Interstrand Crosslinks
-Induction and repair
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Daniel Vare
The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I've found it!), but 'That's funny...'
-Isaac Asimov
Abstract

DNA crosslinking agents exhibit a variety of DNA lesions, such as monoadducts, DNA-DNA interstrand or intrastrand crosslinks or DNA-protein crosslinks. Agents that produce interstrand crosslinks (ICLs) exist naturally and are widely used in chemotherapy. Therefore, it is important to understand how the lesions induced by these agents are repaired. In bacteria, the repair is mainly dependent on nucleotide excision repair (NER) together with homologous recombination (HR) or translesion synthesis (TLS). In human cells, it is not clear how these lesions are repaired, and it is believed to be a more complicated process in which NER does not play as important a role as in prokaryotes. Here, we investigated the repair mechanisms mainly after treatment with psoralen but also with acetaldehyde, cisplatin and mitomycin C in some studies. As expected from studies on plasmids and in bacteria, we used new techniques to confirm that various ICL-inducing agents block replication fork elongation in mammalian cells. We also found that the replication fork was unable to bypass these lesions. We confirmed that ERCC1/XPF and the HR proteins BRCA2 and XRCC2/3 are vital for protection against ICL treatments. These proteins were also found to be equally important for the repair of monoadducts. To better understand ICL repair in mammalian cells, we developed a method to study the induction and unhooking of ICL in human fibroblasts. We found that ICLs were repaired and that 50% of the induced ICLs were unhooked within 3 hours following exposure. Additionally, we determined that XPA, but not XPE, is involved in ICL unhooking, although not affecting lethality. A step in ICL repair is the formation of double-strand breaks (DSBs), and we identified a replication-dependent formation of DSBs following ICL treatment. Furthermore, ERCC1/XPF was not necessary for DSB formation. The repair of these DSBs was performed by HR and involved ERCC1/XPF. Additionally, we were able to quantify the ICL unhooking in human fibroblasts and found that they can unhook ~2500 ICL/h. We also determined that a dose of
approximately 400 ICL/cell is lethal to 50% of the cells, indicating that ICL unhooking is not the most critical step during the repair process.
DNA-skadande ämnen är vanligt i cancerbehandling, då snabbt växande celler, såsom cancerceller, är betydligt känsligare än normala celler för DNA skador. En grupp av ämnen som vanligen används i cancerbehandling är korsbindare av DNA. Dessa ämnen kommer reagera två gånger med DNA och skapa två bindningar mitt emot varandra. DNA strängen, som består av två delar, måste kunna separeras och kopieras (replikation) på ett tillförlitligt sätt för att cellerna ska kunna dela sig och bli flera. DNA strängen måste också kunna dela sig och bli avläst rätt för att nya proteiner ska kunna bildas (transkription). När korsbindarna har bundit till DNA strängarna, hindrar detta deras separation och därigenom förhindras även avläsningen och kopieringen. För att göra undersökningarna av DNA korsbindande ämnen ännu lite svårare, så ger korsbindare flera olika typer av skador. Dels kan det bli flera olika typer av korsbindningar, både mellan två DNA-strängar (ICL) vilket är den farligaste och mest svårreparera typen, men det kan också ske inom samma DNA-sträng (intrastrand crosslink) eller mellan en DNA-sträng och ett protein (DNA-protein crosslink). Korsbindare kan även bilda enbindingsskador (monoadukt), vilket innebär den bara binder en gång till DNA.

Den fösta artikeln i avhandlingen handlar om att försöka reda ut om det är ICLen eller monoaddukten som är orsak till olika effekter som påträffas efter behandling med korsbindande ämnen. Det vi fann var att även om det bara var från ICLs som vi kunde mäta en effekt på replikationen, så fick vi nästan lika stark effekt från monoaddukterna, som från ICL, för en av de vanligast använda markörerna (kännetecknen) för båda DNA strängarna var brutna på samma ställe (dubbelstränsbrott). Detta berodde dock inte på att även monoaddukterna skapade dubbelsträngsbrott, utan på att markören vi använde var ospecifik. Vi fann även att även om ICLs har mycket större effekt än monoaddukten på cellens överlevnad m.m., så kan man inte bortse ifrån effekten av monoaddukten och att den troligen har en betydande roll för de korsbindande ämnen som endast ger en liten del ICLs.

