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MULTI-AUTHOR REVIEW

Using synthetic DNA interstrand crosslinks to elucidate repair pathways and identify new therapeutic targets for cancer chemotherapy

Angelo Guainazzi · Orlando D. Schärer

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Abstract Many cancer chemotherapeutic agents form DNA interstrand crosslinks (ICLs), extremely cytotoxic lesions that form covalent bonds between two opposing DNA strands, blocking DNA replication and transcription. However, cellular responses triggered by ICLs can cause resistance in tumor cells, limiting the efficacy of such treatment. Here we discuss recent advances in our understanding of the mechanisms of ICL repair that cause this resistance. The recent development of strategies for the synthesis of site-specific ICLs greatly contributed to these insights. Key features of repair are similar for all ICLs, but there is increasing evidence that the specifics of lesion recognition and synthesis past ICLs by DNA polymerases are dependent upon the structure of ICLs. These new insights provide a basis for the improvement of antitumor therapy by targeting DNA repair pathways that lead to resistance to treatment with crosslinking agents.

Keywords Interstrand crosslinks · Cancer chemotherapy · DNA repair · Cisplatin · Nitrogen mustards

Abbreviations

ICL	Interstrand crosslink
NM	Nitrogen mustards
NER	Nucleotide excision repair
CENU	Chloro nitroso urea
MMC	Mitomycin C
FA	Fanconi anemia
HR	Homologous recombination
TLS	Translesion synthesis

A. Guainazzi
Departments of Pharmacological Sciences, Chemistry 619, Stony Brook University, Stony Brook, NY 11794-3400, USA

O. D. Schärer (✉)
Departments of Pharmacological Sciences and Chemistry, Chemistry 619, Stony Brook University, Stony Brook, NY 11794-3400, USA
e-mail: orlando@pharm.stonybrook.edu

Introduction

The faster proliferation of cancer cells compared to healthy ones makes cell cycle-dependent DNA metabolism a main target for chemotherapeutic agents. While some agents interfere with chromosome segregation during cell division or with the generation of nucleotide triphosphates needed for DNA synthesis, a large number of drugs used in chemotherapeutic regimens directly target DNA to kill malignant cells [1, 2]. These agents form adducts with DNA that interfere with essential aspects of its metabolism such as DNA replication and transcription, triggering cell death. Among DNA damaging agents, those forming DNA interstrand crosslinks (ICLs) are particularly potent as ICLs make strand separation impossible by the covalent linkage of two DNA strands [3, 4]. ICLs, like most DNA lesions, trigger cellular signaling and repair cascades that are associated with resistance of tumor cells to ICL-forming agents [5]. Due to the genetic make up of tumor cells and the generally higher mutation rates, the levels of resistance to chemotherapeutic agents vary greatly and present a serious problem in finding optimal therapies.

In this review we will discuss our current understanding of the biological responses triggered by ICLs with a focus on the use of synthetic adducts that have facilitated these studies. We will also provide an outlook into how these findings may be translated into the design of therapeutic agents for cancer chemotherapy.

Bifunctional alkylating agents form ICLs as the physiologically most important lesions

DNA crosslinking agents were among the first chemotherapeutic drugs used to treat cancer in the 1940s, after a serendipitous discovery. Mechlorethamine, a nitrogen mustard, was originally designed as a more stable derivative of mustard gas as a chemical warfare agent. An accident led to the exposure of civilians and soldiers to mustard gas during World War II and revealed the lymphotoxic effect of these agents. Together with earlier observations of mustard gas on cell growth, these observations provided the motivation for the first clinical trials and successful treatment of lymphoma patients with nitrogen mustards (NM) [6–8]. Numerous derivatives of mechlorethamine have subsequently been developed and NMs such as chlorambucil, cyclophosphamide, and ifosfamide are still mainstays of antitumor therapy. Only many years later, after the elucidation of the double helical structure of DNA was it established that NMs form ICLs by reacting with the N^7 positions of two guanine residues on two complementary DNA strands [9–11]. ICL formation was subsequently demonstrated for a number of additional antitumor agents, including platinum complexes, mitomycin C, and chloro ethyl nitroso ureas [11, 12]. Crosslinking agents are, however, not limited to cancer chemotherapy and a number of endogenous and environmental agents such malondialdehyde (a lipid peroxidation product), acetaldehyde, and natural products like the furocoumarins (present in plants and cosmetics), can form ICLs [13]. Since such adducts constitute a serious threat to cellular survival, they have provided an evolutionary incentive for organisms to develop the ability to repair ICLs. The importance of these repair pathways is evidenced by the existence of the human cancer-prone disorder Fanconi anemia (FA). Cells from FA patients are specifically sensitive to crosslinking agents, implicating deficient ICL repair as a cause for the genomic instability that leads to carcinogenesis. While these repair pathways are vital for healthy cells, they cause resistance to ICL-forming agents in a therapeutic setting [14, 15].

Generation and structures of ICL-containing DNA

One of the prerequisites for studying ICL repair is the availability of defined ICL-containing oligonucleotides. Initially, this was achieved by the reaction of duplex DNA with crosslinking agents (Fig. 1a). While this approach led to the generation of DNA duplexes with ICLs formed by NM, cisplatin, BCNU, or mitomycin C, it is very inefficient, yielding only about 1–5% of ICL adducts, with monoadducts and intrastrand crosslinks making up the majority of products [3, 4]. This approach can be improved upon to a minor extent by generating first a monoadduct with a single-stranded oligonucleotide, followed by annealing to a complementary strand and ICL formation (Fig. 1b). More targeted strategies for the synthesis of ICLs have recently been developed that make use of site-specific solid-phase DNA synthesis and introduce the ICL either in form of a crosslinked dimer (Fig. 1c) or as ICL precursors that can undergo a specific coupling reaction after incorporation into complementary strands and annealing (Fig. 1d). These approaches are discussed in more detail in conjunction with crosslinking agents and structures of ICL-containing oligonucleotides below.

