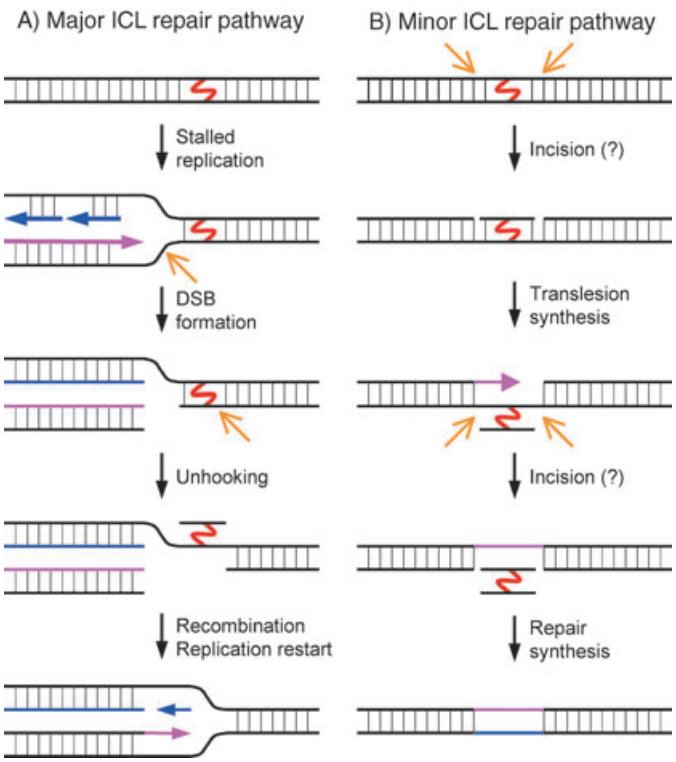


Figure 1. *Cellular responses to ICL formation. The known consequences of ICL formation in mammalian cells are indicated. There are intimate connections between several of the indicated events. See text for details.*

Indeed, the cellular mechanism underlying the cellular decision on when to survive and repair damage to DNA and when to die are not well understood in general. I will focus the discussion on the biological responses to ICLs on two areas, the repair of ICLs and the molecular basis of the inherited disorder Fanconi Anemia, for which the distinctive cellular responses to ICLs are slowly emerging.

Removal of ICLs from DNA

It has been demonstrated that the removal of ICLs is an important mechanism by which tumor cells counteract the effects of crosslinking agents.[24] The understanding of how ICLs are repaired in humans is therefore of great importance for antitumor therapy and could yield new targets for drug design. The repair of ICLs necessarily requires a complex series of steps, as no intact template is available for repair synthesis and the two strands need to be unhooked.[25] Available evidence suggests that the main pathway of ICL repair in mammals is active in the S-Phase of the cell cycle and depends on DNA replication.[22] Upon stalling of a replication fork, a double-strand break (DSB) is introduced (Scheme 3 A).[26] This step probably involves a nuclease-mediated specific incision step at the stalled replication fork. Subsequent steps of this pathway are only ill-defined, but the model shown here accounts for a key role of ERCC1-XPF (a structure-specific endonuclease involved in the nucleotide excision repair (NER) pathway and the only NER protein believed to be essential for ICL repair) and homologous recombination proteins.[27] ERCC1-XPF might incise the ICLs on the other side of the break, thereby unhooking one of the damaged strands.[28] Following the unhooking step, homologous recombination-mediated repair of the DSB as well as a second excision event are hypothesized to take place before replication can resume. This model is a very tentative one and is likely to undergo many modifications in the near future. It should be noted that cell lines with mutations in a number of additional genes are hypersensitive to crosslinking agents. These genes may thus also play a role in this ICL repair pathway. Among these genes are specialized polymerases, helicas-



Scheme 3. *Pathways of ICL repair in mammals. Two pathways of ICL repair have been described in mammals. A) The major ICL-repair pathway depends on DSB formation in response to a stalled replication fork, an unhooking step to release the ICL from one strand, and a homologous recombination event to repair the DSB. B) A minor, recombination-independent ICL-repair pathway depends on genes involved nucleotide-excision repair and translesion synthesis. Neither pathway is understood in detail. Orange arrows indicate potential incision sites.*

es, and nucleases—enzymatic activities that are required for the steps depicted in the model in Scheme 3 A.[29]

A second, recombination- and replication-independent error-prone pathway has also been described in mammals that is dependent on NER and the translesion DNA polymerase η (Scheme 3 B).[30] Although conclusive genetic evidence for this pathway in mammals is missing, closely related pathways have been observed in yeast and bacteria.[31] This pathway could be significant in the absence of replication in stationary cells.

