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Structure-dependent Bypass of DNA Interstrand Crosslinks by Translesion Synthesis Polymerases

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Abstract of the Dissertation

Structure-dependent Bypass of DNA Interstrand Crosslinks by Translesion Synthesis Polymerases

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Bifunctional chemical agents, such as nitrogen mustards or cisplatin, are widely used in cancer chemotherapy and exert their cytotoxic effect by introducing DNA interstrand crosslinks (ICLs). ICLs prevent strand separation of the DNA double helix and therefore interfere with transcription and replication. However, cellular mechanisms such as DNA repair can cause resistance of tumors against treatment with such agents, but the mechanism by which this is accomplished is still not well understood. Models for ICL repair suggest two pathways, one dealing with ICLs during S phase in a replication-dependent

manner and one repairing ICLs outside of S phase. In both pathways, endonucleolytic incisions near the ICL are made to unhook the ICL. In recent years the activity of translesion synthesis (TLS) polymerases was genetically implicated in ICL repair and it was suggested that their role is to bypass an unhooked ICL.

In order to investigate the ability of TLS polymerases to bypass ICLs we used primer extension templates containing a single 1,2 cisplatin or 1,3 nitrogen mustard ICL. In case of the nitrogen mustard ICL, we made use of synthetic substrates available in our laboratory. Hence, we are able generate ICLs with different lengths of the bridge between the two guanines and thereby introducing different amount of distortion in the DNA helix. We found that all ICLs tested could be bypassed by TLS polymerases, most efficiently by Pol η. Bypass was facilitated by resection of the crosslinked non-templating strand and increased linker size of the nitrogen mustard ICL, emphasizing structural constraints as an important factor in the bypass reaction of ICLs.

Table of Contents

List of Figures

List of Tables

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Chapter 1

General Introduction

Cancer Therapy: breaking or gluing DNA

Abnormal growth of tissue was described throughout recorded human history. The earliest descriptions for cancer are dated back to 1600 B.C. and can be found in ancient Egyptian papyrus scrolls found in the late 19th century, describing cauterization of tumors or ulcers. Traces of cancer can be even found in bones of fossilized ancient mummies. The term cancer was coined by Hippocrates (460-370 B.C.) a Greek physician and the “Father of Medicine”. He used the terms *carcinos* and *carcinoma*, which means crab in Greek and referred to the crab like shape of tumors with their emanating blood vessels. Celsus (28-50 B.C.) a Roman physician translated the greek term into the Latin word for crab, *cancer*. Throughout the middle ages the view of cancer was dominated by the medical writings of Galen (129-200 A.D.) a prominent Roman physician who referred to a growth or a tumor as *oncos*. Cancer was considered incurable. Even after removal of the tumor it was known that the tumor could grow back and once it spread through the whole body there was no treatment. Surgical ablation became more effective in the 19th century, especially after the discovery of

anesthesia. But still surgical removal did not guarantee a cure, especially when the cancer was found late and already metastasized (2009a; 2009b; 2009c; Morange, 2003).

Important discoveries were made by the end of the 19th century, which would lead to the development of new tools against cancer. During an evening on November 8th 1895 Wilhelm Conrad Röntgen discovered a new kind of radiation coming from a cathode ray tube, which would penetrate through cardboard but not through lead or platinum (Connell & Hellman, 2009; Heilbron, 1996). Roentgen himself performed the first medical application by using these new kinds of rays to photograph his wife's hand. He named these rays X-rays, for X being the unknown variable in mathematics (reviewed in (2009b; Connell & Hellman, 2009)). A few months later in July 1896 Victor Despeignes used X-rays for the first time therapeutically for a patient who had a stomach tumor (Leszczynski & Boyko, 1997; Tubiana et al, 1996). Claude Regaud demonstrated that repeated small doses of radiation could sterilize ram reticules (Regaud, 1930).

In March 2nd 1896 Henri Becquerel discovered that a uranium-containing mineral could blacken photographic plates without exposing it to sunlight. Two years later Marie and Pierre Curie discovered that pitchblende a uranium ore had greater radiation than pure uranium. These studies led to the discovery of polonium and radium. The Curie's were ultimately responsible for naming the new phenomenon radioactivity (reviewed in (Connell & Hellman, 2009; Kogelnik, 1996; Mazeron & Gerbaulet, 1998; Tubiana et al, 1996; Wendt, 2001)).

Encouraged by Pierre Curie Henri Danlos used radium to treat cases of lupus in 1901. This led to the development of brachytherapy, where radioisotopes are placed inside or near the area to be treated. The first mentioning of usage of radium for treatment of cancer came from Vienna in 1902 (Connell & Hellman, 2009). Goldberg and London were the first to use radium against skin cancer in 1903 (Dutreix et al, 1998). But with these approaches tumors could be rarely cured without extensive normal tissue damage. However, it was not known how X-ray and radioactive material exert their cytotoxic effect.

In a study using fruit fly genetics Muller & Dippel (Muller & Dippel, 1926) found that X-rays could cause chromosome breaks, a structure discovered by Walther Flemming in 1882 (Flemming, 1882) and later was found to bear the hereditary principles (Boveri, 1902; Morgan, 1915; Sutton, 1903). The discovery of DNA being the chemical moiety carrying the genetic information by Avery *et al.* (Avery et al, 1944) and the structural solution for DNA presented by Watson & Crick (Watson & Crick, 1953) suggested that the cytotoxicity exerted by ionizing irradiation was based on the introduction of DNA strand breaks (Connell et al, 2004). In retrospect it is amazing how quickly the discovery of X-rays and radioactivity were communicated and resulted in therapeutic applications, considering that the Internet was a century away.

For the next few decades surgery and radiotherapy would be the only means to deal with cancer. Until Goodman *et al*. reported in 1946 their clinical studies, conducted during the Second World War, with two agents; tris(β-chloroethyl)amine hydrochloride and methyl-bis(β-chloroethyl)amine

hydrochloride (Goodman et al, 1946). These so called nitrogen mustards were developed between and during the World Wars as derivates of mustard gas.

Mustard gas was used as a chemical warfare agent at the end of the First World War. On July 12th 1917 German troops used mustard gas as a new chemical warfare in Ypres (Belgium) against the Allied troops (reviewed in (Hirsch, 2006; Sartori & Marrison, 1939)). During the three weeks of its first use during the First World War it was very effective with 14278 casualties (Hirsch, 2006). The main effect was irritation of exposed areas like skin, eyes and mucous membranes and if not washed away systemic toxicity and ulcer festered. Krumbhaar, a captain in the medical corps, noted that individuals surviving for several days after a mustard gas assault would suffer from suppressed bone marrow and develop leucopenia (Krumbhaar & Krumbhaar, 1919). This was the first indication that mustard gas could be used against lymphomas.

In the years between the two world wars, mustard gas and trifunctional nitrogen mustard were tested in animals and on superficial tumors (Adair & Bagg, 1931). Small doses of intravenously administered mustard gas resulted in leucopenia and impaired antibody response (Kohn, 1996). But systematic clinical antitumor trials began shortly after the Second World War.

Although there was no chemical warfare during the Second World War, a secret incident in Bari, known nowadays as “Little Pearl Harbor”, would lead to new clues for the use of mustard gas as a therapeutic remedy against lymphomas (Hirsch, 2006; Kohn, 1996). In the evening of December 2nd 1943 a large group of German JU88 planes struck the harbor of Bari in a surprise attack.

A large number of ships were destroyed; among them the Liberty ship ‘John Harvey’, secretly loaded with 2000 M47A1 hundred-pound mustard gas bombs. This was a response to concerns of a possible usage of chemical warfare by the Axis powers. Survivors of sinking ships swam ashore through a mix of mustard gas, petrol and water. The difference of this incident to mustard gas exposures during the First World War was that survivors were exposed to a low dose of mustard gas for a prolonged period of time and over the whole body. The medical staff in Bari did not know that they dealt with mustard gas exposure and hence, the victims were systemically intoxicated with mustard gas (Hirsch, 2006).

The observations led to the first systemic clinical trials where nitrogen mustards were used in patients suffering from Hodgkins lymphoma and leukemia (Goodman et al, 1946). Indeed, tumor regression could be observed in these studies marking the beginning of cancer chemotherapy.

It was known from secret research during the war that mustard gas could cause chromosome rearrangement and mutations in *Drosophila melongaster* (Auerbach et al, 1947). Subsequent studies found that sulfur mustard and nitrogen mustards could react with nucleic acid and proteins, which is accompanied with the loss of the chlorines (Barron et al, 1948; Elmore et al, 1948). Haddow and co-workers realized that two chlorine groups were crucial for the cytotoxicity (Haddow et al, 1948) and the ability to introduce chromosome aberration, thus it was suggested that crosslinking of cell components could be the mode of action for nitrogen mustards (Goldacre et al, 1949).

1960 Brookes and Lawley showed that nitrogen mustards alkylate the N7 position of guanine and that it could covalently link two guanines (Brookes & Lawley, 1960; Brookes & Lawley, 1961). About same time in 1961 Geiduschek found that DNA treated with nitrogen mustard could not be irreversibly denatured through heating and realized that the crosslinking of the two strands of DNA was responsible for this “reversibility” (Geiduschek, 1961). This “gluing” of the two strands renders DNA strand separation impossible and is the cause of the cytotoxic effect of interstrand crosslinking agents.

Since regression of tumors treated solely with an ICL forming agents tend to relapse and start to regrow with resistance to ICL forming agents (Goodman et al, 1946), it was suggested early on that there are cellular mechanism to remove ICLs. Further studies confirmed that an elevated repair efficiency of ICLs contributes to the development of tumor resistance to crosslinking agents (Panasci et al, 2002; Spanswick et al, 2002; Yagüe & Raguz, 2005).

Work in the 1970 is in bacteria led to the model proposed by Cole in 1973 (Cole, 1973), which is in its essence still valid for bacteria. ICL repair in eukaryotes is more elaborate and escaped precise identification (Dronkert & Kanaar, 2001; McCabe et al, 2009; Moldovan & D'Andrea, 2009; Niedernhofer et al, 2005; Patel & Joenje, 2007). Only in recent years a clearer picture seems to unravel.

A better comprehension of how ICLs are removed from DNA could help us understand tumor resistance against ICL forming agents and is therefore vital for further improvement of cancer chemotherapy.

Interstrand Crosslinking Agents

There are a large number of compounds discovered and developed since the 1940's, which can introduce ICLs. I will be focusing on two classes of agents, nitrogen mustard and cisplatin, since they are the most relevant crosslinking agents with respect to the work presented here. I will discuss their origin, the target site in DNA, mechanism of action and the structure of the crosslinked DNA adducts.

Nitrogen mustards

Nitrogen mustards were developed in the 1920's and 1930's in an effort to produce more effective chemical warfare agents, which had similar properties to mustard gas. The nitrogen mustards were easier to handle than their sulfur analogues since they could be stored as hydrochloride crystals yet still had similar chemical and biological properties to sulfur mustard (Kohn, 1996; Ward, 1935). Chemically they are bis-(beta-chloroethyl)-amines with different tertiary substituents on the nitrogen atom (Figure 1).

Two of this class of compounds methyl-bis(beta-chloroethyl)amine (mechlorethamine) and tris(beta-chloroethyl)amine (Figure 1) were used in the pioneering clinical trials against Hodgkin's lymphoma (Goodman et al, 1946) and their promising results sparked intensive research on nitrogen mustards and other compounds as chemotherapeutic agents against cancer (DeVita & Chu, 2008; Kohn, 1996).

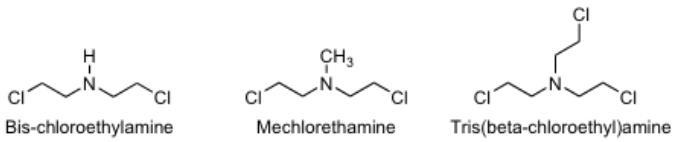


Figure 1: Nitrogen Mustards. Bis-chloroethylamine is the basis of nitrogen mustard drugs. Mechlorethanamine and Tris(beta-chloroethyl)amine were the two compounds used by Goodman et al.

By the end of the 1960's chlorambucil, melphalan and cyclophosphamide (Figure 2) were among the most widely used anticancer drugs and are still in use to this day (Povirk & Shuker, 1994). Some of these compounds like cyclophosphamide need to be metabolically activated by cytochrome P450 in order to react with DNA (Povirk & Shuker, 1994). Research on nitrogen mustard drugs did not end in the 1960's, bendamustine was approved by the FDA in 2008 (Kalaycio, 2009).

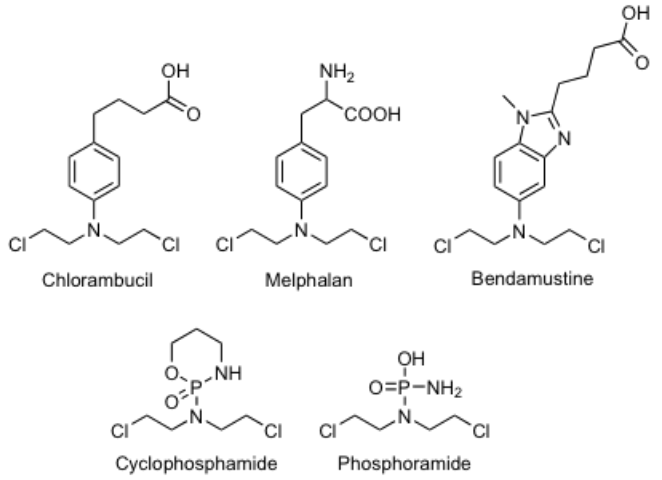


Figure 2: Nitrogen mustard based drugs. Phosphoramide is the metabolic activated form of cyclophosphamide.

Nitrogen mustards react preferentially with the N7 position of guanines and can either form monoadducts, intrastrand crosslinks or ICLs between two guanines (Brookes & Lawley, 1961; Osborne & Lawley, 1992) and depending on

the nitrogen mustard used between 5-20% represent ICLs (Povirk & Shuker, 1994, references therein). Additionally, reaction with the N3 position of adenine can also be observed (Osborne & Lawley, 1993; Pieper & Erickson, 1990; Wang et al, 1991). The formation of nitrogen mustard ICLs depends on a two-step mechanism where an aziridinium ion reacts first to form a monoadduct intermediate and then in a second step forms the ICL (Rink et al, 1993) (Figure 3).

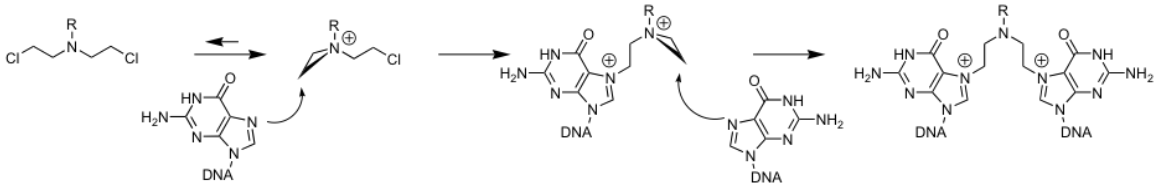


Figure 3: Mechanism of nitrogen mustard with guanine.

Initially, nitrogen mustard ICLs were believed to occur in a GC sequence (Kohn et al, 1987) until the beginning of the 1990's when several studies showed that ICL formation occurs in a GNC sequence, where N denotes any deoxynucleotide (Hopkins et al, 1991; Millard et al, 1990; Ojwang et al, 1989; Rink et al, 1993). This crosslinking specificity has been confirmed for a several nitrogen mustard compounds such as melphalan, cyclophosohamide and chlorambucil (reviewed in (Povirk & Shuker, 1994)).

The introduction of nitrogen mustard ICLs in a GNC sequence causes a significant distortion of the canonical B-form DNA. As a result of the discrepancy of the distance of 8.9 Å between the two guanines and the full length nitrogen

mustard bridge of 7.5 Å, the DNA is slightly bended by 12.4° to 16.8° per lesion, shown by gel retardation assays (Rink & Hopkins, 1995).