Nitrogen mustard ICLs

Nitrogen mustards react with the N^7 position of guanine residues through the reactive *N*,*N*-bis-(2-chloroethyl)amine functional group. NMs preferentially form a 1,3 ICL in a 5′-d(GNC) sequence context [16–18]. It has been shown that NM ICLs induce a slight bend in duplex DNA (∼15°) to best accommodate the bridge between the two strands. In fact, the maximal theoretical length of the five-atom bridge formed by NM (∼7.5 Å), is less than the average distance between the N^7-G sites in 5′-d(GNC) sequence (∼8.9 Å) (Fig. 2) [19, 20]. Initially, NM ICLs were generated by treatment of dsDNA with NMs, yielding only small amounts of ICLs in a non-specific reaction. Additionally, NM ICLs are inherently unstable on a laboratory time scale (in the order of hours to days at room temperature) hindering detailed characterization [21–24]. One way to generate more stable NM ICLs has been to convert them to the stable ring-opened formamidopyrimidine (FAPY) form by treatment with aqueous base enabling initial studies of the repair of NM ICLs in bacteria [16, 25–27], although this approach did not address the problem of selectivity and the final structure of the lesion is significantly different from the original NM ICL.

We recently developed a strategy to synthesize stable analogs of NM ICLs site-specifically with high specificity and in high yield [20, 28]. This approach involved the incorporation of acetaldehyde functionalities at the 7-positions of G residues in complementary oligonucleotides and use of a double reductive amination reaction to generate the ICL (Fig. 1d). dG was substituted with 7-deaza-2′-deoxyguanosine to counteract the inherent lability of the glycosidic bond in N^7-alkylated guanines. This approach not only allowed for the synthesis of ICLs isosteric to the drug-induced adducts but also of ICLs that induce a distinct degree of bending of the DNA helix by variation of the length of the bridge linking the two bases on complementary strands. As discussed in more detail below, evidence is increasing that a number of steps in ICL

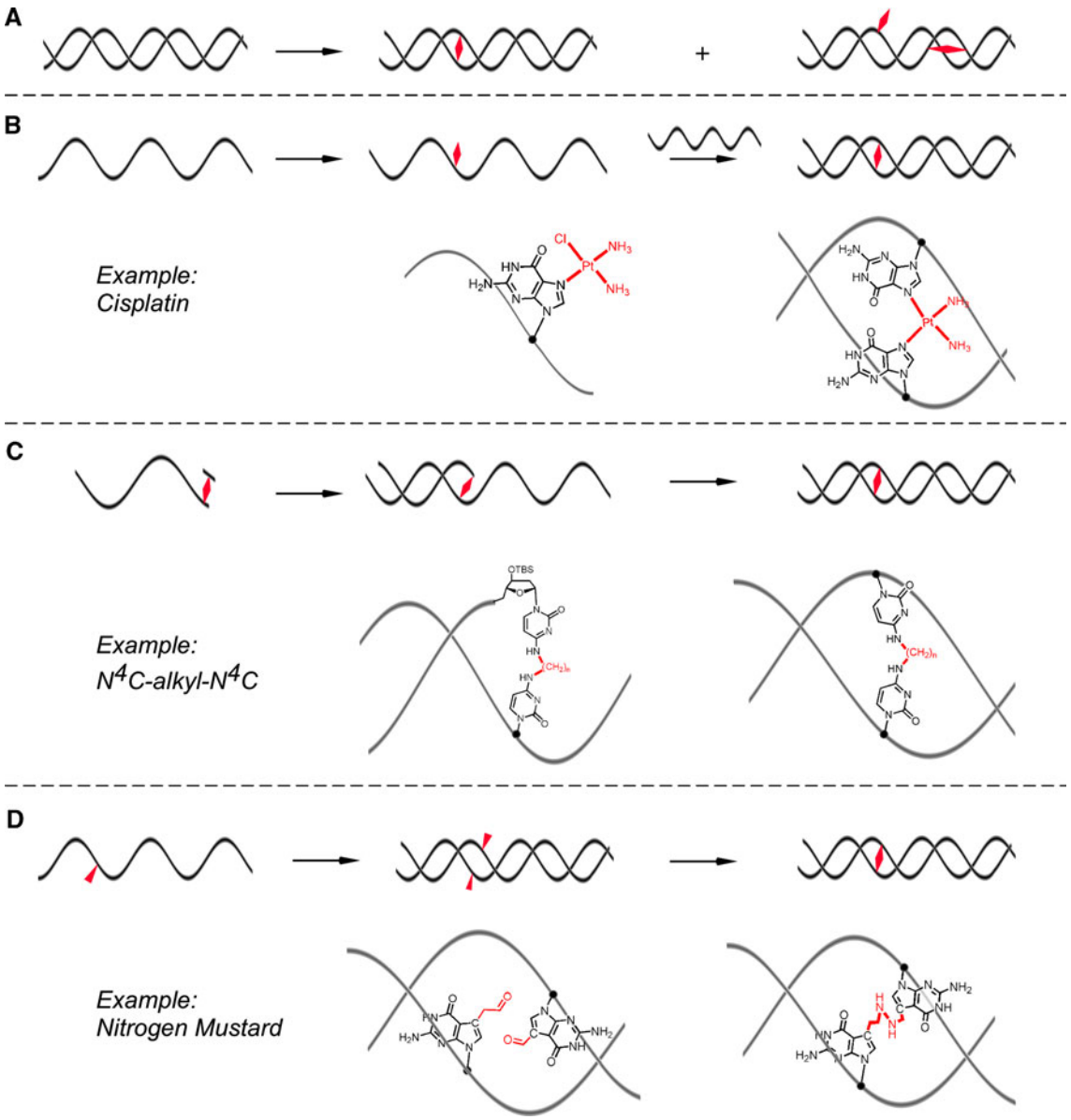


Fig. 1 Methods for the preparation of DNA interstrand crosslinks. **a** Treatment of an oligonucleotide with a bifunctional alkylating agent. This method gives rise to a mixture of products (intra- and interstrand crosslinks, monoadducts) and ICL typically make up less than 5% of all the products. **b** Two-step ICL formation. After reaction of a single strand containing one guanine residue, the monoadduct is purified, annealed with a complementary strand, and activated to react with the other strand. This method is more efficient than **a**, but yields typically do not exceed 20%. **c** Multi-step solid-phase synthesis; the crosslink is chemically synthesized as a nucleotide dimer and incorporated into DNA followed by bidirectional solid-phase DNA synthesis. This approach yields highly specific ICLs. **d** Two crosslink precursors are incorporated into DNA using solid-phase DNA synthesis and the ICL formed by a selective post-synthetic crosslinking reaction. This approach also yields ICLs with high specificity and in high yields. ICLs are shown as *red diamonds*

repair, in particular the initial recognition by cellular proteins and interaction with polymerases, are dependent upon the structure of the ICL.