Fanconi Anemia—Linking ICLs to Inherited Disease and Cancer Predisposition

Cell lines derived from patients with the hereditary disorder Fanconi Anemia (FA) have long been known to display hypersensitivity to crosslinking agents; this suggests a role for the FA genes in ICL repair.[32] At the clinical level, FA is characterized by congenital abnormalities, progressive bone marrow failure and a high incidence of cancer. While the specific sensitivity of FA cells toward crosslinking agents, but not other DNA damaging agents, is still not fully understood, recent studies have linked the FA proteins to homologous recombination events induced by ICLs. To date at least 11 FA genes have been found, a majority of which (FANCA, C, E, F, G, L) assemble to form a stable complex in the nucleus. This FA core complex is responsible for the monoubiquitination of another FA gene, FancD2, in response to DNA damage (Figure 2).[33] Ubiquitinated FancD2 is then targeted to chromatin in the nucleus, where it is responsible for recruiting BRCA2 (a breast cancer susceptibility gene). This recruitment of BRCA2 appears to be essential

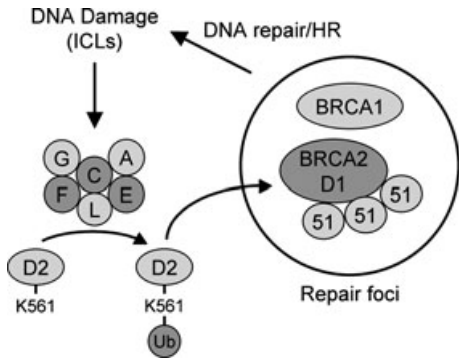


Figure 2. *The Fanconi Anemia pathway regulates DNA repair of ICLs by homologous recombination. At least six FA proteins (A, C, E, F, G, L) form a nuclear complex. In response to DNA damage, the complex monoubiquitinates FANCD2 at Lys561 in a reaction involving the ubiquitin ligase activity of FANCL. Ubiquitinated FANCD2 is targeted to chromatin in foci that also contain BRCA1. FANCD2-Ub recruits BRCA2/FANCD1 to these putative sites of damage and activates DNA repair by homologous recombination.*

for the efficient repair of ICLs by homologous recombination. Interestingly, partial inactivation in BRCA2 can also give rise to FA (FancD1 complementation group).[34] There is some evidence that DSBs are formed normally in response to crosslinking agents in FA cells,[35] indicating that the FA pathway is involved in the processing of ICL-induced DSBs by homologous recombination, including perhaps the unhooking step. An open question is whether the FA core complex directly interacts with ICLs or if an indirect signal is responsible for the activation of its ubiquitin ligase activity.

Recent findings have demonstrated that the status of the FA pathway can determine the sensitivity of tumor cells to crosslinking agents.[36] This information could therefore be an important predictor for the success of treatment of a given tumor with crosslinking agents. Likewise, the inhibition of the FA pathway could provide a way to sensitize tumors to treatment by crosslinkers. The inhibition of the monoubiquitination of FancD2, an enzymatic step of this process, would be a logical starting point to test this concept.

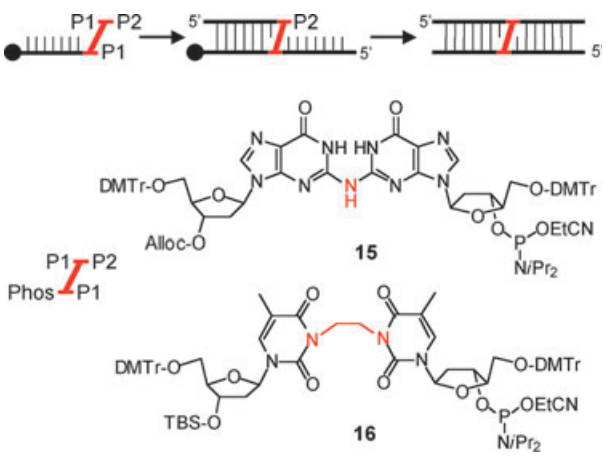
The insight gained into the molecular basis underlying the FA pathway is a nice illustration of how the study of rare genetic diseases can lead to the elucidation of general aspects of cellular metabolism. This is not a novelty in the area of DNA repair, as the study of another rare disorder, Xeroderma Pigmentosum, was instrumental in elucidating the nucleotide excision repair pathway.[37]

Synthetic Approaches toward Defined ICLs

One of the main limitations for studying the biological effects of ICL has been the limited availability of defined ICLs.[38] Fur-

thermore, it has been difficult to distinguish the effects of monoadducts and *intra*- versus *inter*strand crosslinks in studies that involve the treatment of cells with various crosslinking agents, since monoadducts and intrastrand crosslinks are formed more frequently than interstrand crosslink, which however have the stronger biological effects.