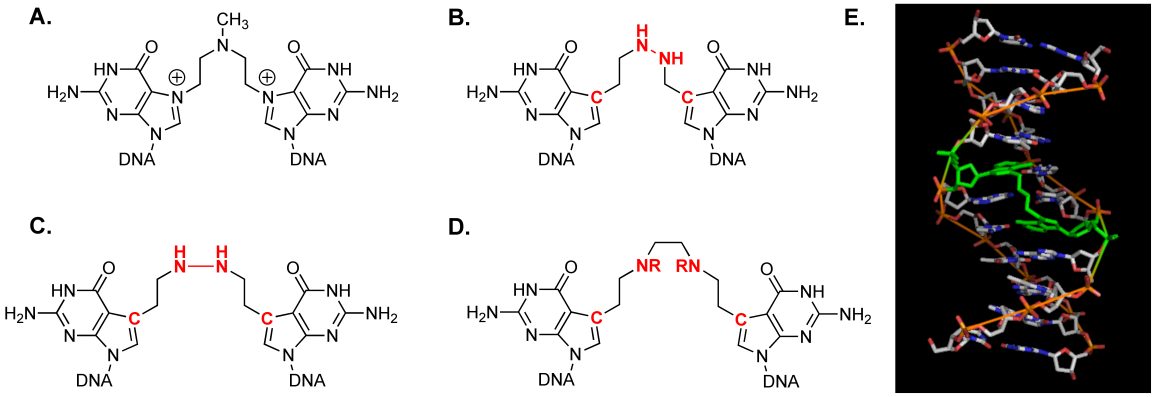
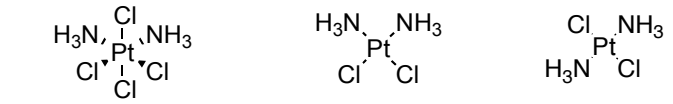


Figure 4: Nitrogen mustard mimic ICLs. **A.** Mechlorethamine ICL 7.5 Å. **B.** Hydrazine crosslinked 7.5 Å mimic. **C.** Hydrazine crosslinked 8.9 Å mimic. **D.** Ethylenediamine or N,N-dimethylethylenediamine crosslinked 11.7 Å mimic. **E.** *In silico* structure of the hydrazine crosslinked 7.5 Å mimic (Guainazzi *et al.*, unpublished)

Furthermore, alkylation at N7 introduces a positive charge rendering nitrogen mustard adducts prone to depurination and it was shown that simultaneous depurination of the two guanines in an ICL occurs, although at a much slower rate than the depurination of monoadducts (Masta et al, 1994). Hence, in the biochemical studies presented herein we made use of synthetic substrates available in our laboratory, which are chemically stable and mimic nitrogen mustard ICLs (Figure 4) (Angelov et al, 2009). Further, by using different coupling reagents we can modulate the length of the bridge of the ICL. *In silico* molecular dynamic comparison confirmed the structural similarity of the mimic to a nitrogen mustard ICL and reveal dynamical constraints in the vicinity of the ICL (Guainazzi et al, unpublished) (Figure 4). Furthermore, these ICL were successfully used in biochemical studies (Räschle et al, 2008).

Platinum complexes

The serendipitous discovery of the cytotoxic effect of platinum complexes is an excellent example of how scientific progress is not always accomplished by goal-oriented research. Dr. Barnett Rosenberg who past away last August at the age of 82 was interested in the influence of electromagnetic dipole fields on cell division (Kelland, 2007; Rosenberg, 1985). The use of seemingly inert platinum electrodes in the growth chamber with *Escherichia coli* (*E. coli*) caused inhibition of cell division and the cells grew to very long filaments without dividing (Rosenberg et al, 1967b; Rosenberg et al, 1965). Subsequent chemical analysis identified that *cis*-diamminetetrachloro platinum (IV) was responsible for this effect (Rosenberg et al, 1967a; Rosenberg et al, 1967b; Rosenberg et al, 1965) (Figure 5).



Cis-diamminetetrachloride Cisplatin Transplatin

Figure 5: First platinum complexes. Cis-diamminetetrachloride and cisplatin were to inhibit cell division. Transplatin is clinically ineffective.

Platinum complexes were then tested in mice bearing a tumor and indeed were shown to cause significant tumor regression (Rosenberg, 1985; Rosenberg & VanCamp, 1970; Rosenberg et al, 1969), among them *cis*-diamminedichloroplatinum(II) (cisplatin) (Figure 5).

Cisplatin is an inorganic, square planar complex that was first synthesized by Peyron in 1844, hence it is also referred to as Peyrone's chloride (Peyrone,

1844; Rybak & Whitworth, 2005), and became the first platinum based antitumor drug. The first patient was treated in 1971 and in 1978 cisplatin was approved by the FDA (Jamieson & Lippard, 1999; Kelland, 2007). Interestingly, its *trans* isomer is clinically ineffective (Jamieson & Lippard, 1999; Kelland, 2007) (Figure 5), but other platinum complexes than cisplatin with chlorides in the *cis* position are used in anticancer therapy (Figure 6).

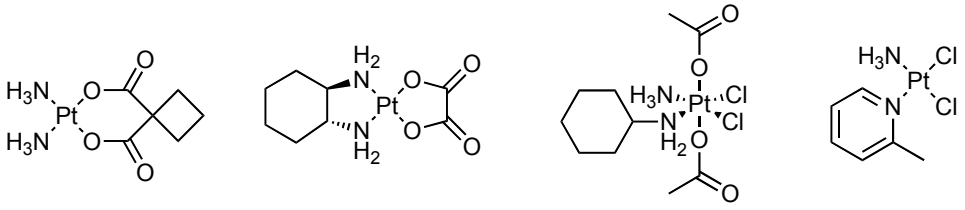


Figure 6: Other platinum based drugs**.** Carboplatin and oxaliplatin are used in anticancer chemotherapy. Satraplatin awaits FDA approval and picoplatin is undergoing phase III clinical trials.

A compelling line of evidence supports the view that cisplatin reacts on DNA in a solvent assisted reaction (Jamieson & Lippard, 1999; Malinge et al, 1999; Siddik, 2003). It is believed that cisplatin has to be activated through a series of spontaneous aquation reaction (Reishus & Martin, 1961), where the chlorid ligands are sequentially replaced by water molecules (Bancroft et al, 1990; Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001; Malinge et al, 1999; Siddik, 2003). This reaction is thought to be facilitated by the low concentration of chlorides within cells. The positively charged mono-aquated complex is a highly reactive species, which preferentially reacts with the nucleophilic N7 position of guanine to form monoadducts (Bancroft et al, 1990; Eastman, 1983; Hopkins et al, 1991). Subsequently the second choride ligand is

also replaced by water to allow the formation of intrastrand or interstrand crosslinked adduct between mainly guanines (Bancroft et al, 1990; Eastman, 1985; Hopkins et al, 1991) (Figure 7). The main products formed are 1,2-GG (<50%) and 1,2-AG (~25%) intrastrand crosslinks with a minor fraction being 1,3-GNG (5-6%) intrastrand crosslinks (Jamieson & Lippard, 1999). Only a small fraction (1-5%) represents cisplatin ICL in a GC sequence (Hansson & Wood, 1989; Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001). In contrast to other ICL forming agents, the intrastrand crosslinks formed are believed to contribute to the cytotoxic effect of cisplatin (Kartalou & Essigmann, 2001).

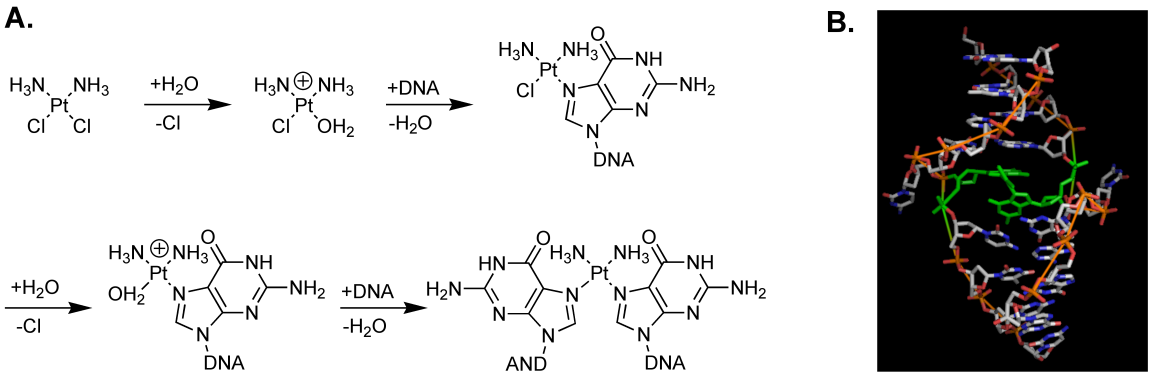


Figure 7: Cisplatin mechanism and structure **A.** Water assisted crosslink formation with cisplatin. **B.** Crystal structure of a cisplatin ICL (Coste *et al*., 1999)

Cisplatin DNA adducts are considered to be stable (Yang & Wang, 1996). However, under physiological conditions ICLs can rearrange to intrastrand crosslinked with a half-life depending on the size of the DNA (Pérez et al, 1997). Thus, the half-life of a ten base pair crosslinked duplex is 29 hours, whereas the stability in a twenty base pair duplex is markedly increased with a half-life of 120 hours.

The formation of cisplatin ICLs introduces a high degree of DNA helix distortion shown by NMR and X-ray studies (Coste et al, 1999; Huang et al, 1995; Paquet et al, 1996) (Figure 7B). The two pairing cytosines are forced out of the helix and no longer pair with the crosslinked guanines. The DNA is unwound by 70-80° and bent by 20-47° towards the minor groove. The N7 atoms of the guanine, which face the major groove in B-form DNA are relocated to the minor groove and the cisplatin bridge lies within the minor groove.

There are still new platinum complexes under investigation for their usefulness in anticancer therapy attested by satraplatin and picoplatin, one is awaiting FDA approval and the other is undergoing phase III clinical trials (Kelland, 2007) (Figure 6).

Chapter 2

Translesion synthesis in the repair of DNA interstrand crosslinks

Foreword

This chapter is intended for publication as a review article in a special issue of Environmental and Molecular Mutagenesis on DNA Interstrand Crosslinks: Repair, Cell Signaling, and Therapeutic Implications. Hence, work presented in chapter 3 is cited in here.

Introduction

Bifunctional agents with the ability to introduce chemical alterations to DNA can form DNA interstrand crosslinks (ICLs). ICLs are highly cytotoxic since they prevent strand separation of duplex DNA and therefore impose a block on essential processes such as transcription and replication. Based on these properties, ICL-forming agents such as cisplatin, nitrogen mustards, chloroethylnitrosoureas or mitomycin C are widely used in cancer chemotherapy (McHugh et al, 2001; Noll et al, 2006; Scharer, 2005).

ICLs represent a formidable challenge to genome maintenance, since the genetic information on both strands is compromised and a straightforward cut and patch mechanism using the information on the non-damaged strand as a template is not feasible. Instead, ICL repair depends on the concerted and coordinated interplay of factors from various DNA repair, damage tolerance and cell cycle checkpoint pathways, such as nucleotide excision repair (NER), transcription-coupled repair (TCR), homologous recombination repair (HRR), mismatch repair (MMR), translesion synthesis (TLS), ataxia telangiectasia and Rad3 related (ATR) and Fanconi anemia pathway (FA) (reviewed in (Lehoczký et al, 2007; Mirchandani & D'Andrea, 2006; Niedernhofer et al, 2005; Noll et al, 2006)).

All current models for ICL repair pathways involve endonucleolytic incisions near the ICL generating so-called unhooked intermediates, in which an oligonucleotide containing the ICL has been separated from one of the two strands (Figure 8). These unhooked intermediates are believed to serve as a substrate for TLS polymerases, which can replicate past the ICL to restore one of the two strands to an intact form. Several polymerases have been implicated in this step, possibly reflecting different ICL repair pathways as well as the structural heterogeneity of unhooked ICLs. Herein, we will discuss our current understanding of how polymerases are involved and regulated in ICL repair.

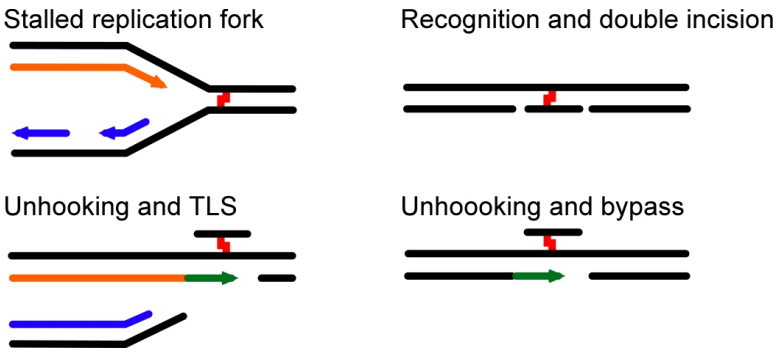


Figure 8: ICL repair intermediates for TLS step.

ICL repair in Escherichia coli and the involvement of polymerases

Removal of ICL in Escherichia coli is dependent on combined action of nucleotide excision repair and homologous recombination (Cole, 1973) or translesion synthesis (reviewed in (Dronkert & Kanaar, 2001; Lage et al, 2003)). Experiments with psoralen ICLs showed that the UvrABC complex, the core NER complex in bacteria, incises on the same strand 5' and 3' to the ICL (Van Houten et al, 1986), which unhooks the ICL. The bacterial HR factor RecA then mediates strand exchange reaction past the ICL (Sladek et al, 1989). Interestingly, it was found that a merely incised ICL was a poor substrate for strand exchange reaction in a in vitro reconstitute system and an additional activity was needed (Sladek et al, 1989). This led to the implication of a polymerase in ICL repair, Pol I a polymerase involved in Okazaki fragment maturation and NER (Caron et al, 1985; Husain et al, 1985; Kumura et al, 1985). However, it was not the

polymerization activity of Pol I, that was needed for the RecA mediated strand exchange reaction, but the 5'-3' exonuclease activity of Pol I. Pol I generates a ssDNA gap 3' to the unhooked ICL, where RecA can be loaded and subsequently mediate strand exchange (Sladek et al, 1989).

Most recently Zietlow et al. showed by in vitro polymerase extension reaction with a template containing a single psoralen ICL that Pol I was able to bypass this ICL (Zietlow & Bessho, 2008), although with an efficiency below 1%. They showed that the Klenow fragment of Pol I with and without the 3'-5' exonuclease (Klenow(-exo)) activity was able to insert a dNMP opposite the adducted thymidine template at high enzyme concentration and that Klenow(-exo) preferentially incorporates dAMP > dGMP > dCMP = dTMP. Interestingly, sequencing of fully bypassed product revealed that no mutation was introduced. The authors argue that dAMP insertion is a prerequisite for successful bypass by Klenow and that most probably extension from insertion products different than dAMP is not favorable. Anyway, the low bypass efficiency of Pol I to bypass a psoralen ICL suggest that indeed the exonuclease activity is most probably the main activity of Pol I contributing to the removal of psoralen ICL.

Studies conducted with a stabilized nitrogen mustard ICL derivative (Grueneberg et al, 1991; Ojwang et al, 1989) revealed a slightly different situation for the repair of nitrogen mustard ICL compared to psoralen ICL. Berardine et al. showed in a RecA deficient background that the activity of Pol II, was essential for the removal of a nitrogen mustard ICL (Berardini et al, 1999; Berardini et al, 1997). The gene for Pol II is damage inducible belonging to the

SOS response genes (Qiu & Goodman, 1997) and is involved in replication restart and NER. Further it was shown that this repair pathway was independent of a 5'-3' exonuclease activity, which is needed in the HR dependent repair of psoralen ICL. The pathway was still dependent on the UvrABC complex, suggesting an additional NER dependent ICL repair pathway, which needed Pol II activity to bypass the unhooked ICL in the absence of HR. Surprisingly the Pol II dependent pathway was accurate and no point, deletion and insertion mutations were detected. Interestingly, the Pol II dependent pathway seemed not to be involved in the removal of psoralen ICL, arguing that structural differences of the two ICL might modulate the way they are preferentially repaired.

Most recently, evidence for the involvement of another bacterial polymerase in ICL repair was found in Stephen Lloyd's group and even excluded Pol II in the bypass of a structurally distinct ICL from psoralen and nitrogen mustard ICLs (Kumari et al, 2008). In this study they used a N2-N2 guanine ICL, which is a model ICL for malondialdehyd and acrolein induced ICLs (Dooley et al, 2001) and found that Pol II was unable to bypass the N2-N2 guanine ICL. Further, reactivation of a plasmid containing a single N2-N2 guanine ICL was comparable to wild type cells, suggesting repair of this ICL was independent of Pol II. On the other hand they found that Pol IV, a Y-family polymerase involved in TLS in bacteria, was able to bypass and was needed for reactivation of a plasmid containing N2-N2 guanine ICL. As with Pol II and nitrogen mustard ICL, Pol IV dependent repair was accurate and no point mutation, deletion or insertion were detected.