Cisplatin ICLs

Cisplatin was serendipitously discovered as a compound with cytotoxic activity in 1965 [29, 30]. It is still widely used and is particularly successful against testicular cancer. Based on the initial success, many analogous of cisplatin (e.g., carboplatin, oxaliplatin, satraplatin, and picoplatin) have been generated and others are in development to improve the pharmacological properties [31]. Cisplatin is a planar coordination complex of Pt(II) with two chloride ligands in *cis*-position. Once the molecule enters the cell, the chlorides are displaced by two molecules of water, producing an activated form of cisplatin that is capable of

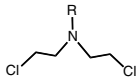
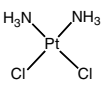
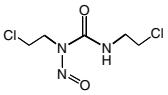
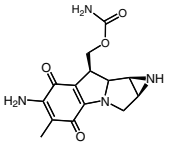
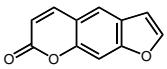
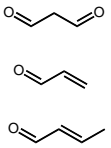
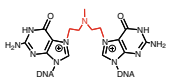
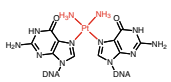
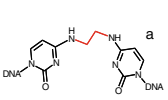
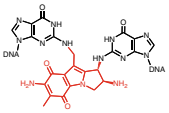
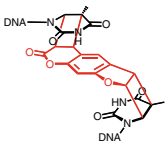
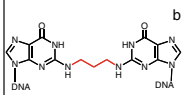
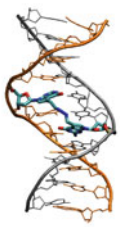
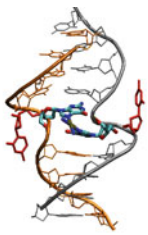
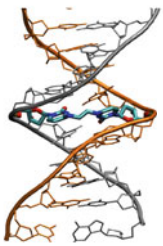
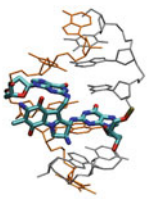
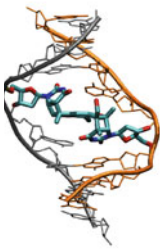
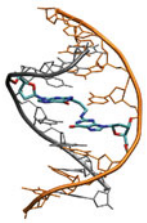
ICL-forming agent	Nitrogen Mustard	Cisplatin	Carmustine	Mitomycin C	Psoralen	Malondialdehyde
Target Sequence	5'-GNC	5'-GC	G-C basepair	5'-CG	5'-TA	5'-GC/5'-CG
Alkylating agent						
ICL structure[a]						
DNA structure						
PDB file	N/A[c]	1DDP	1N4B	Ref [50][d]	204D	1HZ2
Distortion	-15° bend -Minor local distortion	-Major distortion -Two Cs opposing the crosslinked Gs are extrahelical	Very minor local distortion[e]	Minor widening of MG	Minor local distortion	5'GC: Minor 5'CG: Major

Fig. 2 Common ICL-forming agents and their DNA adducts. *a* Although native BCNU adducts have not yet been synthesized, various mimics have been synthesized and used to study repair. The structure of the ICL and the pdb file refer to one of these mimics. *b* This ICL has been synthesized in two sequence contexts. This ICL is a reduced and stabilized form of the crosslinks formed by malondialdehyde. *c* The structure shown here is based on a molecular modeling study [28]. *d* The coordinates for the mitomycin C ICL were kindly provided by Suse Broyde based on Ref [51]. *e* The distortion refers to the mimic showed here. The exact distortion provoked by BCNU is not known and is likely to be different than the mimic

forming adducts with proteins, RNA, and DNA [32]. Like NMs, cisplatin preferentially reacts with the nucleophilic N^7 position of guanine, forming a monoadduct and subsequently forming intra- and interstrand crosslinks. Among these, the 1,2-intrastrand crosslinks (65% at 5′-GG and 25% at 5′-AG) are formed most frequently while 1,3-intrastrand crosslinks (up to 5% at 5′-GNG) and 1,2-interstrand crosslink (up to 8% at 5′-GC) are also formed in significant amounts [32]. The cisplatin interstrand crosslink severely distorts the double helix, due to the shortened distance between two guanines on complementary strands (Fig. 2). The cytosines opposite to the connected guanosines are flipped out into an extrahelical conformation and the DNA is considerably bent [33, 34]. Although no fully synthetic approach to cisplatin ICLs has been reported, these adducts have been prepared relatively efficiently by hybridization directed crosslinking (Fig. 1b). Activated cisplatin (*cis*-[$Pt(NH_3)_2(H_2O)Cl$]) is first incubated with an oligonucleotide containing a unique G in a 5′-d(GC) site to generate a monoadduct. Following purification, annealing to a complementary strand results in ICL formation [35]. We have employed this strategy in our laboratory for the preparation of oligonucleotides with a single defined cisplatin ICLs that were inserted into plasmids [36] to study replication-dependent repair of ICLs (see below).

Zhu and Lippard [37] synthesized a derivative of a cisplatin ICL in which one of the amines was conjugated to

a photoreactive benzophenone moiety. This photoreactive substrate was used to identify proteins that bind to the ICL in cell extracts revealing important differences in which proteins bind to inter- versus intrastrand crosslinks.