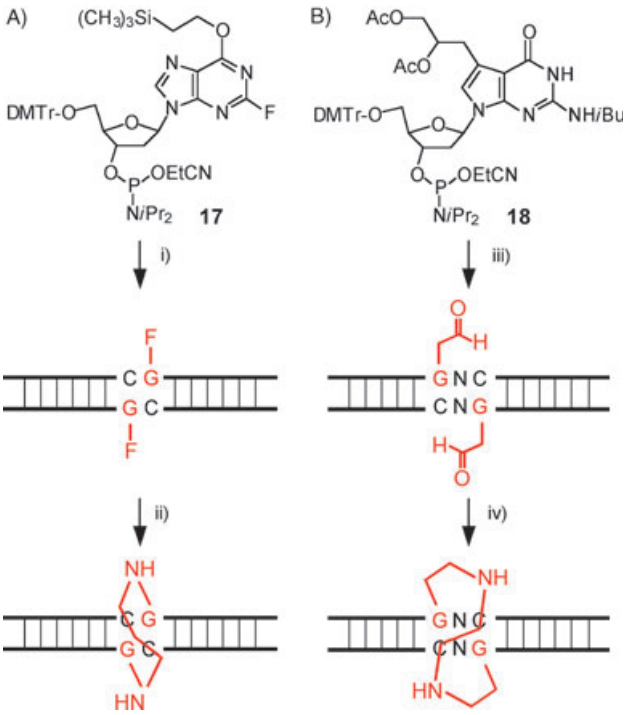
The synthesis of ICLs was initially accomplished by treating double-stranded oligonucleotides with crosslinking agents and subsequent isolation of the ICL from the reaction mixture.[6,9,39] Since ICLs are only a minor component of all the products formed (typically 1–5%) and monoadducts and intrastrand crosslinks make up the vast majority of products, this approach is of limited preparative use. The de novo chemical synthesis of ICLs has been explored by two different strategies to ensure the specific formation of ICLs. One is based on the crosslinking of two nucleosides outside of DNA and the introduction of the crosslinked dimer into DNA by using solid-phase DNA synthesis after appropriate functionalization and protection (Scheme 4). A differential protection scheme of the nucleoside



Scheme 4. *ICL synthesis by using a presynthesized crosslinked nucleotide dimer. Nitrous acid and alkyl ICLs were synthesized through incorporation of the phosphoramidites of the crosslinked dimer building blocks* ***15*** *and* ***16****. The use of two orthogonal protecting groups (denoted P1: DMTr and P2: Alloc or TBS) allowed the synthesis of palindromic sequences containing site-specific ICLs.*[40,42]

dimer was developed that allowed for the stepwise construction of the oligonucleotide flanking the ICL. Although this method of ICL synthesis imposes certain limitations in terms of accessible sequences and lengths of the ICL-containing oligonucleotides, it has led to the synthesis of ICLs formed by nitric oxide,[40] mimics of ICLs formed by alkylating agents[41,42] and artificial crosslinked base-pairs[43] in sufficient amounts for detailed structural characterization.

A second approach consists of the site-specific incorporation of crosslink precursor or post-synthetically modifiable nucleotides on opposing strands of DNA, annealing of the two single strands, and subsequent use of a specific coupling reaction to furnish the ICL (Scheme 5). This concept has been used for the synthesis of disulfide ICLs in the major and minor grooves,[44] psoralen ICLs,[45] and stable mimics of malondialdehyde[17] and in nitrogen mustard ICLs.[46]



Scheme 5. *ICL synthesis with postsynthetically modifiable ICL precursors. A) Malondialdehyde ICL mimic: i) A phosphoramidite containing a guanosine with a fluoride instead of an amine at the 2′ position (****17****) is incorporated on two complementary oligonucleotides.*[17] *ii) The two strands are condensed with diaminopropane to form an ICL in the minor groove. B) Nitrogen mustard ICL mimic: iii) A phosphoramidite containing a protected propan-2,3-diol side chain at the 7-position of deazaguanosine (****18****) is incorporated on complementary strands of DNA. Under basic deprotection conditions, the corresponding diol is formed and oxidized to an ethanal with* $NaIO_4$*. iv) Reductive amination with diaminoethane in the presence of* $NaBH_3CN$ *yields an ICL in the major groove of DNA.*[46]

The latter approach allows the synthesis of ICLs of any length and sequence and can easily be adapted for the synthesis of intrastrand crosslinks or monoadducts. It therefore provides access to defined substrates to investigate the different biological effects of various adducts formed by crosslinking agents in a controlled fashion.

Concluding Remarks

ICLs are complex DNA lesions that are not easily removed from the genomes of living organisms. The study of the cellular responses to ICL formation has already yielded fascinating insights into the fields of DNA-damage signaling and DNA repair. The further elucidation of the ICL-repair and FA pathways should have profound implications for the understanding of the mechanisms of resistance in antitumor therapy and the development of new drugs designed to be used in combination with current treatments. Recent progress in the synthesis of defined ICL adducts has provided an important advance on the way to the detailed biochemical and cell biological characterization of the ICL induced repair and signaling pathways.

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