These studies indicate that the TLS step in bacteria might be fulfilled by not only one polymerase (Table 1) but with the help of multiple polymerases. Similar to the two-step bypass model for one stranded lesions suggested for eukaryotes (Prakash & Prakash, 2002) there are also evidence for a multi step lesion bypass in prokayotes (Fuchs & Fujii, 2007). Hence, it is conceivable that in case of ICLs, structural differences might need the activity of one or multiple polymerases. Surprisingly, the results so far indicate that ICL repair dependent on a TLS step in bacteria seemed to be accurate and in all the studies discussed here, no mutations were introduced by the polymerases *in vivo*.

Table 1

E. coli polymerases involved in ICL repair			
Polymerases	Family	*in vitro* bypass ICL	References
Pol I	A	yes	{Zietlow, 2008 #646; Smeaton, 2009 #917}
Pol II	B	no	{Kumari, 2008 #947}
Pol IV	Y	yes	{Kumari, 2008 #947}

So far Pol III, the bacterial replicative polymerase, which was shown to be able to bypass certain lesions (Kokubo et al, 2005) and Pol V, another Y-family polymerase in bacteria (Tang et al, 1999), was not shown to be involved in ICL repair, although it is possible that these two polymerases might also be involved in the bypass of structurally distinct ICLs.

ICL repair in yeast and the involvement of polymerases

Genetic studies in yeast Saccharmyces cerevisiae identified numerous mutants, which were sensitive to DNA damaging UV and ionizing irradiation. These studies established three major epistasis groups for DNA repair in yeast, the RAD3 (NER), RAD52 (HR) and RAD6 (damage tolerance or post-replication repair) (Cox & Game, 1974). The repair of ICL in yeast depends on all three epistasis groups (reviewed in (Dronkert & Kanaar, 2001; Lehoczký et al, 2007; Noll et al, 2006)) and hence undermines the epistatic relationship between these groups in respect to ICL repair. One of the main differences from bacteria to yeast is the prominent appearance of double strand breaks as repair intermediates during ICL repair in exponentially growing cells (Dardalhon & Averbeck, 1995; Jachymczyk et al, 1981; Magana-Schwencke et al, 1982), which is greatly reduced in stationary cells (Magana-Schwencke et al, 1982; McHugh et al, 2000) suggesting that DSBs arise from collapsed replication forks at ICLs. The formation of DSBs depends on NER (Miller et al, 1982; Saeki & Machida, 1991), although there is evidence for an NER independent formation of DSBs in exponentially growing cells (McHugh et al, 2000). This incised intermediate is then either processed by HR in S/G2 phase or TLS in G1/G0 phase (McHugh et al, 2000; Saeki & Machida, 1991; Sarkar et al, 2006).

In an early genetic study, using UV-A irradiation to trigger photoaddition of psoralen to DNA, Henriques & Moustacchi (Henriques & Moustacchi, 1980)

isolated the pso1-1 mutant. This mutant was later on found to be allelic to the rev3-1 mutant (Cassier-Chauvat & Moustacchi, 1988), a mutant found earlier in a screen for reversion of an auxotrophic marker after UV irradiation (Lemontt, 1971). Rev3 was found to be the catalytic subunit of a nonessential polymerase (Morrison et al, 1989) and forms together with the accessory subunit Rev7 Pol ζ (Lawrence et al, 1985; Nelson et al, 1996b; Torpey et al, 1994b). Pol ζ belongs to the B-family of polymerases, which includes the replicative DNA polymerases Pol δ, ε, and α (Morrison et al, 1989). But unlike the other members of the family, Pol δ and ε, Pol ζ lacks the 3'-5' proofreading exonuclease activity, has a low processivity and a higher error rate on undamaged DNA (McCulloch & Kunkel, 2008; Prakash et al, 2005; Waters et al, 2009). Pol ζ was found to have a distinct ability to replicate damaged DNA, which would stall replicative polymerases. Although it was shown that Pol ζ by itself is able to bypass certain DNA lesion such as cis-syn TT dimers and thymine glycol (Johnson et al, 2003; Nelson et al, 1996b), its foremost ability is to extend from distorted primer template termini, resulting from base mismatches or lesions such as 6-4 photoproduct, 7,8-dihydro-8-oxoguanine, O6-methylguanine, thymine glycol, γ–hydroxy-1-N2-propano-2'deoxyguanine and actylaminofluorene-adducted guanine (Baynton et al, 1999; Haracska et al, 2003; Haracska et al, 2001c; Johnson et al, 2003; Washington et al, 2004a).

Cells lacking Pol ζ were shown not only to be sensitive to psoralen plus UVA treatment (Henriques & Moustacchi, 1980) but as well to other ICL forming agents, such as cisplatin, nitrogen mustards and mitomycin C (Beljanski et al,

2004; Grossmann et al, 1999; McHugh et al, 2000; Simon et al, 2000; Wu et al, 2004b). Intriguingly, cells lacking Pol ζ would undergo normal S phase when treated with cisplatin and nitrogen mustard indicating that S phase checkpoints were not triggered, but the cells would arrest permanently in G2 (Beljanski et al, 2004; Grossmann et al, 2000). This indicates that these ICLs do not interfere with the bulk of replication but activate a checkpoint after replication is complete or nearly complete in absence of Pol ζ. In contrast to cisplatin and nitrogen mustard, mitomycin C and bis-chloronitrosourea do not trigger a permanent G2 arrest, indicating that the need Pol ζ activity might depend on the nature of the ICL (Beljanski et al, 2004).

Other studies confined Pol ζ activity in ICL repair to G1/G0 phase of the cell cycle. Pol ζ deficient cells, which were kept in G1/G0, were more sensitive to nitrogen mustard treatment than exponentially growing cells (McHugh & Sarkar, 2006; McHugh et al, 2000). This suggests that Pol ζ is important for removing nitrogen mustard ICLs in the absence of an available sister chromatid, which might allow repair to occur through HR. Evidence for this was found in the up-regulation of Rad51 in Rev3 deficient cells after treated with psoralen plus UVA (Cohen et al, 2002).

Pol ζ dependent repair pathway involves another polymerase Rev1, which behaves epistatic to Rev3 with respect to ICL repair (Sarkar et al, 2006). Rev1 was found in the same UV induced auxotrophic gene reversion screen as Rev3 (Lawrence & Christensen, 1978; Lemontt, 1971). The gene for Rev1 was isolated and sequence comparisons revealed that it had sequence homology to the

bacterial gene UmuC, which is a subunit of Pol V (Larimer et al, 1989), placing Rev1 in the Y-family of polymerases (Ohmori et al, 2001).

Although purified Rev1 does not have a canonical polymerase activity and has a template dependent deoxycytidyl-transferase activity, which inserts specifically deoxycytosine opposite to guanine, abasic sites (Nelson et al, 1996a), 7,8-dihydro-8-oxoguanine, 6-methyl guanine (Haracska et al, 2002a; Howell et al, 2007) and N2 guanine adducts (Nair et al, 2008; Washington et al, 2004a). Rev1 does this by carrying its own template (Nair et al, 2005b) and insertion products can be successfully extended by Pol ζ (Washington et al, 2004a). Moreover, Rev1 interacts directly with Pol ζ thereby enhancing the ability of Pol ζ to extend from distorted primer template termini (Acharya et al, 2005; Acharya et al, 2006; D'Souza & Walker, 2006). Furthermore, the C-terminal end of Rev1 interacts not only with Pol ζ but also with Pol δ and Pol η (Acharya et al, 2005; Acharya et al, 2007; Acharya et al, 2009; Acharya et al, 2006). Hence, Rev1 might act as a structural scaffold, independent of its catalytic activity (Nelson et al, 2000), linking the replicative Pol δ with Pol ζ and might be involved in polymerase recruitment and switching at the replication fork (Acharya et al, 2009; D'Souza & Walker, 2006; Haracska et al, 2001c). In contrast, cell cycle analysis showed that expression of Rev1 peaks G2/M and not in S phase, suggesting Rev1 activity is needed after the bulk of replication is completed (Sabbioneda et al, 2007; Waters & Walker, 2006).

Regarding ICL repair Rev1 role is thought to recruit Pol ζ to the site of stalled Pol δ in G1/G0 (McHugh & Sarkar, 2006). It is still under debate if the

catalytic activity of Rev1 is involved in the bypass process. Since ICL inducing agents often react with guanine in DNA, like cisplatin and nitrogen mustards (Lemaire et al, 1991; Ojwang et al, 1989), Rev1 catalytic activity might be able to insert a nucleotide opposite the adducted guanine and Pol ζ would, analogous to one sided lesions, extend from these intermediates (Washington et al, 2004a).

However, recent biochemical studies using primer extension reaction with templates containing structurally distinct ICLs linking guanines showed various degree of bypass by Pol ζ (Ho et al, unpublished; Minko et al, 2008a). Pol ζ was able to approach the ICL and stalls one nucleotide before the insertion step opposite to the crosslinked base. In case of the dialdehyde derived ICL this block was absolute (Minko et al, 2008a). However, Pol ζ could extend from the insertion product, albeit extension from a “mismatched” insertion was more efficient (Minko et al, 2008a). In contrast Rev1 was able to specifically insert a dCTP opposite the dialdehyde derived ICL. This argues against a sequential action of Pol ζ and Rev1 in case of a dialdehyde derived ICL.

Shortening of the cisplatin crosslinked non templating strand allowed some insertion and bypass by Pol ζ (Ho et al, unpublished) (Figure 9). Interestingly, the limited insertion product was non mutagenic and the correct dCTP was incorporated.

The nitrogen mustard-like ICLs were efficiently bypassed when the length of the bridge between the guanines was increased (Ho et al, unpublished) (Figure 9). Similar to the cisplatin ICL insertion opposite the nitrogen mustard-like ICL by Pol ζ was accurate.

These in vitro studies show that bypass of an unhooked ICL is not easily accomplished and apparently needs additional factors *in vivo*. One of these missing factors might be an additional polymerase. Yeast has another TLS polymerase, which is able to bypass certain DNA lesions (Table 2). Pol η is the gene product of RAD30 and was found by its homology to bacterial polymerase Pol IV and Pol V (McDonald et al, 1997), hence belonging to the Y-family of polymerases (Ohmori et al, 2001).

Table 2

S. cerevisiae polymerases involved in ICL repair				
Polymerases	Family	Gene	*in vitro* bypass ICL	References
Pol ζ	B	REV3/REV7	no	{Zietlow, 2008 #646; Ho, unpublished}
REV1	Y	REV1	no	{Zietlow, 2008 #646; Ho, unpublished}
Pol η	Y	RAD30	no	

In humans the lack of Pol η leads to a cancer-prone syndrome known as xeroderma pigmentosum variant (XPV). Yeast's Pol η is able to bypass or insert nucleotides opposite to a number of structurally distinct DNA lesions including single base lesions such as 7,8-dihydro-8-oxoguanine (Haracska et al, 2000b), acetylaminofluorene-adducted guanine (Yuan et al, 2000), O6-methylguanine (Haracska et al, 2000a), N2-guanine adducts (Minko et al, 2001; Zhao et al, 2006) and intrastrand crosslinks like cis-syn cyclobutane pyrimidine dimers (CPDs) (Johnson et al, 1999), cisplatin (Alt et al, 2007), guanine thymine (Jiang et al, 2007) and guanine cytosine intrastrand crosslinks (Gu & Wang, 2004). This structural diversity of lesion suggests the notion that Pol η may also be able to bypass an unhooked ICL. However, genetic studies ruled out a major role for Pol

η in yeast's ICL repair. Asynchronously growing and G1/G0 arrested rad30 cells were not hypersensitive to ICL inducing agents, such as psoralen, cisplatin or nitrogen mustard (Grossmann et al, 2001; Sarkar et al, 2006; Wu et al, 2004b). Furthermore, double mutant rev3rad30 cells, lacking both TLS polymerases, were not more sensitive than single rev3 cells suggesting that Pol η is not needed for ICL repair in yeast. This argues that other factors are involved to assist Pol ζ dependent bypass of ICLs.

Based on this, ICL repair in G1/G0 initiates with the incision by NER, since NER deficient cells are sensitive to nitrogen mustard regardless of the cell cycle (Barber et al, 2005). The repair synthesis machinery is recruited the incision site and Pol δ tries to fill-in the apparent gap and stalls at the ICL triggering the recruitment Pol ζ through ubiquitinated PCNA (McHugh & Sarkar, 2006; Sarkar et al, 2006). These latter steps might as well take place during a replication dependent bypass of ICLs, since Pol δ is also one of the replicative polymerases and might bump into ICLs during normal replication. Furthermore, the non-essential Pol32 subunit of Pol δ (Gerik et al, 1998) was shown to be required for Pol ζ translesion synthesis (Hanna et al, 2007). The necessity of this interaction and the lack of bypass in primer extension reactions by Pol ζ raise another possibility for Pol δ being the polymerase to insert a nucleotide opposite the adducted base, since it was shown that Pol δ is able to insert a dATP opposite an abasic site, which can be extended by Pol ζ (Haracska et al, 2001c). Although, an abasic site arguably is a complete different structure than an unhooked ICL.

ICL repair in vertebrates and the involvement of polymerases

Like yeast, vertebrates are able to repair ICLs in an S-Phase and replication-depedent and independent manner, revealing that many basic features of ICL repair process are conserved from yeast to vertebrates. S-Phase dependent ICL repair involves the formation of DSBs (Akkari et al, 2000) in response to replication. It involves the action of structure-specific endonucleases such as ERCC1-XPF and MUS81-EME1 and proteins involved in HR and TLS, but in contrast to yeast NER genes other than ERCC1-XPF do not appear to be involved (reviewed in (Dronkert & Kanaar, 2001; McCabe et al, 2009; Noll et al, 2006)). In vertebrates, the Fanconi anemia (FA) pathway has an important role in ICL repair in response to replication, providing a level of regulation that is absent in *S. cerevisiae* (reviewed in (Mirchandani & D'Andrea, 2006; Moldovan & D'Andrea, 2009; Patel & Joenje, 2007; Wang, 2007)). In addition to the major S-phase dependent ICL repair pathway, there is at least one additional replication and HR-independent pathway that appears to recognize ICLs in a NER-dependent fashion (Muniandy et al, 2009; Wang et al, 2001; Zheng et al, 2003). A key common feature for all ICL repair pathways is the need to unhook the ICL resulting in an intermediate, in which the ICL attached to a fragment of an oligonucleotide provides a substrate for TLS polymerases to bypass the unhooked ICL and restore one of the two strands damaged by the ICL (Figure 8).

Compared to yeast and bacteria, vertebrates have a vastly increased number of polymerases that are able to bypass lesions and many of those have been shown genetically to play a conferring resistance to ICL forming agents or have the ability to bypass ICLs *in vitro* (Table 3). Here, we will discuss what is known about the involvement of individual TLS polymerases with respect to ICL repair. Finally, we will discuss our current understanding of how TLS activity might be regulated in the context of ICL repair.

Table 3

Polymerases in higher eukaryotes involved in ICL repair				
Polymerases	Family	Gene	*in vitro* bypass ICL	References
Pol ζ	B	REV3L/REV7	no	
REV1	Y	REV1	no	
Pol η	Y	POLH, XPV, RAD30A	yes	{Ho, unpublished}
Pol κ	Y	POLK, DINB1	yes	{Zietlow, 2008 #646; Ho, unpublished}
Pol ι	Y	POLI, RAD30B	yes	{Ho, unpublished}
Pol θ	A	POLQ, MUS308	no	
Pol ν	A	POLN	no	

Pol ζ and Rev1

Analogous to yeast, vertebrate cells deficient in Pol ζ are exquisitely sensitive to ICL forming agents (Gan et al, 2008). Pol ζ consist, as its yeast counterpart, of two subunit, the catalytic subunit Rev3 (Gibbs et al, 1998; Lin et al, 1999a; Xiao et al, 1998) and the accessory subunit Rev7 (Murakumo et al, 2000). Rev3 null mice were shown to be embryonic lethal (Bemark et al, 2000; Esposito et al, 2000; Van Sloun et al, 2002; Wittschieben et al, 2000), possibly due to an essential role in tolerating endogenous DNA damage. Studies of Pol ζ in mammals have therefore relied on either knock down approaches or the

rescue of cellular lethality by deleting REV3 in a p53-/- background. Additional information was obtained from knockout studies in DT40 chicken cells. These studies revealed a pronounced sensitivity of REV3 as well as REV7-deficient cells toward exposure toward cisplatin, nitrogen mustards and mitomycin C (Cheung et al, 2006; Okada et al, 2005; Sonoda et al, 2003; Wittschieben et al, 2006; Wu et al, 2004a). Intriguingly, REV3 knockout chicken DT40 cells are the most sensitive cells toward ICL forming agents. REV3 mutants are more sensitive than mutations in Fanconi genes such as FANCC and deletion of FANCC does not further sensitize REV3 mutants indicating an epistatic relationship. (Niedzwiedz et al, 2004; Nojima et al, 2005). These observations suggest that Pol ζ plays a role in the FA and replication-recombination dependent ICL removal pathway and that it has additional, FA-independent roles in ICL repair. Consistent with this observation and described roles for Pol ζ in S. cerevisiae, this polymerase has a role in a recombination independent ICL repair pathway shown in host cell reactivation assays using plasmids containing either a single mitomycin C or psoralen ICL (Shen et al, 2006; Zhang et al, 2007).