Chloro ethyl nitroso urea ICLs

Chloro ethyl nitroso ureas (CENUs), in particularly carmustine (BCNU), are widely used in cancer chemotherapy and are particularly useful for the treatment of brain tumors due to their ability to pass the blood–brain barrier [38]. The mechanism by which CENUs form ICLs is distinct from that of NMs and cisplatin. CENUs initially alkylate the O^6 position of dG and rapidly react again to form O^6-ethanoguanine, which slowly rearranges to an ICL formed between N^3 of dC and N^1 of dG [39]. Although CENU ICL-containing oligonucleotides have been generated by treatment of oligonucleotides with BCNU [40], strategies for the efficient site-specific generation of this adduct on a larger scale have so far remained elusive. A number of BCNU-like adducts have been synthesized either by incorporation of a precursor (a chloroethyl modified thymine) that can form an ICL with a guanine residue [41] or by incorporation of crosslinked nucleotide dimer into DNA using solid-phase synthesis (Fig. 1c) [42–44]. This later strategy allowed the synthesis of bridges with different lengths and different structures. Although these synthetic adducts are distinct from the CENU ICLs, some of them have started to yield insight into how the repair of ICL that are linked through their Watson–Crick pairing surfaces may differ from those that are linked through either major or minor groves [45, 46].

Mitomycin C: ICLs induced by a natural product

Mitomycin C (MMC) belongs to a class of antibiotics originally isolated from *Streptomyces caespitosus* and it is widely used in chemotherapy against gastrointestinal tumors, gastric, pancreatic, biliary tract, colorectal, and anal cancer [47]. Mitomycin C only forms adducts with DNA after reduction of its quinone ring reacting mainly with exocyclic amines of dG. After rearrangement and alkylation of a second guanine, it forms ICLs in a 5′-(GC) sequence in addition to intrastrand crosslinks and monoadducts [48, 49]. MMC ICLs are formed in the minor groove, where they induce a moderate widening to accommodate the MMC heterocycle [50, 51]. Since ICLs are formed relatively efficiently by treatment of a duplex with MMC, this agent has been used in cellular ICL repair studies as well as for some biochemical studies [52–54].

Psoralen–ICL formation can be induced by UV light

Psoralens are linear furocumarins isolated from plants and fungi and are used in the treatment of skin disease such as vitiligo or psoriasis. Psoralens intercalate into DNA and can be photoactivated with UV-A radiation to form covalent adducts with thymidine bases, forming interstrand crosslinks (Fig. 2) [55]. This ability to control the activity of psoralen by photoactivation is why this drug is specifically useful for the treatment of skin disease [56] and has made psoralen one of the most useful agents to generate ICL-containing oligonucleotides. Treatment of oligonucleotides containing a specific 5′-AT sequence with psoralen and UV can be controlled to produce mostly ICLs without excessive formation of byproducts such as monoadducts or intrastrand crosslinks. The efficiency of ICL formation has been additionally increased by the use of high-intensity lasers to specifically produce first the monoadduct and then the ICL [57] and by the chemical synthesis and incorporation into DNA by solid-phase synthesis of the monoadduct followed by the UV irradiation to obtain the ICL [58]. Psoralen ICLs have been structurally characterized by NMR [59, 60] and X-ray crystallography [61], revealing that these ICLs locally constrain and distort the DNA at the site of the intercalation but do not change the overall DNA structure (Fig. 2).

Due to the relative ease of preparation and stability, psoralen ICLs have been the most frequently used substrate in investigations of ICL repair in bacteria, yeast, and mammalian cells. More recently, Seidman and coworkers [62] developed a method for the induction of spatially defined ICLs in living cells by introduction of psoralen conjugates into cells and generation of ICLs using UV laser irradiation at defined positions in the cell nuclei. In this approach, psoralen was conjugated to a digoxigenin, allowing for the detection of the psoralen ICL by immunofluorescence. This approach allows the study of the recruitment of repair proteins to sites of ICLs [63] and provides a powerful new tool for studying ICL repair.

ICLs formed by endogenous and environmental compounds

Whereas most of the current interest in crosslinking agents stems from their use in cancer chemotherapy, the many endogenous and environmental ICL-forming agents must have been the driving force behind the evolution of the responses they trigger. One large group of agents that can form ICLs includes bifunctional aldehydes that are formed in cellular metabolic processes such as lipid peroxidation [13]. A prototypical bifunctional electrophilic compound is malondialdehyde, which can crosslink DNA via two

exocyclic guanine amino groups [64]. A number of additional aldehydes (formaldehyde and acetaldehyde) and α,β-unsaturated aldehydes (acrolein, crotonaldehyde), that are found in food, pesticides, and tobacco smoke and can also form DNA ICLs [65, 66]. One characteristic of aldehyde-induced ICLs is that they are intrinsically reversible, which can complicate biochemical and cellular studies. To circumvent this issue, Harris, Rizzo and coworkers [67] devised a way to synthesize a trimethylene ICL connecting two G residues in the minor groove, representing a reduced and stabilized form of aldehyde ICLs. The structures of these ICLs in 5′-GC and 5′-CG sequence contexts were characterized by NMR [67, 68]. The 5′-GC ICL is readily accommodated in the minor groove and causes little distortion, while the 5′-CG destabilizes the duplex and induces a bend and a twist in the double helix (Fig. 2).

Interstrand crosslinks can be formed from additional potentially endogenous sources. For example, nitric oxide has the ability to form ICLs through diazotization of the exocyclic amine of a G and subsequent ICL formation by reaction with an adjacent G on a complementary strand. This reaction yields an adduct in which two guanine residues are directly linked through a single amine group in the minor groove [69–71]. Such NO-induced ICLs have been incorporated into oligonucleotides as dimers using a solid-phase synthesis strategy [72, 73]. Structural characterization of these ICLs by NMR revealed that although they do not bend the DNA they induce distortion of the double helix by everting the cytosines paired with the crosslinked guanines into an extrahelical position in the minor groove [72].