Biochemical studies of mammalian Rev3 have not yet been reported, presumably because the protein is difficulty to purify due to is a large predicted size of 353 kDa (Gan et al, 2008). Thus, there is no direct biochemical evidence for its polymerase activity. Nonetheless, studies using a cell free replication assay in Xenopus laevis egg extracts revealed a direct role for Pol ζ in ICL repair. These extract were shown to be proficient in removing a single cisplatin or nitrogen mustard-like ICL from a plasmid in a replication dependent manner and

allowed for the temporal resolution of a number of steps involved (Figure 8) (Räschle et al, 2008). In this system, a replication fork pauses 20-40 nucleotides before the ICL, then approaches the ICL, stalling again one nucleotide before the ICL, then inserting a nucleotide opposite the ICL and finally extending past the ICL. Rev7 depleted extracts were proficient in inserting a nucleotide opposite the cisplatin ICL, but were deficient in extending to the full-length product from these intermediates. This study provided direct evidence that Pol ζ is required for the extension step in TLS past and ICL, consistent with observations in yeast showing that Pol ζ is a good extender polymerase (Prakash et al, 2005).

Rev1 is a tight interaction partner of Pol ζ (Murakumo et al, 2001) and the REV1 gene displays almost identical properties to REV3 and REV7 at the genetic level. REV1 disruption in chicken DT40 cells renders them sensitive to cisplatin and it bears the same epistatic relationship to FANCC (Niedzwiedz et al, 2004; Okada et al, 2005; Simpson & Sale, 2003). In addition to its role in FA- and replication-dependent ICL repair, REV1 also appears to have a defect in replication-independent repair of psoralen or mitomycin C ICLs, possibly explaining the strong sensitivity to ICL-forming agents that is also seen in the case of REV3 (Shen et al, 2006).

Human Rev1 has a deoxycytidyl-transferase activity and preferentially inserts a deoxycytosine opposite a guanosine, uracil and abasic site, similar to the yeast enzyme (Lin et al, 1999b; Masuda & Kamiya, 2002). The importance of this catalytic activity for ICL repair remains to be established, as it is not essential for involvement in damage tolerance (Ross et al, 2005). The ability of Rev1 to

interact with Pol η, ι and κ, supports the notion that Rev1 might be a scaffolding protein involved in coordinating the activity of various polymerases at DNA lesions (Guo et al, 2003; Tissier et al, 2004).

Pol η

Pol η was identified as the gene deficient in xeroderma pigmentosum variant (XPV) patient cells and was shown to be responsible for the error-free bypass of UV induced CPDs (Cordeiro-Stone et al, 1997; Lehmann et al, 1975; Masutani et al, 1999a; Masutani et al, 2000; Masutani et al, 1999b). In addition to its role in preventing the formation of UV-induced mutations, Pol η is involved in TLS of a variety of DNA lesions (reviewed in (Guo et al, 2009; Prakash et al, 2005; Waters et al, 2009)).

In contrast to yeast, where RAD30 deficient cells were shown to be moderately sensitive to ICL forming agents (Grossmann et al, 2001; Sarkar et al, 2006; Wu et al, 2004b), XPV patient cells were shown to be hypersensitive to ICL-forming agents cisplatin and psoralen (Albertella et al, 2005; Chen et al, 2006; Misra & Vos, 1993). While it has not been shown whether the sensitivity of cisplatin results from intra- or interstrand adducts, direct evidence suggest that psoralen interstrand adducts are responsible for this effect, since DSBs are specifically induced in XPV cells under conditions that favor interstrand crosslink formation (Mogi et al, 2007).

Pol η may also have a role in replication-independent ICL repair, since the repair of mitomycin C and to a lesser extent psoralen ICL in a host cell reactivation assay was affected in XP-V cells (Wang et al, 2001; Zheng et al, 2003). These studies suggested that the involvement of a TLS polymerase in ICL repair also depends on the structure of an ICL. Work in our lab has addressed this issue using a series of cisplatin and nitrogen mustard-like ICL that form ICL between two guanine residues in the major groove of DNA and introduce various degrees of distortion (Figure 9). This work showed that human Pol η is able to bypass structurally distinct ICLs such as cisplatin and nitrogen mustard like ICLs (Ho et al, unpublished) (Figure 9). Furthermore, increasing the length of the bridge between two crosslinked guanines (releasing distortion) facilitated the bypass ICLs, probably reflecting an easier displacement of the crosslinked strand. Bypass by Pol η was further improved by reducing the double stranded character of the template around the ICL, which reduces the need for a strand displacement activity of Pol η. Although the bypass of the cisplatin or the nitrogen mustard ICLs by Pol η was error-prone these studies demonstrate that Pol η has the ability to bypass ICLs efficiently.

Pol κ

A third Y-family polymerases found in higher eukaryotes, Pol κ (Ohmori et al, 2001), was identified in a homology search for eukaryotic orthologs of E. coli dinB gene (Gerlach et al, 2001; Johnson et al, 2000a; Ogi et al, 1999). Pol κ was

found to be the most accurate Y-family polymerase on undamaged DNA and has a misinsertion frequency of about 1 in 102 to 103 nucleotides (Johnson et al, 2000a; Ohashi et al, 2000a) and has non-TLS role in the repair synthesis step in NER along with Pol δ (Ogi & Lehmann, 2006).

In primer extension reaction Pol κ can insert nucleotides opposite to a variety of DNA bases and has a particular propensity to bypass minor groove adducts that form at the N2-position of dG. (Fischhaber et al, 2002; Gerlach et al, 2001; Jarosz et al, 2006; Minko et al, 2008b; Ohashi et al, 2000b; Rechkoblit et al, 2002; Suzuki et al, 2004a; Suzuki et al, 2001; Suzuki et al, 2004b; Yuan et al, 2008; Zhang et al, 2000a).

Based on the strong preference of Pol κ for N2 dG adducts Minko et al. studied the ability of this polymerase to bypass N2-N2 guanine interstrand crosslinks, which are formed by antitumor drugs such as mitomycin C or reactive aldehyde compounds formed by lipid peroxidation (Minko et al, 2008a). This work demonstrated that Pol κ was indeed able to bypass such minor groove ICLs and that the efficiency of this bypass increased with shortening of the nontemplating crosslinked strand. If the ICL was only part of to a two nucleotide dsDNA fragment, bypass efficiency approached that of a nondamaged template, suggesting that such a structure is not more obstructive than other N2 deoxyguanine adducts. Consistent with this observation, knock-down of Pol κ lead to hypersensitivity of cells toward treatment with Mitomycin C, suggesting a role for Pol κ for the bypass of minor groove ICLs in cells.

Using a similar approach to study lesions bypass, we showed that Pol κ was able to insert a dNTP opposite cisplatin and nitrogen mustard ICLs and was even able to efficiently bypass some of the less distorting major groove ICLs (Figure 9) (Ho et al, unpublished). Although the biological significance of the bypass of major groove ICLs by Pol κ has not yet been established, it suggests that Pol κ might be able to bypass such lesion in vivo under certain conditions.

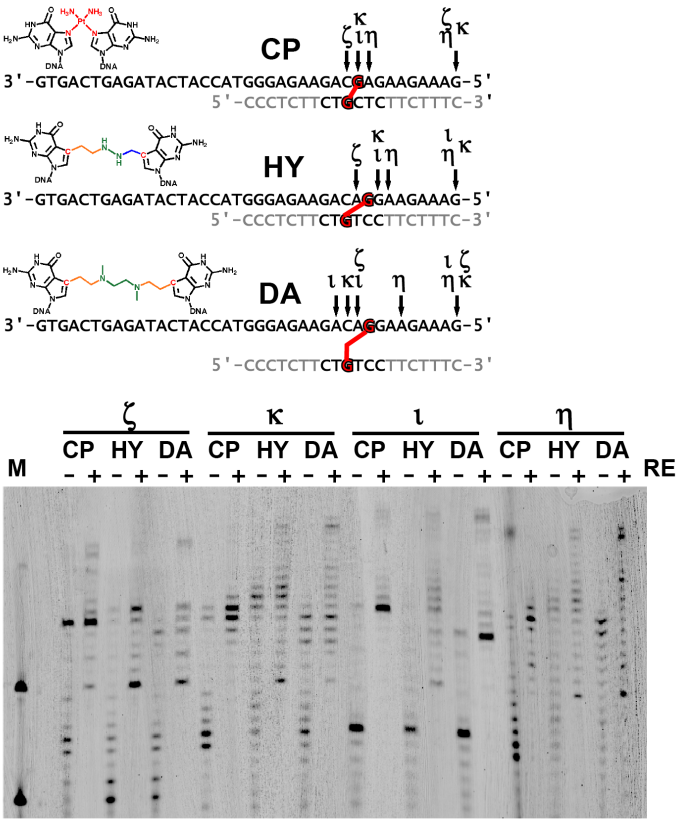


Figure 9: Primer extension reactions with distinct ICLs. TLS past cisplatin (CP), nitrogen mustard-like hydrazine ICL (HY) and nitrogen mustard-like dimethylethylendiamine ICL (DA).

Pol ι

Pol ι is a second homolog to yeast RAD30 (McDonald et al, 1999; Tissier et al, 2000). Pol ι is a highly error-prone polymerase on undamaged DNA, especially opposite to pyrimidines (Bebenek et al, 2001; Frank et al, 2001; Tissier et al, 2000; Zhang et al, 2000b). It can accommodate both Watson-Crick and Hoogsteen base pairs in the active site and has the ability to bypass a variety of DNA adducts, such as abasic sites (Frank & Woodgate, 2007; Nair et al, 2005a; Nair et al, 2009; Nair et al, 2004; Washington et al, 2004b). Additionally, it has been suggested that Pol ι is involved in base excision repair, counteracting the effects of oxidative damage (Bebenek et al, 2001; Petta et al, 2008).

Although genetic analysis has not yet implicated Pol ι in ICL repair, we found that Pol ι is able to insert a dNTP opposite a guanine residue in a cisplatin ICL (Figure 9) (Ho et al, unpublished). Furthermore, we found that Pol ι can bypass a nitrogen mustard-like crosslink in a fashion similar to Pol κ and Pol η with greatly enhanced bypass resulting from reducing the length of the double strand segment around the ICL and increasing length of the bridge between the two guanine residues (Ho et al, unpublished).

While these biochemical results for TLS of nitrogen mustard ICLs do not prove that the Y-family polymerases Pol η, Pol κ and Pol ι are involved in TLS of ICLs *in vivo*, they show that they can bypass ICLs efficiently under certain conditions and there might be redundancy of TLS polymerases of major groove

ICLs. This redundancy could be one reason why cells deficient in Pol κ and Pol ι are not sensitive to treatment by agents that form ICLs in the major groove.

Pol θ and Pol ν

Drosophila melanogaster mutated in the gene MUS308 are specifically hypersensitive to ICL inducing agents such as nitrogen mustard and cisplatin (Boyd et al, 1981; Boyd et al, 1990) and was shown to encode a nuclear protein, which contains a N-terminal helicase-like domain and a C-terminal A-family polymerase domain (Harris et al, 1996; Oshige et al, 1999; Pang et al, 2005). In vertebrates there are three genes identified to be homologous to fly MUS308; POLQ, POLN and HEL308.

POLQ encodes for Pol θ, which has an A-family polymerase domain and a helicase-like domain (Seki et al, 2003; Sharief et al, 1999). The role of Pol θ in vertebrates is not well understood. In chicken DT40 cells Pol θ seems to be exclusively involved BER and deals with oxidative damages and shows no hypersensitivity to crosslinking agents (Yoshimura et al, 2006).

In mice Pol θ was suggested to play a role in somatic hypermutation shown by a couple of groups (Franklin & Blanden, 2007; Masuda et al, 2007; Masuda et al, 2005; Zan et al, 2005), although a recent report suggest a marginal role of Pol θ in this process (Martomo et al, 2008). Mouse Pol θ was also proposed to be a candidate for a chaos1 (chosmosome aberration occurring spontaneously 1) that leads to spontaneous, radiation and mitomycin C induced

chromosome instability observed by micronuclei formation and was shown to genetically interact with ataxia telangiectasia mutated kinase (ATM), an important DSB repair signaling kinase (Goff et al, 2009; Shima et al, 2003; Shima et al, 2004). But chaos1 mutant cells were not hypersensitive to killing by mitomycin C treatment, suggesting that Pol θ is not essential for murine ICL repair (Shima et al, 2004).

Human Pol θ is preferentially expressed in lymphoid tissue and upregulated in tumors with poor clinical outcome (Kawamura et al, 2004). Purified Human Pol θ is a low fidelity polymerase and is able to bypass abasic sites and thymine glycols (Arana et al, 2008; Seki et al, 2004). Furthermore, Pol θ can extend from mismatched termini and bases incorporated opposite to CPD and 6-4 photoproduct (Seki & Wood, 2008). However, there is no evidence for sensitivity against ICL forming agents in human cells lacking Pol θ.

As stated above, there are two more MUS308 homologs in vertebrates, POLN and HEL308. POLN is homologous to the polymerase part of Drosophila melongaster's MUS308 gene and encodes for an A-family polymerase, Pol ν (Marini et al, 2003; Seki et al, 2003). Pol ν is a low fidelity polymerases on undamaged DNA and was able to bypass thymine glycols in vitro (Takata et al, 2006). Interestingly, Pol ν has a remarkable strand displacement activity (Takata et al, 2006), which makes it an attractive candidate to act on an unhooked ICL. Recently, it was reported that Pol ν is able to bypass a psoralen ICL (Zietlow et al, 2009). However, bypass was very inefficient.

HEL308 was shown to be homologous to the helicase-like domain of MUS308 and was shown to possess a 3' to 5' helicase activity (Marini & Wood, 2002; Seki et al, 2003). The archeal homolog was shown to specifically bind replication fork structures and unwinds the lagging strand (Guy & Bolt, 2005). It is not know if HEL308 plays a role in ICL repair.

To this point it is not clear what the role of MUS308 homologs in vertebrates with respect to ICL repair is. All homologs identified so far were not especially sensitive to ICL forming agents, even in double mutants in these genes, shown in chicken DT40 cells (Yoshimura et al, 2006). However, there is the possibility of other homologs in vertebrates fulfilling the function of Drosophila melanogaster MUS308 and the lack of these would render vertebrate cells also sensitive to ICL forming agents. Supporting this notion comes from a recent report in Caenorhabditis elegans, where it was shown that two MUS308 homologs in worms, POLQ-1 and HEL308 were sensitive to nitrogen mustard and operating in distinct ICL repair pathways (Muzzini et al, 2008).

Regulation and facilitation of TLS step during ICL repair

Since TLS polymerases are low fidelity polymerases on undamaged DNA, their access to DNA is restricted and has to be regulated. A key component in this process is proliferating cell nuclear antigen (PCNA), the replicative sliding clamp, which is suggested to serve as central scaffold for the polymerase switching process (Guo et al, 2009; Lee & Myung, 2008; Lehmann et al, 2007; Prakash et al, 2005; Waters et al, 2009).

It is generally believed hat PCNA gets monoubiquitinated at sites of stalled replication forks. Monoubiquitination involves the ubiquitin conjugating enzyme Rad6 and its interacting partner the ubiquitin ligase Rad18 (Hoege et al, 2002; Kannouche et al, 2004; Stelter & Ulrich, 2003; Watanabe et al, 2004). The uncoupling of the polymerases and helicase activity produces stretches of single-stranded DNA coated with RPA (replication protein A), which recruits Rad18 to site of stalled replication forks and subsequently monoubiquitinates PCNA at lysin164 (Chang et al, 2006; Davies et al, 2008; Niimi et al, 2008; Tsuji et al, 2008).

All Y-family polymerases have a basal affinity to unmodified PCNA either through their PIP (PCNA interacting peptide) domain (Haracska et al, 2001a; Haracska et al, 2001b; Haracska et al, 2002b) or through the BRCT (breast cancer 1 C-terminus) domain in case of Rev1 (Guo et al, 2006). Furthermore, all of them possess ubiquitin binding motifs, either UBM (ubiquitine binding motif) or UBZ (ubiquitine binding zinc finger) domains, which strengthen their interaction with ubiquitinated PCNA (Bienko et al, 2005) and thought to facilitate the switch between replicative polymerases and TLS polymerases (Lehmann et al, 2007; Waters et al, 2009).

PCNA was shown to be required for psoralen ICL induced DNA synthesis in a mammalian cell-free system (Li et al, 1999; Li et al, 2000). Furthermore, PCNA stimulates incision near psoralen ICLs, demonstrated in a partially reconstituted system (Zhang et al, 2003) and binding of the mismatch repair complex MutSβ to a psoralen ICL (Zhang et al, 2002). Importantly, ubiquitination

of PCNA is necessary for reactivation of a reporter construct containing a mitomycin C ICL in chicken DT40 (Shen et al, 2006) and a screen in chicken DT40 cells revealed that cells lacking Rad18 are sensitive to crosslinking agents such as cisplatin, nitrogen mustard and mitomycin C, suggesting ubiquitination of PCNA in response to ICL inducing agents is dependent on Rad18 E3 ligase. Additionally, in yeast PCNA mono-ubiquitination in G1 phase is specific for ICL inducing agents and is required for ICL removal in G1 phase acting upstream of Pol ζ (Sarkar et al, 2006).

These studies place PCNA and its ubiquitination in ICL repair upstream from the TLS step. Moreover, it was shown that RPA is needed for ICL induced DNA synthesis (Li et al, 2000) indicating a similar mechanism as in one-stranded lesions where single-strand DNA coated with RPA is needed for the recruitment of Rad18 (Chang et al, 2006; Davies et al, 2008; Niimi et al, 2008; Tsuji et al, 2008). Although, the origin of single-stranded DNA cannot be explained by uncoupling of helicase and polymerase activity in the case of ICLs.

One possibility may be the activity of exonucleases, which might resect the initially unhooked ICL thereby generating single stranded DNA (Figure 10). A resection of the crosslinked strand is also desirable for the TLS polymerases, since in *in vitro* extension studies bypass of ICLs was facilitated by shortening of the crosslinked strand (Ho et al, unpublished; Kumari et al, 2008; Minko et al, 2008a). An obvious candidate for such an exonuclease activity specific for ICL repair is the PSO2/SNM1 gene, isolated in the same psoralen sensitivity screen

as Pol ζ (Cassier-Chauvat & Moustacchi, 1988; Henriques et al, 1997; Henriques & Moustacchi, 1980).

PSO2/SNM1 mutants are specifically sensitive to ICL forming agents, although they are not epistatic to Rev3 and Rad51 in respect to crosslinking agent sensitivity (Grossmann et al, 2001). Interestingly, PSO2/SNM1 was found to have overlapping functions with mismatch repair factor MutS and EXO1 in dealing with ICL during S phase (Barber et al, 2005). Yeast Pso2/Snm1 protein was found to possess a metallo-β–lactamase domain and belongs to the β-CASP family of nucleic acid processing enzymes (Callebaut et al, 2002). The Pso2/Snm1 metallo-β-lactamase fold is important for processing of DSB arising during treatment with psoralen (Li & Moses, 2003). Pso/Snm1 has 5' to 3' exonuclease activity, which enables it to digest single stranded and double stranded DNA (Li et al, 2005).

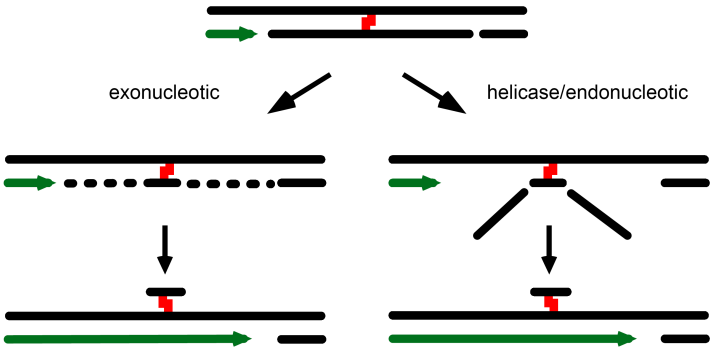


Figure 10: Resection mechanism. Possible mechanism for strand resection by either exonucleolytic or endonucleolytic cleavage.

There are a couple of homologs for SNM1 in mammals, among others SNM1A, SNM1B/Apollo and SNM1C/Artemis (Callebaut et al, 2002). SNM1A

harbors a 5' to 3' exonuclease activity specific for single stranded DNA (Hazrati et al, 2008; Hejna et al, 2007). SNMB/Apollo is involved in replication fork collapse in response to ICLs (Bae et al, 2008). SNMC/Artemis is involved in nonhomologous endjoining and VDJ recombination and has exonucleotic and a modulated endonucleotic activity (Ma et al, 2002). Interestingly, only SNM1A was able to partially rescue a yeast pso2/snm1 mutant from treatment with nitrogen mustard and cisplatin (Hazrati et al, 2008). It is quite conceivable that one of these nucleases might be responsible for the 5' to 3' resection of the crosslinked strand.

Resection from the 3' end was also shown to be beneficial for lesion bypass by TLS polymerases (Ho et al, unpublished; Minko et al, 2008a). This reaction could be catalyzed by ERCC1-XPF in the presence of RPA as shown in cell free assays (Mu et al, 2000). Furthermore, there are other nucleases, which were suggested to be involved in ICL repair such as Exo1 (Barber et al, 2005) and Mre11 (McHugh et al, 2000; Pichierri et al, 2002).

The FA pathway is another mechanism suggested to regulate TLS of ICLs. The FA is a rare autosomal recessive genetic disorder involving chromosome instability. The hallmark of FA is that cells derived from patients are exquisitely sensitive to ICL inducing agents and thirteen complementation groups are known so far (most recent reviews (Alpi & Patel, 2009; Moldovan & D'Andrea, 2009; Thompson & Hinz, 2009)). Eight proteins of these complementation groups FancA, -B, -C, E, -F, G, L and M together with FAAP100 and FAAP24 form a core complex, which includes the FancL E3 ligase activity.

This core complex is targeted to the site of DNA damage by its FancM-FAAP24 subunits where it is responsible for mono-ubiquitination of the FancD2-FancI heterodimer (Kim et al, 2008). FancD2-FancI is monoubiquitinated constitutively during S phase and especially during damage response (Garcia-Higuera et al, 2001; Sims et al, 2007; Smogorzewska et al, 2007). Ubiquitinated FancD2-FancI colocalizes with DNA repair factors such as BRCA2, Rad51 and PCNA, among others (Garcia-Higuera et al, 2001; Taniguchi et al, 2002). In chicken DT40 cells FANCC was shown to be epistatic to REV1 and Pol ζ with respect to crosslinking agent sensitivity (Niedzwiedz et al, 2004; Nojima et al, 2005). Moreover, Rev1 foci formation requires FA core complex in human cells (Mirchandani et al, 2008) placing the FA pathway upstream of TLS during ICL repair.

As mentioned earlier a replication fork stalled at one-stranded lesion differs from replication forks stalled at ICLs by the fact that uncoupling of polymerase and helicase activity is not possible. Thus, the later replication fork has to undergo major structural changes such as removal of the replicative helicase and/or fork regression to grant access to the ICL. Indication for such a process were found in a study in *Xenopus laevis* egg extract where the replication fork initially stalls 20 to 40 nucleotides in front of the ICL before advancing to the ICL (Räschle et al, 2008). The FA pathway is triggered during this process shown by ubiquitination of FancD2 (Räschle et al, 2008), suggesting a role of the FA pathway in coordinating processes at ICL stalled replication forks.

Recently, it was shown that FancM, a member of the core complex has a DNA translocase activity (Meetei et al, 2005; Mosedale et al, 2005) that may be able to catalyze fork regression (Gari et al, 2008) linking the ubiquitination activity of the core complex to stalled replication fork structures (Xue et al, 2008). In addition, another FA factor FancJ was shown to have a 5'-3' helicase activity and preferentially binds and unwinds fork like structures (Cantor et al, 2004; Cantor et al, 2001; Kumaraswamy & Shiekhattar, 2007). Although initially found to interact with Brca1, it exerts its function in ICL repair independently of Brca1 and downstream of FancD2-FancI ubiquitination (Bridge et al, 2005). Interestingly, FancJ interacts with mismatch repair factor MutLα and this interaction is necessary for its function in ICL repair (Peng et al, 2007), although it is still unclear how this interaction promotes ICL repair.

Most recently it was reported that FancD2-FancI ubiquitination promotes incision and TLS of ICL (Knipscheer et al, 2009), supporting the notion that the FA pathway is involved in remodeling of ICL stalled replication forks to prepare them for incision near the ICL, exonucleolytic resection of the crosslinked strand (Figure 8), regulating the recruitment of TLS polymerases by interacting with PCNA and eventual bypass of the ICL by TLS polymerases.

Chapter 3

Structure-dependent Bypass of DNA Interstrand Crosslinks by Translesion Synthesis Polymerases

Introduction

Bifunctional chemical agents such as cisplatin or nitrogen mustards are highly cytotoxicity due to their ability to form DNA interstrand crosslinks (ICLs) and are commonly used in cancer chemotherapy. ICLs render the two strand of a DNA helix inseparable and thereby inhibit transient division of the two strands, providing a complete block to DNA replication and transcription (Dronkert & Kanaar, 2001; McHugh et al, 2001; Noll et al, 2006; Scharer, 2005).

ICLs can also be induced by endogenous aldehydes formed by lipid peroxidation (Niedernhofer et al, 2003), providing an evolutionary incentive for a cellular ICL repair mechanism. Since ICLs simultaneously affect both strands of DNA, a mechanism for ICL removal based on a straightforward cut and patch mechanism is not possible. Instead ICL repair has been genetically linked to factors of multiple DNA repair pathways, including those involved in nucleotide

excision repair (NER), homologous recombination (HR), translesion synthesis (TLS). ICL repair additionally involves the activity of a number of endonucleases and the Fanconi Anemia pathway (reviewed in (Dronkert & Kanaar, 2001; Mirchandani & D'Andrea, 2006; Moldovan & D'Andrea, 2009; Niedernhofer et al, 2005; Noll et al, 2006; Scharer, 2005; Thompson & Hinz, 2009)). How these factors mechanistically work together to remove ICLs is still poorly understood.

Currently, ICL repair is believed to occur by at least two pathways; one operates in the S Phase and is triggered by a stalling of a replication fork at an ICL, and one outside of S phase that may be initiated by NER or a stalled RNA polymerase. Common feature of both pathways include an endonucleolytic unhooking step that releases the ICL from one of the two strands of the duplex and translesion synthesis (TLS) step that bypasses the unhooked ICL and restores one of the two strands, providing one intact template to complete the repair process.

At least 15 DNA polymerases are known in humans and many of those have the ability to bypass a number of structurally diverse lesions and are therefore candidates to be involved in the bypass of ICLs (Loeb & Monnat, 2008; Sweasy et al, 2006). There is strong genetic (Beljanski et al, 2004; Grossmann et al, 2001; Henriques & Moustacchi, 1980; McHugh et al, 2000; Mogi et al, 2007; Nojima et al, 2005; Richards et al, 2005; Sarkar et al, 2006; Shen et al, 2006; Simon et al, 2000; Sonoda et al, 2003; Wu et al, 2004b) and biochemical (Räschle et al, 2008) evidence for a key role for Pol ζ in ICL repair, as cells deficient in Pol ζ display the most pronounced hypersensitivity to ICL forming

agents. Pol ζ is a heterodimeric B-family polymerase, consisting of the catalytic subunit Rev3 and Rev7 (Morrison et al, 1989; Nelson et al, 1996b; Torpey et al, 1994a). It has the ability to insert dNTPs opposite various DNA lesions and in particular to carry out the extension step following insertion catalyzed by a variety of polymerases (reviewed in (Gan et al, 2008; Prakash et al, 2005))

A second polymerase, Rev1, is epistatic to Pol ζ for ICL repair (Niedzwiedz et al, 2004; Sarkar et al, 2006; Simpson & Sale, 2003) and interacts with Pol ζ via its Rev7 subunit (Acharya et al, 2005; Murakumo et al, 2001) Rev1 belongs to the Y-family polymerases (Ohmori et al, 2001) and has an atypical template dependent deoxycytidyl transferase activity (Gibbs et al, 2000; Haracska et al, 2002a; Lin et al, 1999b; Nelson et al, 1996a). Rev1 and Pol ζ can cooperate to bypass certain DNA damages and it has been suggested that they may do so in the context of ICL repair (Acharya et al, 2006; Räschle et al, 2008; Shen et al, 2006; Washington et al, 2004a).

To what extent other Y-family polymerases with the ability to bypass various DNA lesions (McCulloch & Kunkel, 2008; Ohmori et al, 2001; Prakash et al, 2005; Waters et al, 2009) are involved in ICL repair is less clear-cut. For example, human XP-V cells, which lack Pol η (Masutani et al, 1999b), were shown to be moderately hypersensitive to ICL-forming agent cisplatin and psoralen (Albertella et al, 2005; Chen et al, 2006; Misra & Vos, 1993) and to be unable to reactivate a gene in a reporter plasmid containing a Mitomycin C ICL (Zheng et al, 2003), indicating a possible role for Pol η in ICL repair.

More recently, biochemical and cellular studies have strongly implicated Pol κ in the repair of minor groove ICLs, such as those formed by mitomycin C and bifunctional dialdehydes that are formed by lipid peroxidation (Minko et al, 2008a).

Since TLS polymerases bypass structural distinct DNA lesion with various efficiencies (reviewed in (McCulloch & Kunkel, 2008; Prakash et al, 2005; Waters et al, 2009)) and ICLs induced by various agents are structurally heterogeneous (Noll et al, 2006; Scharer, 2005), it is likely that the requirement for polymerases for TLS past ICLs changes with the structure of ICLs.

In this study we took advantage of the ability of our laboratory to generate a series of major groove ICLs that induce various amounts of distortion in the DNA helix to systematically investigate how structural variation affects the activity of TLS polymerases. We found that two factors greatly affect the efficiency of lesion bypass; the amount of dsDNA flanking an ICL and the distance of the ICL linker between the two crosslinked guanosine residues. Our studies show that major groove ICLs can be bypassed by a number of TLS polymerases and that the structure of the ICL dramatically affects the efficiency of the bypass.

Results

Preparation of major groove cisplatin and nitrogen mustard ICL templates for primer extension studies

Previous investigations of lesion bypass of ICLs have used psoralen and dialdehyde substrates, which form adducts between the bases or in the minor groove (Noll et al, 2006). By contrast, ICLs formed by cisplatin (CP) and nitrogen mustards (NM), the two most commonly used ICL-forming agents in anti-tumor therapy, react preferentially with the N7 position of guanine forming a bridge in the major groove of DNA (Figure 11A).

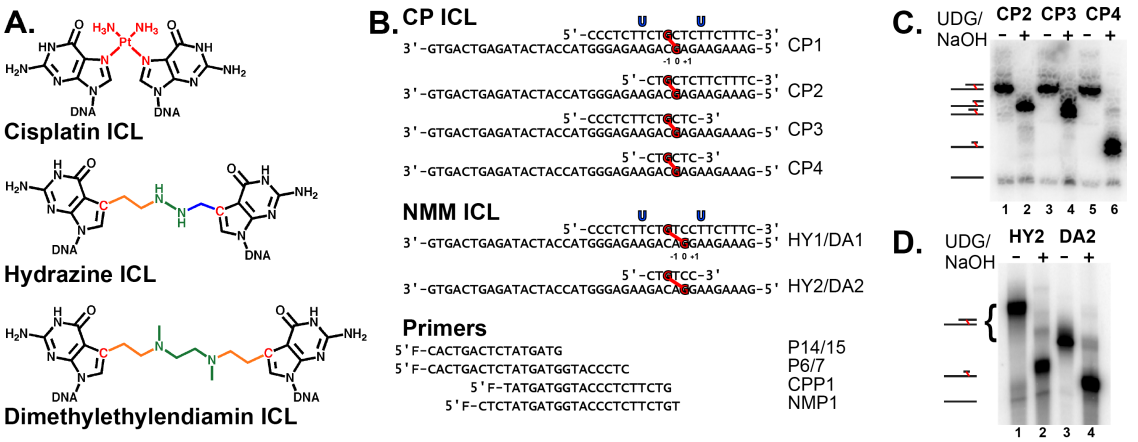


Figure 11: TLS polymerase templates. **A.** Chemical structure of the ICLs. Cisplatin (CP ICL), Hydrazine ICL (HY ICL) and Dimethylethylendiamine ICL (DA ICL). **B.** Sequence of the crosslinked oligonucleotides with the location of the CP, HY and DA ICL. Primers were labeled 5' with either 6-FAM or HEX fluorophores. The positions are marked as 0 for the insertion, minus before and plus after the insertion position. **C.** CP ICL containing substrates before and after cleavage, 5' labeled with ^{32}P. **D.** HY and DA ICL containing substrates before and after cleavage, 5' labeled with ^{32}P.