Finally, the Greenberg laboratory has demonstrated that radicals such as the ones formed at the methyl group of thymidine can from ICLs, pointing to yet another potential endogenous source of ICLs [74, 75].

ICLs pose a difficult problem for the DNA repair machinery

Interstrand crosslinks are uniquely complex lesions, as they need to be removed from both strands of DNA. An intact template for the regeneration of the original DNA sequence is therefore not available as it is for monoadducts that are repaired by base excision or nucleotide excision repair. It became apparent from the first studies of ICL repair in *E. coli* by Cole [76, 77] that this process requires the interplay of multiple pathways, including nucleotide excision repair (NER) and homologous recombination (HR). The situation is even more complex in eukaryotes, where additional layers of regulation are present and ICLs are repaired by distinct pathways in S and G0/G1 phases of the cell cycle. Thus in addition to NER and HR, repair and signaling pathways like FA, mismatch repair (MMR) and translesion synthesis (TLS) have been implicated in ICL repair.

Replication forks stall at ICLs and trigger their repair

ICLs exhibit the highest levels of toxicity when they block strand separation and polymerase activity of the replication machinery and it is believed that the arrest of the replication fork is the trigger to initiate ICL repair [78]. Any details of replication-dependent repair have remained elusive until very recently, but several classes of proteins have been shown to be involved in ICL repair based on the sensitivity of cell lines with deficiencies in the corresponding genes. Such studies implicated endonucleases, including ERCC1–XPF [79–81] and MUS81–EME1 [82, 83], translesion synthesis polymerases, in particular Polζ and Rev1 [84–86] and proteins involved in homologous recombination, such as Rad54, XRCC2, and XRCC3 [87, 88] in ICL repair. In vertebrates, cells deficient in one of the at least 13 proteins associated with the cancer-prone inherited disorder FA pathway are specifically hypersensitive to ICL-forming agents. The mechanism by which the FA pathway is involved in mediating and regulating ICL repair is slowly emerging [89–91].

A recent breakthrough in our understanding of ICL repair came from studies in cell-free *Xenopus* egg extracts, a biochemical system that supports efficient replication of plasmids [92]. To adapt this system to study ICL repair, short oligonucleotides containing site-specific cisplatin or nitrogen mustard-like ICL prepared as described above were ligated into plasmids [36]. Incubation of these ICL-containing plasmids with the extracts allows plasmid replication under physiological conditions. Upon initiation of replication, two forks approach the crosslink from opposite directions, initially stalling at ∼20–40 nucleotides from the ICL (Fig. 3). After a delay of ∼20 min, one of the leading strands is extended to within one nucleotide of the ICL. At this point, dual incision around the ICL of the lagging strand template leads to unhooking of the ICL, and translesion synthesis leads to bypass of the lesion and full extension of the nascent leading strand. Although it has not yet been experimentally demonstrated, the ICL-remnant may then be removed by NER and the unhooked strand reengaged in replication by homologous recombination in a way similar as for double-strand breaks formed during replication.

Regulation of ICL repair by the FA pathway

The various steps and factors involved in replication-dependent ICL repair will be discussed here in the