CP preferentially reacts with two guanines in a GC (Coste et al, 1999; Huang et al, 1995; Lemaire et al, 1991), while NMs prefer a GNC sequence

(Hopkins et al, 1991; Millard et al, 1990; Ojwang et al, 1989; Rink et al, 1993). The two ICL affect the structure of the DNA duplex in distinct ways: A CP ICL unwinds the DNA by 70° and forces the two cytosines that pair with the crosslinked guanines out of the double helix, relocating the N7 position of the guanines to the minor groove and introducing a high degree of helix distortion (Figure 11A)(Coste et al, 1999; Huang et al, 1995).

By contrast, NM ICLs and a stable mimic (HY) recently synthesized in our laboratory (Angelov et al, 2009) only introduce a minor bend into the helix and retain mostly B-form DNA (Figure 11A) (Guainazzi et al, unpublished; Rink & Hopkins, 1995; Rink et al, 1993). Furthermore, our synthetic approach allowed for the synthesis of nitrogen mustard-like ICLs (NMM ICLs) in which the length of the bridge between the crosslinked ICLs is increased, resulting in an ICLs that we predict to be free of any distortion (Figure 11A). The availability of these substrates allowed us to study systematically how major groove ICLs affects translesion synthesis.

The CP ICL containing template was prepared (Figure 11A and B) following a published procedure by Hofr et al. (Hofr & Brabec, 2001). A 20mer containing a single guanosine (Figure 11B CP1 upper strand) was reacted with CP generating the monoadducts, which was subsequently purified by anion exchange column. The monoadducted strand was then annealed and crosslinked to 39mer (Figure 11B CP1 lower strand) generating a double stranded crosslinked oligonucleotide with a 3’ overhang, which was purified by denaturing

anion exchange column resulting in crosslinked template of >93% purity (Figure 11C, lane 1, 3 and 5).

The NMM ICLs were synthesized and prepared according to a procedures established in our laboratory (Angelov et al, 2009). The chemical structures of the NMM ICLs are depicted in Figure 11A. A 20mer and 39mer were synthesized containing site specifically a 7-deazaguanosine with carbon linkers, which are functionalized with an aldehyde group. The two strands are annealed and coupled by reductive amination using either hydrazine or dimethylethylendiamine (DA). The crosslinked templates were purified by denaturing polyacrylamide gel electrophoresis (PAGE) yielding the HY1 and DA1 ICL with a purity of > 95% (Figure 11D, lane 1 and 3). Interestingly, the HY crosslinked templates migrated faster in a denaturing PAGE gel than the DA crosslinked ICL, most probably reflecting structural differences between these two adducts (Figure 11D, compare lane 1 with lane 3 and lane 2 with lane 4). The sequence of the CP and NMM ICL differ only at the crosslinked site to accommodate a 1,2 and 1,3 ICL, respectively.

CP1, HY1 and DA1 were annealed to a fluorescently labeled primer, P14/15, which leaves a three nucleotides gap to the crosslinked strand and fourteen and fifteen nucleotides to the insertion site, respectively. We tested these substrates with Klenow(-exo) and indeed found that Klenow(-exo) stalled one nucleotide before the insertion opposite the adducted guanine confirming the presence of an ICL at the predicted site (data not shown).

Since it was shown that resection of the crosslinked strand was beneficial for TLS by Pol κ (Minko et al, 2008a), we further prepared templates in which the amount of dsDNA next to the ICL was shortened on the 5', 3' or both sides (Figure 11B) by incorporating uracils instead of thymines into the 20mer at specific sites. After treating these crosslinked oligonucleotides with uracil-DNA glycosylase (Lindahl et al, 1977) and cleaving the generated abasic site under alkaline condition we could form CP2, CP3, CP4, HY2 and DA2 (Figure 11B) with more than 90% purity (Figure 11C, lane 2, 4 and 6 and Figure 11D, lane 2 and 4).

Insertion opposite CP ICL by Y-family polymerases

In a first set of experiments we tested the activity of a variety of polymerases on the CP1 substrate. An analogous duplex without an ICL was used as a control to monitor strand displacement synthesis. Incubation of Pol ζ with the non-crosslinked control substrate revealed that the polymerase stalled upon encountering the beginning of the duplex, but was able to displace the downstream strand at the highest concentration used (Figure 12, Pol ζ lane 3). The primer was extended until two nucleotides before the end of the template strand, in agreement with previous results (Nelson et al, 1996b).

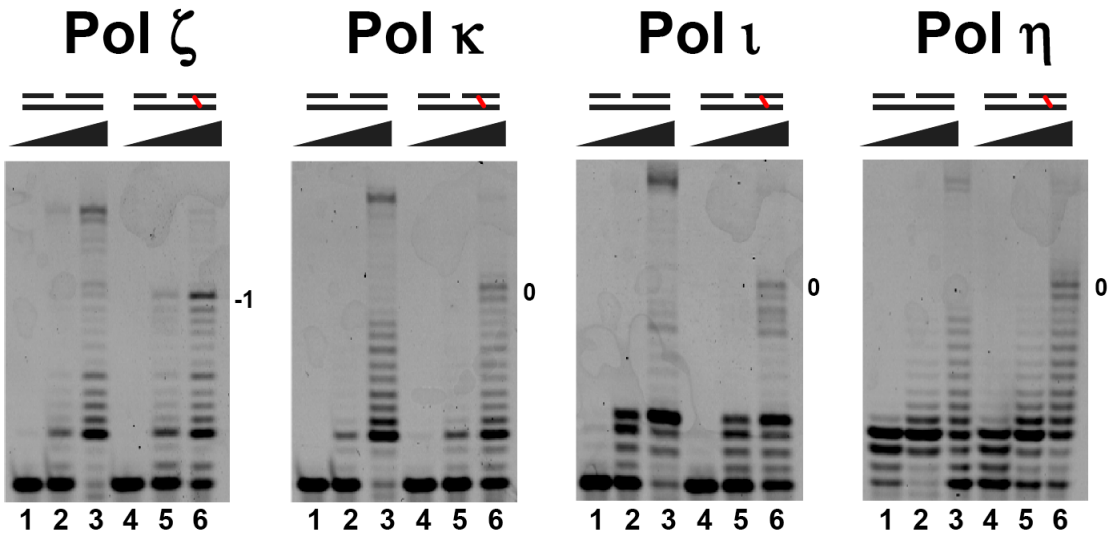


Figure 12: Cisplatin templates. Primer extension reaction of CP ICL containing template with Pol ζ, Pol κ, Pol ι and Pol η. Lanes 1-3 of each gel represent reactions with increasing polymerase concentration with a template, which has a strand annealed 3' to the primer for monitoring strand displacement synthesis. Lanes 4-6 of each gel represent reactions with a template containing a CP ICL. Pol ζ stalls at the -1 position before the insertion opposite to the adducted guanine. Pol κ, Pol ι and Pol η stall at the 0 position after inserting a nucleotide opposite to the adducted guanine.

On the ICL-containing CP1 substrate, the p14/15 primer was extended until one nucleotide before the adducted guanine (Figure 12, Pol ζ lane 6), which we will refer to as 0 position (Figure 11B). Thus, Pol ζ is able to displace the crosslinked strand and polymerize until the CP ICL, but fails to insert a nucleotide opposite the adducted guanine.

The Y-family polymerases, Pol κ, Pol ι and Pol η, were able to perform strand displacement synthesis on the control substrate with various efficiencies (Figure 12, compare lane 3 of Pol κ, ι and η). Surprisingly, Pol κ, Pol ι and Pol η were able to extend the primer on CP1 one step further than Pol ζ and stalled at the 0 position, which meant that they could insert a dNTP opposite the crosslinked guanine (Figure 12, lanes 6 of Pol κ, ι and η). These results indicate

that the insertion of a dNTP opposite an ICL can be accomplished by multiple polymerases.

Trimming of the crosslinked strand facilitates insertion and bypass

None of polymerases were able to completely bypass the cisplatin ICL. Since the flanking of the CP ICL in CP1 by 9-10 nucleotides on either side necessitates a strand displacement reaction, and causes pausing of the polymerase activity at the start of duplex DNA (Figure 11 and Figure 12), we reasoned that reducing the amount of dsDNA around the ICL might facilitate lesion bypass as previously shown by Minko et al. (Minko et al, 2008a).

We tested the activity of the polymerases on substrates having only three nucleotides 5' to the ICL (CP2, Figure 11B), or only two nucleotides 3' to the ICL (CP3, Figure 11B), or with the duplex shortened on both sides of the ICL (CP4, Figure 11B) so that the ICL is embedded in a duple of six nucleotide (Figure 11B).

Using these substrates with the primers P14/15 and P6/7, which leave a three and two nucleotide gap, respectively, we addressed the question to what extent the necessity of strand displacement is inhibitory for complete bypass of CP ICL (Figure 11B).

Resection of the duplex 5' and 3' to the ICL did not improve bypass by Pol ζ and stalling occurred at the -1 position (Figure 13A, lane 1-6). However, resection on both sides of the ICL (CP4), allowed for some insertion opposite the

ICL and bypass, while the -1 position was still the major stalling point (Figure 13A, lane 9). The insertion and bypass products accounted together for approximately 18% of all extension products, significantly more than the uncrosslinked impurity in CP ICL preparation in CP4 (4%).

Extension to the CP ICL by Pol κ was facilitated by 5' resection, as seen with CP2 and CP4 (Figure 13B, lane 1-3 and 7-9). Curiously, in contrast to the CP1 template Pol κ mainly stalled at the -1 position on all resected templates (Figure 13B, lane 1-9). We have no explanation for this observation. Apparently the resection of the CP ICL leads to a structure that is not a good fit for the Pol κ active site. However, on the CP4 template we could still observe some insertion and bypass product.

As with Pol κ 5' resection markedly facilitated the approach of Pol ι to the CP ICL (Figure 13C, lane 1-3), but additional 3' resection did not result in bypass of the cisplatin lesion (Figure 13C, lane 7-9). Extension by Pol ι on all resected templates stalled at the 0 position after inserting a dNTP opposite the crosslinked guanine as seen with the fully double-stranded CP1 substrate (Figure 12).

In contrast to the moderate effect of dsDNA resection around the CP ICL observed for Pol ζ, Pol κ and Pol ι, the shortening of the dsDNA stretch around the ICL altered dramatically the ability of Pol η to insert a dNTP opposite the CP ICL and to bypass it. Even 3' end resection allowed Pol η to reach +2 site, indicating complete bypass of the CP ICL (Figure 13D, lane 4-6). The major stalling site on CP4 template was the +2 position (Figure 13D lane 9), suggesting

that Pol η could efficiently bypass CP ICL when the duplex around the ICL is reduced to six nucleotides.

These studies show that if the resection of the duplex around the CP ICL facilitates insertion opposite the adducted guanine by all polymerases tested, and in the case of Pol η greatly facilitate complete bypass.

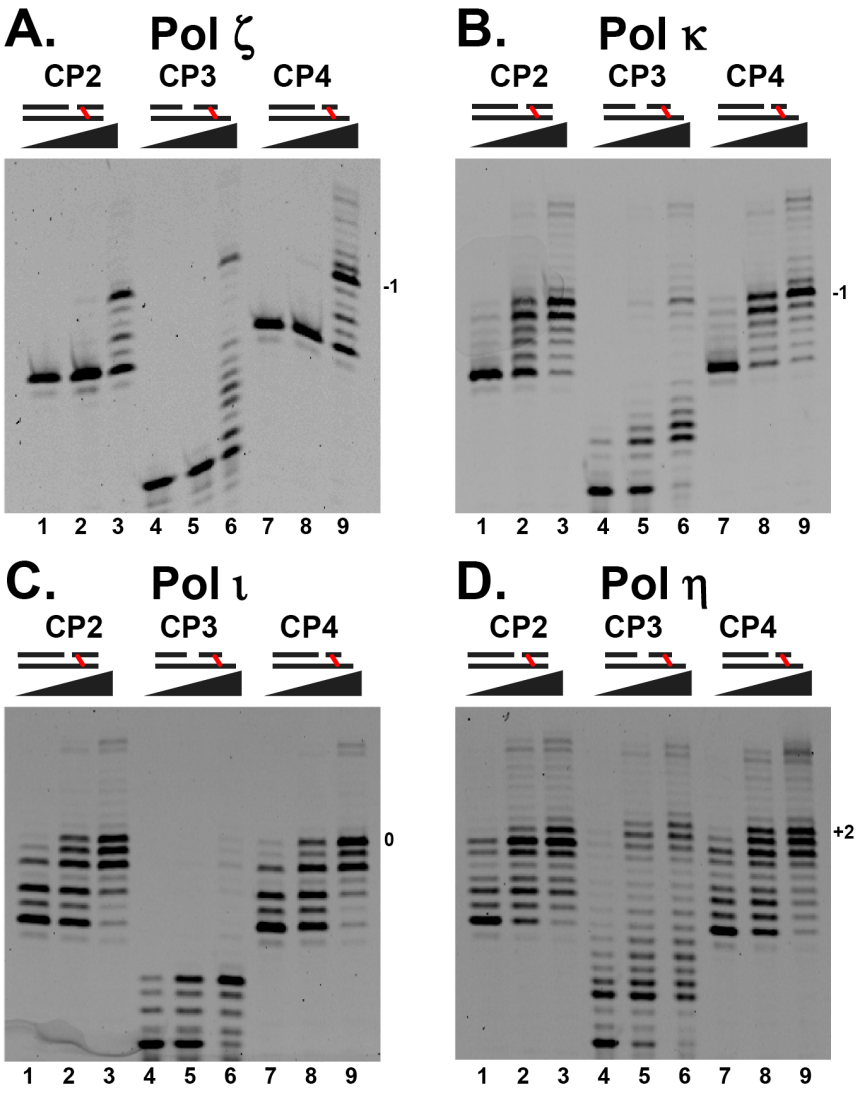


Figure 13: Extension reactions of resected CP ICLs. **A.** Pol ζ extension reactions on 5' (lanes 1-3), 3' (lanes 4-6) and 5'-3' resected ends (lanes 7-9) stall at -1 position. **B.** Pol κ extension reaction on 5' (lanes 1-3), 3' (lanes 4-6) and 5'-3' resected ends (lanes 7-9) stall at -1 position. **C.** Pol ι extension reaction on 5' (lanes 1-3), 3' (lanes 4-6) and 5'-3' resected ends (lanes 7-9) stall at 0 position. **D.** Pol η extension reaction on 5' (lanes 1-3), 3' (lanes 4-6) and 5'-3' resected ends (lanes 7-9) stall at +2 position past the CP ICL.

Rev1 does not stimulate Pol ζ to bypass a cisplatin ICL and a complex of Pol ζ/Rev1 is unable to extend insertion products of the Y-family polymerases

Rev1 can stimulate the ability of Pol ζ to bypass certain bulky DNA adducts (Acharya et al, 2006; Guo et al, 2003; Washington et al, 2004a) and therefore we wanted to test if a complex of Rev1/Pol ζ would be more proficient to bypass a CP ICL. Since CP forms an ICL between two guanines, we hypothesized that Rev1 could perform the insertion step followed by an extension by Pol ζ, (Haracska et al, 2001c; Johnson et al, 2001; Johnson et al, 2000b). However, the extension reaction with a Pol ζ/Rev1 complex did not result in more bypass product compared to Pol ζ alone (Compare Figure 12 with Figure 14A) and the complex also stalled at the -1 position (Figure 14A, lane 1-9). Apparently, and consistent with previous observations (Minko et al, 2008a), Pol ζ and Rev1 fail to interact together productively under our experimental conditions.