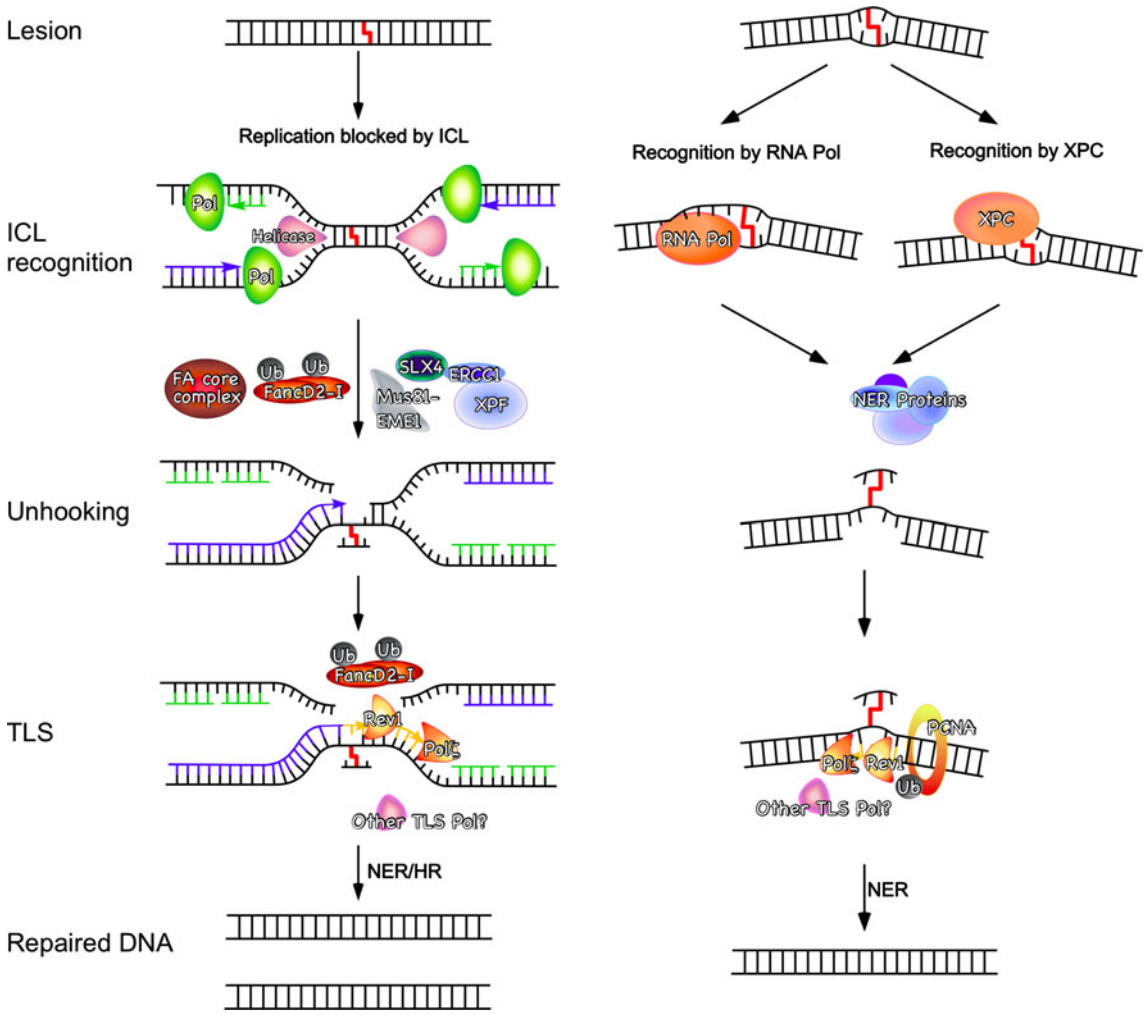


Fig. 3 Models for replication-dependent (*left*) and -independent (*right*) ICL repair pathways. In the replication-dependent pathway, the lesion is detected when replication forks are blocked by the ICL. The replication fork initially pauses 20–40 nucleotides from the ICLs, and then approaches to the ICL, activating the FA pathway. The FA core complex ubiquitinates the FANCD2–FANCI complex, a step required for the endonucleases to incise the lagging strand on both sides of the ICL and REV1 and Polζ to extend the leading strand past the ICL. It is believed that HR and possibly NER then complete the process and restore the intact DNA sequence. In G0/G1, the ICL can also be recognized by an RNA polymerase during transcription or, depending on its structure by the NER damage recognition factor XPC-RAD23B. NER proteins are thought to be responsible to unhook the ICL initiating repair synthesis and carrying out TLS past the unhooked ICL in a manner dependent on ubiquitinated PCNA and a TLS polymerase (probably Rev1 and Polζ to bypass the lesion in an potentially error-prone manner. Finally, it is believed that the NER machinery eliminates the ICL remnant restoring the intact DNA

framework provided by this study. The stalling of replication forks can trigger many pathways and we will limit discussion here to the FA pathway, which is most relevant to ICL repair. Indeed, the FA pathway is activated in S-phase in particular after exposure to crosslinking agents and this activation is also fully recapitulated in the cell-free *Xenopus* system. The FA pathway contains three components; the first one is the core complex, which contains eight FANC proteins (A, B, C, E, F, G, L, M) and at least five associated proteins (FAAP100, FAAP24, HES1, MHF1 and MHF2) [91, 93, 94]. Only two proteins of the FA core complex have known catalytic activities: FANCM is a DNA translocase and interacts tightly with MHF1-2 and FAAP24 [93–96]. It has the ability to remodel stalled replication fork structures and it is likely to play a role in loading the FA core complex at replication forks that are stalled at ICLs. FANCM also has a role in activating checkpoint signaling in the absence of the FA core complex [97].

The other protein with known catalytic activity is FANCL, which is a ubiquitin ligase [98, 99] and its activity is required for the monoubiquitination of the FANCD2–FANCI proteins, the second component of the FA pathway [100, 101]. Ubiquitination of FANCD2–FANCI leads to colocalization of the heterodimer with other DNA repair factors in chromatin and is essential for mediating cellular

resistance to crosslinking agents. A matter of debate for a long time, the direct role of FANCD2–FANCI in ICL repair was recently demonstrated in a cell-free *Xenopus* system using a site-specific cisplatin ICL [102]. In the absence of FANCD2–FANCI or in the presence of a mutant FANCD2 protein that can no longer be ubiquitinated (K562R), the replication fork was still able to approach the ICL to the −1 position, but both the unhooking and translesion synthesis steps were blocked. This result thus suggests that ubiquitinated FANCD2–FANCI complex has a direct role in recruiting nucleases and/or translesion synthesis polymerases to the sites of ICLs (see below).

The remaining FA proteins, FANCD1 (better known as BRCA2), FANCJ (a helicase, also known as BACH1 or BRIP1) and FANCN (also know as PALB2) act downstream of FANCD2–FANCI are believed to have a role in mediating recombination later in the pathway [91].

Recently, it has been suggested that the FA pathway may also function outside of replication, but the mechanisms by which this might occur are not yet clear [103, 104].

Which endonucleases are involved in unhooking the ICL?

An important feature of ICL repair in eukaryotes is that double-stranded breaks (DSBs) are induced at stalled replication forks [78, 80, 105]. They are likely probably induced in an unhooking step immediately before the replication machinery bypasses the lesion (Fig. 3). Two structure-specific endonucleases involved in ICL repair are ERCC1–XPF and MUS81–EME1, since cells defective in these enzymes are hypersensitive to crosslinking agents [79, 81, 82, 106]. Both of these enzymes cleave ss/dsDNA junction with 5′ ssDNA overhangs and therefore have the wrong polarity to make the first cut to generate DSBs in ICL repair [107]. In line with this view, studies have shown that neither ERCC1–XPF nor MUS81–EME1 are absolutely required for the formation of DSBs in response to crosslinking agents during S-phase as evidenced by the formation of γ-H2AX-foci, cellular markers of DSBs [81, 106]. Although dissenting views have been voiced as well [82, 108], it is most likely that the these two nucleases are instead involved in mediating the second incision in ICL repair [80, 109, 110] or perhaps also in resolving intermediates further downstream in the pathway during the homologous recombination step [111]. Both proteins seem to be recruited to the site of ICL repair by interaction with the SLX4 protein, which can also form a Holliday junction resolvase with another nuclease SLX1. Knock-down of SLX4 renders cells sensitive to crosslinking agents and SLX4 may therefore have a special role in coordinating nuclease activities at ICL [112–115]. Since SLX4 also contains a ubiquitin-binding (UBZ) domain, this may happen by interaction with a ubiquitinated protein such as FANCD2–FANCI.

Despite the implication of a number of nucleases in unhooking ICLs, it is likely that the protein(s) that makes the first incision on the lagging strand has not yet been identified.

Translesion synthesis restores the leading strand during ICL repair

Translesion synthesis is an essential step in replication-dependent (and replication-independent, see below) repair as it restores one of the two strands affected by the ICL (Fig. 3). Human cells contain at least 15 polymerases, many of which have the ability to bypass various types of DNA damage [116, 117]. Among these, there is a striking sensitivity of cells deficient in Polζ (Rev3–Rev7) and Rev1 to crosslinking agents [86]. Chicken DT40 cells deficient in Rev3 are in fact more sensitive than any other cell line to crosslinking agents pointing to a key role of this polymerase in ICL repair. The polymerase activity of Rev1 is limited to the insertion of a single dCTP opposite a damaged base while Polζ is generally considered to be a good extender polymerase and the two polymerases often act in concert [118]. The kinetics of ICL repair in the *Xenopus* egg extracts system suggest that one polymerase may insert a dNTP opposite the crosslinked nucleotide, while a second polymerase may responsible for the extension past the ICL [36]. Depletion of Rev7 from the *Xenopus* extracts leads to an additional stalling of the replication fork at the 0 position at a cisplatin ICLs, after insertion of a dNTP opposite the ICL, strongly suggesting that Polζ is responsible for the extension step past the ICL.

While these observations cumulatively suggest that Rev1 and Polζ are the key players for the TLS step in ICL repair, there is increasing evidence that additional polymerases take part in this process. For example, while the bypass of the helix-distorting cisplatin ICLs was dependent on Rev7 in *Xenopus* egg extracts, a nitrogen-mustard-like ICL that does not distort the DNA helix was bypassed with comparable efficiency in the presence and absence of Rev7. This indicates that other polymerases are able to bypass this ICL. Indeed, other TLS polymerases such as Polη, Polκ, or Polι are able to bypass such non-distorting ICLs in vitro under certain conditions (Ho T.V., A.G., O.D.S., unpublished observations). Lloyd and coworkers have studied the ability of minor groove trimethylene ICLs (Fig. 2) or non-distorting major groove ICLs. They found that these minor groove ICLs were readily and specifically bypassed by Polκ [66], whereas the major groove ICL were

specifically bypassed by Pol*v* [119]. The studies suggest that the bypass of ICLs by polymerases is highly dependent of the structure of the ICL and that a number of polymerases aside from Rev1 and Polζ may contribute to the process.

The completion of replication-dependent ICL repair involves homologous recombination and possibly nucleotide excision repair. The unhooked crosslink now resembles a "common" NER substrate, affecting only one strand, however genetic experiments showed only mild sensitivity to cells defective in NER genes other than ERCC1–XPF. On the other hand, loss of HR proteins showed substantially increased sensitivity to crosslinking agents. The role of HR in ICL repair has been recently reviewed by Hinz [120].

Replication-independent ICL repair

While most evidence points to replication-dependent repair being the main mechanism of dealing with ICLs, there is now robust support for ICL repair mechanisms in G0/G1 phases of the cell cycle (reviewed in [121]). The contribution of replication-independent pathways to ICL repair has not been fully appreciated, primarily because the extreme toxicity of ICLs is most apparent when a replication fork is blocked. Therefore, cells with deficiencies in genes that contribute to ICL repair during the S phase display higher toxicity when exposed to crosslinking agents.

Conclusive evidence for a replication-independent pathway stems from several experimental systems. Genetic studies in the yeast *S. cerevisiae* have revealed that NER and TLS pathways are essential for the repair of nitrogen mustard ICLs in the G0/G1 phase of the cell cycle [122]. The use of reporter assays has suggested similar pathways in mammalian cells [54, 123, 124]. In these studies, oligonucleotides containing site-specific ICLs were ligated into the promoter region of a gene of a plasmid. Activation of gene expression required the repair of the ICLs in mammalian cells. These studies showed that, consistently with the studies in *S. cerevisiae*, ICLs were repaired by a pathway involving NER and TLS. A number of ICLs were repaired in this way, including those formed by psoralen [123], mitomycin C [54] N^4C-N^4C and N^3T-N^3T alkyl ICLs [125], and cisplatin (Enoiu M., O.D.S., unpublished observations). As it has been shown for replication-dependent ICL repair, the TLS polymerases Rev1 and Polζ are of particular importance for the bypass of ICLs [124], in line with the observation that deletions in Rev1 and Polζ render DT40 cells more sensitive to crosslinking agents than deletions in any other gene [126]. The TLS polymerases are recruited to bypass the ICLs in G0/G1 in dependence of ubiquitination of PCNA by Rad18 [122, 124], similar to TLS of single-stranded lesions.

Interestingly, the recognition of some ICLs (psoralen, MMC) occurs by global genome NER (GG-NER) involving XPC-RAD23B [54, 123], while the repair of others (alkyl ICLs, cisplatin) was found to be dependent on transcription-coupled NER (TC-NER) [125] (Enoiu M., O.D.S., unpublished observations). The efficiency of NER of lesions affecting one strand of DNA is believed to be roughly proportional to the amount of thermodynamic destabilization induced by a lesion [127]. By contrast, it is believed that all bulky adducts can stall an RNA polymerase and trigger TC-NER (in the transcribed strands of active genes) [128]. An example of a lesion that is addressed mainly by TC-NER is the cyclopyrimidine dimer (CPD), which induces only minor distortion in the DNA [54, 127].

Although NER clearly plays a role in repairing ICLs, our understanding of how these lesions interact with NER proteins is limited. Although it has been shown that XPC-RAD23B is likely to be the first NER protein to arrive at psoralen ICLs in living cells [129], very little is known about how various ICLs are recognized by NER proteins. It is intriguing that cisplatin ICLs, which are highly helix-distorting [33], are not repaired by GG-NER [130], whereas psoralen or MMC ICLs, which are less distorting, are processed by GG-NER [54, 123, 129]. It therefore appears that the rules of damage recognition in NER and binding by XPC-RAD23B as they have been defined for lesions on one strand of DNA simply do not apply for ICLs [131–133]. It has been suggested that the mismatch repair proteins MutSβ (MSH2–MSH3) may play a role in facilitating the recognition of ICLs by NER and perhaps also independently of NER [134, 135]. The structural features that trigger NER of ICLs therefore remain to be determined.