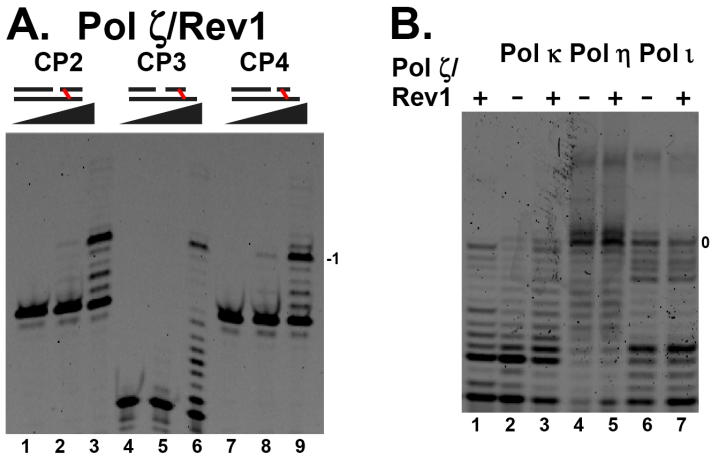


Figure 14: Two polymerases reactions. **A.** Extension reaction of Pol ζ/Rev1 complex extension reaction on 5' (lanes 1-3), 3' (lanes 4-6) and 5'-3' resected ends (lanes 7-9) stall at -1 position. **B.** Pol ζ/Rev1 does not increase bypass efficiency of Pol κ (lane 2 and 3), Pol η (lane 4 and 5) and Pol ι (lane 6 and 7).

Next we wanted to investigate if a complex of Pol ζ/Rev1 would be able to extend the products generated by Pol κ, Pol ι and Pol η on the CP1 template. All these polymerases were able to insert a dNTP opposite the crosslinked guanine probably resulting in a distorted primer-template end, which we speculate could be extended by Pol ζ. We pre-incubated CP1 with Pol κ, Pol ι or Pol η for ten minutes and then added a complex of Pol ζ/Rev1 and compared this reaction to equivalent ones where no Pol ζ/Rev1 was added (Figure 14B). Under the condition used, we did not detect a difference between reaction in the presence or absence of Pol ζ/Rev1. This result suggests that extension of an ICL insertion product generated by Pol κ, ι or η is not readily extended by a Pol ζ/Rev1 complex in vitro. It is likely that the activities of Pol ζ/Rev1 are coordinated by additional factors in vivo to perform TLS at CP ICLs (Shen et al, 2006).

Only Pol ζ and Rev1 have a preference to insert the correct dNTP opposite a cisplatin ICL

Since all polymerases tested were able to insert a deoxynucleotide opposite a CP ICL on the CP4 substrate, albeit with varying efficiencies, we wanted to determine the accuracy of dNTP insertion opposite the adducted guanine. Therefore, we carried out single nucleotide incorporation experiments by annealing the CP4 to the CPP1 primer (Figure 11B) and monitored the incorporation of the individual dNTPs by the different polymerases.

Pol ζ incorporated dCMP but none of the other dNTPs opposite the crosslinked guanine (Figure 15), indicating that the enzyme accurately bypasses a cisplatin ICL. Rev1 also specifically incorporated dCMP opposite the cisplatin ICL, which is in agreement with the reported deoxycytidyltransferase activity of Rev1 (Nair et al, 2005b; Nelson et al, 1996a). However, the insertion opposite the crosslinked guanine was very inefficient (Figure 15). A complex of Pol ζ/Rev1 gave the same result as Pol ζ alone (Figure 15) indicating that most of the activity comes from Pol ζ, confirming our results with templates CP2-4 (Figure 14A).

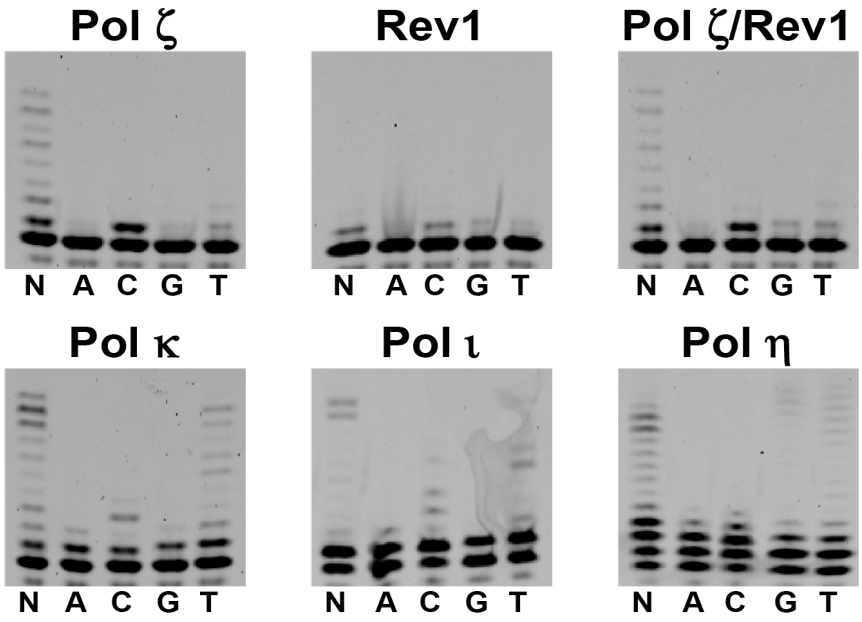


Figure 15: Insertion reactions opposite to CP ICL. Pol ζ, Rev1 and Pol ζ/Rev1 insert the right dCMP opposite the CP ICL. Pol κ, Pol ι and Pol η incorporating any of the provided dNTPs.

The Y-family polymerases proved to be promiscuous in terms of inserting deoxynucleotides opposite the crosslinked guanine (Figure 15) and had the

ability to insert any of the individual dNTPs present in the reaction. Even Pol κ, which is known to be the most accurate polymerase in the Y-family (Johnson et al, 2000a; Ohashi et al, 2000a), would insert any of the dNTPs opposite the adducted guanine. Interestingly, both the insertion and first extension step by Pol η were highly mutagenic and all dNTPs were incorporated with similar efficiency at the 0 and the +1 position (Figure 15).

The length ICL bridge influences bypass of nitrogen mustard-like ICLs

Having shown that bypass of a highly distorting CP ICL is possible after resection of the dsDNA around the ICL, we wished to compare these results to those of two of our nitrogen mustard-like ICLs. The hydrazine ICL (HY), which slightly bends the DNA, while retaining mostly B-form DNA and the dimethylethylenediamine ICL (DA), which we expect to be free of any distortion (Figure 11A). We used the HY1 and DA1 substrates, in which the ICL is part of a 20mer duplex, and the HY2 and DA2 substrates, in which the duplex portion around the ICL is shortened to six nucleotides to investigate the influence of the ICL bridge length on bypass by TLS polymerases. Pol ζ stalled one nucleotide before the insertion opposite to the adducted guanine on HY1 and DA1 templates (Figure 16A, lane 1-12), similar to what we observed with the CP1 substrate. Interestingly, there was a marked difference in how Pol ζ reacted with the resected HY2 and DA2 substrates. The extension reaction was completely

blocked at the -1 position with HY2 (Figure 16A lane 6), leading to an even lower degree of insertion and bypass than with CP ICL. On the other hand Pol ζ could bypass the DA2 substrate (Figure 16A lane 12), indicating that increasing the length of the link between the guanines greatly facilitated bypass by Pol ζ.

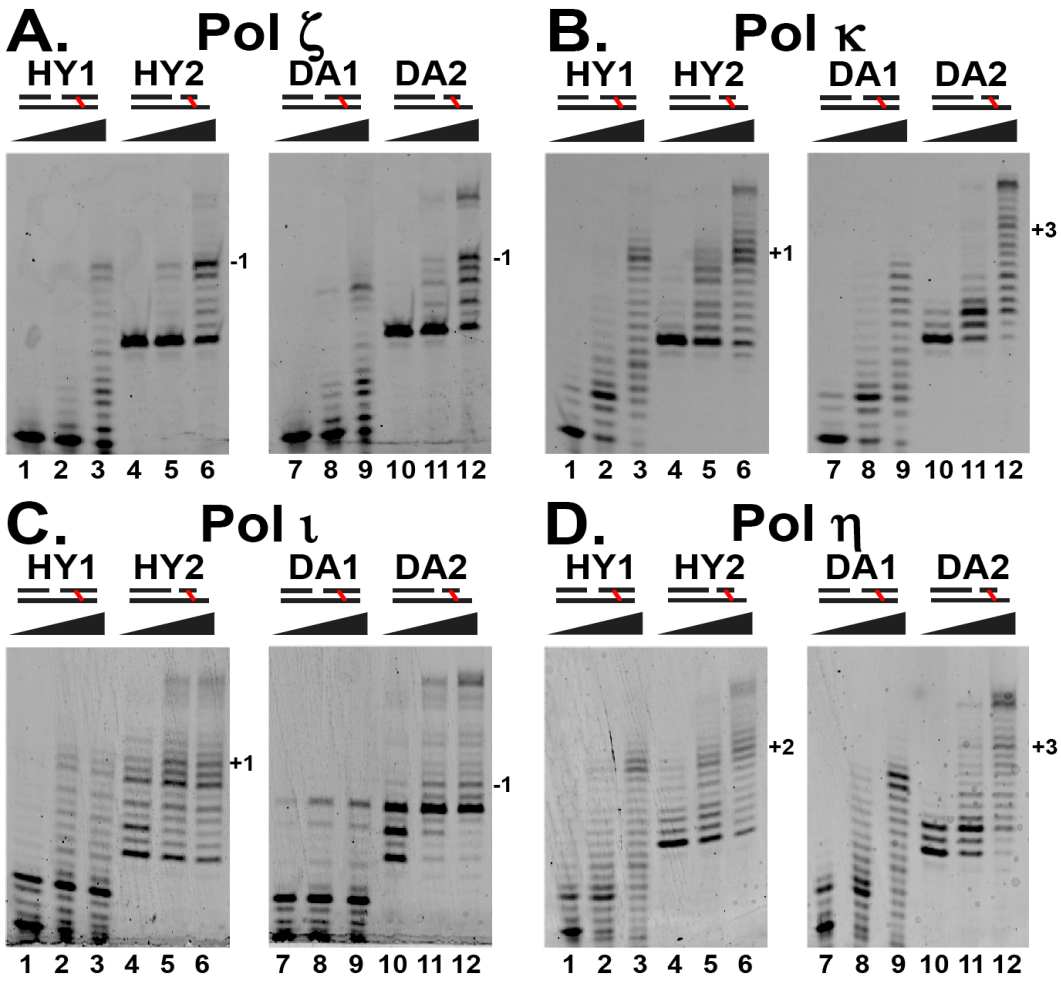


Figure 16: Extension reaction of NMM ICLs. **A.** Pol ζ extension reactions of non-resected and 5'-3' resected HY ICL stall at -1 (lanes 1-6). DA ICL reaction stall at position -3 (lanes 7-9) and at -1 on 5'-3' resected template with significant bypass (lanes 10-12). **B.** Pol κ reaction pauses at +1 site on HY ICL templates with bypass (lanes 1-6) and bypasses DA ICL once resected (lane 12). **C.** Pol ι bypasses HY and DA ICL when resected with pausing sites at +1 and -3, respectively (lane 5, 6, 11 and 12). D. Pol η bypasses HY and DA ICL once resected with pausing sites at +2 on the HY ICL (lanes 6 and 12).

The Y family polymerases were more efficient in bypassing NMM ICLs than CP ICL and resection of the dsDNA portion around the ICL markedly facilitated approaching, insertion and bypass of the HY and DA ICLs. Thus, Pol κ had pausing sites on the HY2 template at the 0, +1 and +2 position, but was able to fully extend a notable fraction of the HY ICLs (Figure 16B, lane 6). The bypass of the ICL in DA2 template was even more efficient and proceeded without any pronounced pausing site at the ICL, indicating that the DA ICL in a 6mer duplex does not inhibit insertion and bypass by Pol κ (Figure 16B, compare lane 9 with lane 12). Interestingly, strand displacement was inhibited to a greater extent by a DA than a HY ICL (Figure 16B, compare lane 5 with lane 11). It therefore appears that helical distortion by the ICL facilitates strand displacement, while increased length of the ICL facilitates bypass.

Similar results were observed with Pol ι, which bypasses the DA and HY ICLs, and is only slowed down by the HY ICL with pausing sites at the -1, 0 and +1 positions (Figure 16C, lane 1-6). On the DA2 template strand displacement was a bigger obstacle than on the HY2 template and pausing sites were observed before start of duplex DNA and at the -3 position (Figure 16C, lane 10). Since these pausing sites were not observed with the HY2 template, it reinforces the notion that a more stable duplex structure near the DA ICL can inhibit the strand displacement reaction.

Pol η was the most efficient Y family polymerase in bypassing the NM ICLs. It was even able to bypass efficiently the fully double stranded substrate HY1 stalling at the +2 position (Figure 16D, lane 3). On the resected HY2

substrate Pol η was also only slowed down with pausing site at the +1, +2 and +3 position (Figure 16D, lane 6). In contrast, Pol η was barely able to approach the DA ICL in the DA1 template stalling at the -1 position (Figure 16D, lane 9). By contrast Pol η was able to efficiently bypass the resected DA2 substrate. Again, pausing sites at the beginning of the crosslinked strand were observed, which were specific for the DA ICL (Figure 16D, lane 5 and 11). But once the strand is displaced, Pol η was able to extend to the end with no additional major pausing sites, similar to Pol κ (compare Figure 16, B and C lanes 12).

Insertion opposite the NMM ICL by Pol ζ is very inefficient and shows a different pattern to cisplatin ICL in regard of the Y-family polymerases

Analogous to the CP ICL we carried out single insertion studies using the HY2 and DA2 substrate annealed to the NMP1 primer (Figure 11B) to study the accuracy of the insertion opposite the NMM ICLs.

As seen before the insertion at the HY ICL by Pol ζ was very inefficient (Figure 16A) and this was confirmed in this single insertion reaction. Although very little insertion product could be seen, the insertion was specific for dCMP and since the sequence contains two consecutive guanosines, two dCMP were incorporated (Figure 17). In contrast, insertion by Pol ζ opposite a DA ICL was comparable to CP ICL and was also specific for dCMP, suggesting that the structure of DA ICL is more accessible than the HY ICL for Pol ζ (Figure 17).

Interestingly, Rev1 was unable to perform any insertion opposite either of the two NMM ICLs and also failed to stimulate Pol ζ (Figure 17).

Pol κ inserted any dNTP opposite the HY ICL, but interestingly only extended with the right dCMP (Figure 17). On the other hand dCMP insertion was preferentially catalyzed by Pol κ opposite the DA ICL, indicating that the longer linker allows Pol κ to insert the correct base (Figure 17).

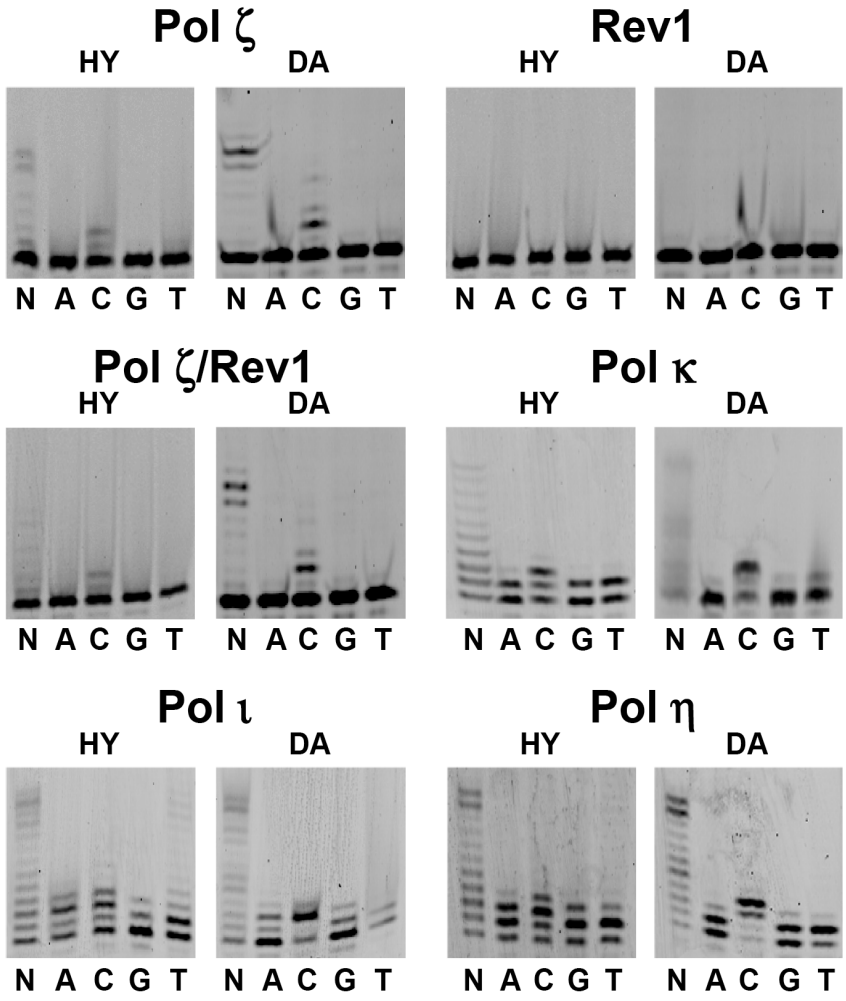


Figure 17: Insertion reaction opposite NMM ICLs. Pol ζ inserts the right dCMP opposite to HY and DA ICL, with DA ICL insertion being more efficient. Rev1 is unable to insert opposite to HY and DA ICL in the presence of any dNTPs and does not enhance Pol ζ insertion reaction. Pol κ, Pol ι and Pol η are inaccurate opposite HY ICL. Pol κ and Pol ι have a preference for dCMP insertion opposite to DA ICL. Pol η does not show a dNTP preference for insertion opposite DA ICL template.

The insertion reaction opposite the HY ICL with Pol ι showed that any of the dNTPs were inserted, with dGMP being the least efficient. Additionally, the insertion product was efficiently extended by one and two nucleotides in presence of dATP and dCTP, repectively (Figure 17). In case of the DA ICL, Pol ι had a clear preference for dCMP and incorporated dCMP at the 0 and +1 positions (Figure 17). The reactions with the other dNTPs were less efficient (Figure 17).

Insertion by Pol η was highly inaccurate opposite both NMM ICLs and any of the dNTPs were incorporated (Figure 17). However, extension from the insertion product with a dNTP other than dCMP was more prominent with HY ICL than with the DA ICL. As seen before with Pol κ and Pol ι the extension reactions are more accurate for DA ICLs than for HY ICLs and occurred almost exclusively in the presence of dCTP.

Discussion

Current models for ICL repair suggest replication dependent or independent incisions in the vicinity of an ICL followed by TLS past the unhooked ICL and restoration of the DNA by removing the ICL remnant by excision repair (Moldovan & D'Andrea, 2009; Niedernhofer et al, 2005; Noll et al, 2006; Patel & Joenje, 2007). Pol ζ and Rev1 were genetically implicated in tolerance against ICL forming agents (Cheung et al, 2006; Nojima et al, 2005; Simpson & Sale, 2003; Wittschieben et al, 2006; Wu et al, 2004a) and recently the activity of Pol ζ

was shown to be necessary to extend from a insertion product across a CP ICL in *Xenopus laevis* egg extract (Räschle et al, 2008). Although, in a recent study using primer extension reaction with a template containing an acrolein derived N2-N2 guanine ICL and yeast Pol ζ and Rev1, it was shown that Pol ζ was unable to bypass such an ICL in presence or absence of Rev1 (Minko et al, 2008a). Though, Rev1 was able to insert the right deoxynucleotide opposite the adducted guanine (Minko et al, 2008a).

There are several explanations for this apparent discrepancy. One of the reasons might be the structural difference between CP ICL and the N2-N2 guanine ICL used in these studies. Indication for structure specific bypass of an ICL comes from the above mentioned *Xenopus laevis* egg extract study where the dependency on Pol ζ for the extension was specific for CP ICL but not for NMM ICL (Räschle et al, 2008) suggesting that other polymerases may act on structurally distinct ICLs. Supporting this notion is the ability of Pol κ to bypass an N2-N2 guanine ICL once the crosslinked strand was trimmed to a two-nucleotide ICL remnant (Minko et al, 2008a). Also, work with bacterial polymerases Pol I and Pol IV suggests that there is more than one polymerase, which is able to bypass ICLs (Kumari et al, 2008; Smeaton et al, 2009; Zietlow & Bessho, 2008).

To address these issues we were in the unique position to compare the bypass reaction of several TLS polymerases on three structurally distinct ICLs, which exhibit various degree of helix distortion with CP ICL being the most and DA ICL being the least distorting ICL.

Our results suggest that there are two factors, which are beneficial for bypass of an ICL by a polymerase, resection of the crosslinked nonreplicating strand and the length of the bridge of the crosslink. As shown previously (Minko et al, 2008a), resection of the nonreplicated crosslinked strand to few nucleotides markedly facilitates advancement of all polymerases to the ICL regardless of the structure of the ICL and even promote bypass of ICLs, indicating strand displacement to be a major limiting factor. Thus, Pol ζ was able to perform limited insertion and bypass of CP ICL and complete bypass of DA ICL, once the crosslinked strand was shorten to six nucleotides (Figure 13A, Figure 15, Figure 16A and Figure 17). Interestingly, Pol ζ was unable to bypass an HY ICL even after resection, indicating that the immediate structure at the HY ICL does not support insertion and bypass by Pol ζ (Figure 16A and Figure 17). Pol ζ also failed to extend from insertion products generated by the Y-family polymerases (Figure 14B), the suggested role for Pol ζ in bypassing lesions (Prakash et al, 2005; Waters et al, 2009) and in particular ICLs (Räschle et al, 2008).

All the Y family polymerases were able to insert a deoxynucleotides opposite to CP ICL, although only Pol η was efficient in bypassing it (Figure 13B, C and D). In contrast TLS was observed for both NMM ICLs with DA ICL being more readily bypassed once the crosslink strand was resected (Figure 16B, C and D). Interestingly, the DA ICL caused a more prominent pausing site at the start of duplex DNA than the HY ICL. Since the DA ICL has a longer crosslinking bridge we speculate that the duplex DNA is more stable when containing a DA ICL compared to a HY ICL and therefore causing this prominent pausing site at

the start of the nonreplicated strand. Furthermore, we could not detect any distinctive pausing site around the DA ICL, whereas there are clear stalling sites around the HY ICL (Figure 16B, C and D). We presume that once the DA ICL is displaced, it interferes less with the polymerization reaction by the Y-family polymerases due to its long crosslinking bridge.

A surprising result was the amount of redundancy we observed within the Y-family polymerases in mutagenic bypass of the three ICLs with Pol η being the most proficient polymerase. This might explain the lack of genetic data implicating Pol κ and Pol ι in ICL repair whereas cells lacking Pol η (XPV) were shown to be sensitive to exposure to cisplatin and psoralen (Albertella et al, 2005; Chen et al, 2006; Misra & Vos, 1993; Mogi et al, 2007). Furthermore, shown in host cell reactivation assays XPV cells fail to reactivate reporter constructs containing a mitomycin C ICL and to a lesser extent a psoralen ICL (Wang et al, 2001; Zheng et al, 2003), again suggesting a role of the structure of the ICL being important for bypass and might reflect the need for other polymerases to bypass structurally distinct ICLs. Hence, further clarification for the *in viv*o role of Pol κ and Pol ι is certainly necessary.

In contrast to the genetic data, Pol ζ and Pol ζ/Rev1 were only marginally able to bypass CP ICL and were only efficient in bypassing the least obstructing DA ICL, although interestingly in contrast to the Y-family polymerases, Pol ζ inserts the right dCMP. The *in vivo* activity of Pol ζ and Rev1 may be tied to auxiliary factors such as PCNA, which was shown to be required for ICL repair (Li et al, 1999; Li et al, 2000). Furthermore, a recent report suggests a role for FA

pathway; specifically FancD2-FancI ubiquitination was shown to promote incision and TLS over a CP ICL (Knipscheer et al, 2009).

Although we did not observe a preference of 5' or 3' resection as reported previously (Minko et al, 2008a) nonetheless it was essential for efficient bypass. A candidate for such an activity on the 5' end is Snm1, which has 5' to 3' exonuclease activity (Hejna et al, 2007; Li et al, 2005). On the 3' end the structure specific endonuclease Xpf/Ercc1 was shown to have 3' to 5' exonuclease activity in the presence of RPA (Mu et al, 2000). Importantly, both SNM1 and XPF/ERCC1 were genetically implicated in the repair of ICLs (Bergstralh & Sekelsky, 2008; Hemphill et al, 2008; Lam et al, 2008; Niedernhofer et al, 2004).

Materials and Methods

Preparation of cisplatin ICL templates

HPLC purified oligonucleotides for cisplatination were purchased from Integrated DNA Technologies Incorporation. Cisplatin was purchased from Sigma-Aldrich.

Templates containing a defined CP ICL were prepared according to Hofr et al. (Hofr & Brabec, 2001). A 20mer containing one single guanine (Figure 11B, upper strand of CP1) at 125 μM concentration was reacted with three fold excess of monoaquamonochloro cisplatin in 10 mM $NaClO_4$ pH 5.2 for 12 minutes at 37° C. The monoaquamonochloro cisplatin was generated by precipitating chlorine

with 0.9 molar equivalent of AgNO3. The monoadduct was purified on a GE Healthcare Mono Q 5/50 GL column using a gradient from 0.1 M to 0.8 M NaCl in 10 mM TrisHCl pH 7.4. The monoadducts was then annealed to a complementary 39mer (Figure 11B) and crosslink formation was promoted by exchanging the buffer to 100 mM $NaClO_4$ pH 5.2. The crosslinked oligonucleotides were purified under denaturing condition (10 mM NaOH) on a GE Healthcare Mono Q 5/50 GL column with a gradient from 0.1 M to 0.8 M NaCl. Amicon Ultra-4 Centrifugal Filters (3000 NWML) were used to exchange buffer (10 mM TrisHCl pH 7.5, 10 mM NaClO4) and to up concentrate the crosslinked oligonucleotides.

For the trimmed templates, the upper strand of CP1 contained deoxyuracils at the indicated sites (Figure 11B). 600 pmol of purified crosslinked products were first treated with excess amount of uracil DNA glycoslase for 10 minutes at 37° C to generated abasic sites at the uracils and then incubated in 100 mM NaOH at room temperature for 2 hours to cleave the abasic sites. The reaction was neutralized by adding equimolar amount of acetic acid and the buffer was exchanged to 10 mM Tris HCl pH 8.0, 1 mM EDTA using Amicon Ultra-4 Centrifugal Filters (3000 NWML).

To analyze the crosslinked oligonucleotides, they were 5' labeled by polynucleotide kinase with ^{32}P and analyze on a 10% 7 molar urea PAGE gel. The dried gel was exposed to phosphor imager screens and scanned on a Typhoon 9400 (GE Healthcare).

Preparation of nitrogen mustard mimic ICL templates

The template containing a NMM ICLs were prepared as described in Angelov et al. and Guainazzi et al. (Angelov et al, 2009).

A 20mer containing a 7-deazaguanosine with a one carbon or two carbon linker precursor for HY and DA preparation, respectively, was annealed to a 39mer containing a 7-deazaguanosine with a two carbon linker precursor at 0.2 mM concentration (Figure 11). The linkers are functionalized with an aldehyde group to couple the two strands by reductive amination (35 mM $NaCNBH_3$, reducing agent) by using either hydrazine or demethylethylendiamine at 0.35 mM concentration for HY or DA ICLs, respectively at room temperature over night. The crosslinked oligonucleotides were purified on a 15% denaturing PAGE gel followed by electroelution using a Schleicher & Schuell BIOTRAP BT 1000. The electrophoresis buffer was exchanged to 10 mM Tris, 1 mM EDTA buffer by using Amicon Ultra-4 Centrifugal Filters (3000 NWML). To obtain the trimmed templates the same method was used as with the cisplatin ICL templates.

To analyze the crosslinked oligonucleotides, they were 5' labeled by polynucleotide kinase with 32P and analyze on a 15% 7 M urea PAGE gel. The dried gels were exposed to phosphor imager screens and scanned on a Typhoon 9400 (GE Healthcare).

Polymerase assay

The templates were annealed to 6-FAM or HEX labeled primers in a primer to template ratio of 1:2 – 1:3 in annealing buffer (10 mM Tris HCl pH 8.0,

50 mM NaCl). To avoid decrosslinking, annealing was performed at room temperature over night. The annealing reaction was checked on a native 20% PAGE gel (data not shown). For the strand displacement control substrate, primer, template and downstream strand were annealed in a 1:2:5 ratio.

Human Pol κ and *S. cerevisiae* Pol ζ and Rev1 were purchased from Enzymax. Human Pol η and Pol ι were kindly provided by R. Woodgate.

The primer extension reactions were performed in 10 µl volume containing 5 nM primer-template with respect to the primer and 100 µM dNTPs. Reaction buffer for Pol κ, Pol ι and Pol η contained; 40 mM Tris HCl pH 8.0, 0.1 mg/ml bovine serum albumin, 10 mM dithiothreitol and 2.5 % glycerol (Frank & Woodgate, 2007). Pol κ and Pol η reactions contained 5 mM and 250 µM $MgCl_2$, respectively (Frank & Woodgate, 2007). Pol ι reactions contained 100 µM $MnCl_2$ (Frank & Woodgate, 2007). Concentrations for the polymerases were varied. For Pol κ 0.2 nM, 2 nM and 20 nM were used. For Pol ι and Pol η concentration of 4 nM, 40 nM and 400 nM were used.

Reaction buffer for Pol ζ and Rev1 contained; 25 mM potassium phosphate pH 7.0, 0.1 mg/ml bovine serum albumin, 25 mM dithiothreitol, 5 mM $MgCl_2$ and 5% glycerol (Guo et al, 2001). The concentration of Pol ζ in the reaction was 0.4, nM 4 nM and 35 nM. Standard reaction time was 10 minutes for all polymerases used. For all insertion reaction the highest enzyme concentration was used.

The reactions were stopped by adding an equal amount of formamide loading buffer (80% formamide, 1 mM EDTA) and heated up to 95° C for 3-4

minutes. The extension products were separated on an 8–10% 7 M urea PAGE gel. The fluorescence of the labeled primer was detected using a Typhoon 9400 (GE Healthcare).

Conclusion and Perspectives

The aim of this thesis was to investigate the ill defined mechanism by which DNA ICLs are removed from the DNA. These lesions are caused by a range of chemotherapeutic drugs such as cisplatin and nitrogen mustards and present a formidable challenge to the cells by inhibiting replication and transcription. We were specifically interested in an intermediate step during repair of ICL, where the unhooked ICL is bypassed by TLS polymerases.

For this purpose we designed and synthesized templates for primer extension reaction, which contain chemically and structurally well-defined ICLs. We were in the unique position to draw the comparison between a CP ICL, which is highly helix distorting and two NMM ICL which differ in the length of the crosslink bridge, hence differ in the amount of helix distortion. By using a set of TLS polymerases we were able to assess the ability of these different polymerases to bypass these structural distinct ICLs.

We could conclude that two factors were important for successful bypass of ICL by polymerases. First, reducing the amount of dsDNA around the ICL obliterated the need for strand displacement synthesis and hence greatly facilitated advancement of polymerases to the ICL and eventual bypass of the ICL. Second, we found that having a less distorting and an ICL with longer bridge length was beneficial for a complete bypass by the tested polymerases. Further,

we were able to show that Y-family polymerases in most cases would bypass the ICL inaccurately and introduce mutations. Only Pol ζ was inserting the right deoxynucleotide opposite to all three ICLs, although with very low efficiency.

Future work in a bottom-up approach will have to include different factors in the reaction such as PCNA, exonucleases and/or endonucleases. PCNA was shown to stimulate TLS polymerases to bypass certain lesion. Exonucleases and endonucleases such as Snm1, Mre11, Exo1, Xpf/Ercc1, Fen1 or Mus81/Eme1 could promote TLS on the non-resected templates by in situ cleaving the displaced strand and resecting the nontemplating crosslinked strand.

In a complementing top-down approach we are also able to integrate oligonucleotides containing an ICL into plasmid DNA for the use in cell-based and cell-free assays. To this end our lab has already successfully expanded into host cell reactivation assays, where we used transformed plasmids containing ICLs into cells and monitor the reactivation of a reporter gene and found that transcription-coupled repair can also be involved in removing ICLs. Furthermore, Pol ζ and Rev1 were shown to be necessary in this pathway, which is not mutagenic. Furthermore, studies in cell-free *Xenopus leavis* egg extract in the group of Johannes Walter using our constructs successfully revealed details of the replication dependent ICL repair pathway and substantially clarified the molecular mechanism of ICL repair (Knipscheer et al, 2009; Räschle et al, 2008). Analogous to the work presented here, the future work in this cell-free system will be focusing on examine the different NMM ICLs. Furthermore, depletion studies

in this system could further define the role of exonucleases/endonucleases in ICL removal.

Finally, structural studies using ICL containing oligonucleotides with a polymerase trapped at the ICL would certainly help understand how an ICL can be bypassed. We already explored this possibility by using Dpo4 archaeal Y-family polymerase, which was already successfully used in crystallographic studies (Ling et al, 2003; Ling et al, 2001; Wang & Broyde, 2006; Wong et al, 2008). We found that it had a similar ICL bypass ability to Pol η and hence it would be interesting to explore the possibility of generating a quaternary structure with Dpo4, primer, incoming nucleotide and ICL containing template.

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