In vitro studies of processing of ICLs by NER have revealed additional features that differ from canonical repair. NER proteins have been shown to make two incisions on one side of a psoralen or N^4C-ethyl-N^4C ICL, resulting in a gap 5′ to an ICL [45, 136, 137]. Such a gap could in principle facilitate TLS past the ICL, perhaps in conjunction with an incision 3′ to the lesion mediated by a nuclease that is not involved in NER, but the physiological relevance of this observation remains to be established.

Toward the discovery of clinically useful inhibitors of ICL repair

As it has been shown that the repair of ICLs in tumor cells leads to resistance to treatment with crosslinking agents such as cisplatin or NMs [138, 139], the inhibition of these repair pathways is an important goal for anti-tumor

therapy. The recent demonstration that the inhibition of ssDNA break repair protein PARP in BRCA2-deficient tumors is synthetically lethal, demonstrates that DNA repair inhibitors can be effective even in the absence of DNA-damaging agents [140–142].

As discussed above, the repair of ICLs is a complex affair and involves players from many different DNA repair pathways. To ensure specific and efficient inhibitory effects in conjunction with the treatment of crosslinking agents, it would be desirable to target those pathways that are specifically responsible for the repair of ICLs. With the advances of our understanding of ICL repair, proteins and pathways that may be targeted to inhibit ICL repair are emerging.

So far, the FA pathway has received most of the attention as a target due to the specific sensitivity of FA-deficient cells to crosslinking agents. Indeed, preclinical model studies in mice have revealed that disruption of FA core complex proteins FANCC and FANCG in adenocarcinoma cell lines abrogates FANCD2 monoubiquitylation and renders cells sensitive to agents such oxaliplatin and melphalan [143, 144]. To date, screens for inhibitors of the FA pathway have mostly focused on the most clearly discernable step, the ubiquitination of FANCD2–FANCI and the concomitant translocation of these two proteins into cellular foci and chromatin. D'Andrea and coworkers [145] used a cellular screen to identify compounds that inhibit the translocation of a GFP-labeled FANCD2 to nuclear foci following genotoxic treatment. The Hoatlin laboratory used *Xenopus laevis* cell-free extracts to screen for small molecules that inhibit FANCD2 ubiquitination upon incubation with different DNA molecules [146]. These two studies identified inhibitors of the FA pathway, including the natural product curcumin and derivatives thereof, that render cells sensitive to treatment with crosslinking agents. Gallmeier et al. [144] carried out a different type of screen to identify compounds that are selectively toxic to FA-deficient cells, yielding a number of interesting lead compounds. These studies raise the possibility that FA inhibitors with clinically useful properties may be found, both as a chemosensitizers in cisplatin-based cancer treatment and as synthetic-lethal agents in tumors with defects in the FA genes. Further development of FA pathway inhibitors will require a more detailed understanding of the mechanisms by which the identified compounds operate. Furthermore, emerging structural studies of FA proteins [99, 147] and a better understanding of the mechanisms by which FA proteins such as FANCM contribute to ICL repair will provide additional opportunities for inhibitors that target FA proteins.

As discussed above, another group of proteins that have ICL repair-specific functions are the endonucleases that make incisions around ICLs during replication-dependent repair. Although proteins like ERCC1–XPF have a number of functions, recent studies suggest that this protein is recruited to sites of ICL repair through specific interaction with partners such as SLX4 [112–115]. Importantly, cells expressing mutant ERCC1–XPF with a specific defect in NER are not sensitive to crosslinking agents such as cisplatin or MMC [148], suggesting that the identification and characterization of interaction sites that recruit nucleases to carry out their function in ICL repair may be fruitful targets for therapeutic intervention.

Concluding remarks

The repair of ICLs is accomplished by an assembly of many components from different repair pathways. Our understanding of the intricate network of interaction between signaling and repair pathways triggered by ICLs is still far from complete. The development of new methods to synthesize oligonucleotides containing site-specific ICLs has been an important advance for the field. These oligonucleotides have been incorporated into plasmids and used in cell-free extracts and living cells to study ICL repair pathways. Together with cell-biology and genetic approaches, this will aid the understanding of the molecular basis of how ICL repair differs from repair pathways of lesions that only affect one strand of DNA. These insights should provide opportunities to find new targets for drug developments to increase the therapeutic efficiency of crosslinking agents and to target tumor cells with specific defects in ICL repair.

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Note added in proof A candidate nuclease to make the first incision in the lagging strand in replication-dependent ICL repair has recently been discovered. This protein, FAN1 (Fanconi-associated nuclease 1) interacts with ubiquitination FancD2-FancI, has the appropriate polarity of incision, and knock-down of FAN1 renders cells sensitive to crosslinking agents. See McKay C, Declais AC, Lundin C, Agostino A, Deans AJ, MacArtney TJ, Hofamn K, Gartner A, West SC, Helleday T, Lilley DM, Rouse J (2010) Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. Cell 142:65–76; Kratz K, Schöpf B, Kaden S, Sendoel A, Eberhard R, Lademann C, Cannavó E, Sartori AA, Hengartner MO, Jiricny J (2010) Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. Cell 142:77–88; Smogorzewska A, Desetty R, Saito TT, Schlabach M, Lach FP, Sowa ME, Clark AB, Kunkel TA, Harper JW, Colaiácovo MP, Elledge SJ (2010) A genetic screen identifies FAN1, a Facnoni anemia-associated nuclease necessary for DNA interstrand crosslink repair. Mol Cell 39:36–47. Liu T, Ghosal G, Yuan J, Chen J, Huang J (2010) FAN1 acts with FANCI-FANCD2 to promote DNA interstrand crosslink repair. Science 329:693–696.

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