I artikel två har vi utvecklat en ny metod, som gör det möjligt att mäta hur många ICLs som bildas vid en viss dos av de korsbindande ämnen vi undersöker. Vi kan även mäta hur fort ICLerna kan repareras i mänskliga celler med hjälp av metoden. Tack vare en kombination av våra mätningar och med hjälp av datorsimuleringar, kunde vi räkna ut hur många ICLs som bildades per dos för tre vanliga korsbindare. Vi kunde även visa att 50 % av ICLen har påbörjat reparationen och kommit så långt att de var bortklippta från ena stängen inom 3 timmar efter behandlingen.

I artikel tre undersöker vi vilka proteiner som är inblandade i den tidiga delen av ICL reparationen, alltså fram till och med att celler klipper ut korsbindningen på båda sidorna om skadan i ena strängen. Här visar vi att celler som är defekta i reparationsprotein kallat XPA, har en betydligt långsammare borttagning av ICLer än vad båda normala celler och celler defekta i reparationsprotein XPE har. Vi visar även att detta inte påverkar cellens replikationshastighet, eller har någon effekt på cellens överlevnad.

I den femte och sista artikeln i avhandlingen undersöker vi ett av de vanligast föreslagna proteinen för att sköta klippningen av DNA (ERCC1/XPF) och hur den är inblandad i reparationen av korsbindningar. Vi kan här visa att även det krosbindande ämnet mitomycin C bromsar replikationshastigheter och att ERCC1/XPF är nödvändigt för att kunna fullfölja homolog rekombination av ICLs.
List of publications

This thesis is based on the following work:


Paper II Vare D, Johansson F, Persson J-O, Erixon K, Jenssen D. The quantification and repair of psoralen-induced interstrand crosslinks in human cells. Submitted

Paper III Vare D, Erixon K, Johansson F, Jenssen D. XPA has a key role in the unhooking step of ICL repair in intact mammalian cells. Manuscript

Paper IV Natalia Kotova, Daniel Vare, Niklas Schultz, Dobrosława Gradecka Meesters, Maciej Stępnik, Jan Grawé, Thomas Hellday and Dag Jenssen. Genotoxicity of alcohol is linked to DNA replication-associated damage and homologous recombination repair. Revised submitted

Publications not included in this thesis


4. Katarina Vielfort, Niklas Söderholm, Linda Weyler, Daniel Vare, Sonja Löfmark, and Helena Aro Neisseria gonorrhoeae infection causes DNA damage and affects p21, p27 and p53 expression in non-tumour epithelial cells. Revised submitted
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>ADU</td>
<td>Alkaline DNA unwinding</td>
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<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand break</td>
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<td>FA</td>
<td>Fanconi anemia</td>
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<td>GGR</td>
<td>Global genome repair (NER)</td>
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<td>Homologous recombination</td>
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<td>ICL</td>
<td>Interstrand crosslinks</td>
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<td>MMR</td>
<td>Mismatch repair</td>
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<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
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<tr>
<td>NHEJ</td>
<td>Non-homologous end-joining</td>
</tr>
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<td>Pol</td>
<td>DNA Polymerase</td>
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<td>PUVA</td>
<td>Psoralen activated by UVA</td>
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<td>SSB</td>
<td>Single-strand breaks</td>
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<tr>
<td>TLS</td>
<td>Translesion synthesis</td>
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<tr>
<td>UVA/C</td>
<td>Ultraviolet radiation A/C</td>
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Cellular DNA is constantly exposed to various endogenous and exogenous sources of DNA-modifying agents. Replication errors may also contribute to the induction of DNA lesions. DNA damage occurs spontaneously, generating abasic sites and causing deamination, largely by hydrolysis (Lindahl, 1993; Sander et al., 2005). In addition, metabolism generates a variety of reactive oxygen species and nitrogen moieties that may damage DNA (De Bont and van Larebeke, 2004). The types of DNA lesions that frequently occur include methylations, oxidations of the bases or of the DNA backbone, single-strand breaks (SSBs), double-strand breaks (DSBs), photoproducts, bulky adducts and interstrand, intrastrand or DNA-protein crosslinks, among others. If not repaired before replication or if repaired incorrectly, these DNA lesions can be converted into mutations and/or aberrations. The mutations may be base substitutions, frameshift mutations, deletions or insertions. Genotoxic agents that modify the DNA are present in food, cigarette smoke, car exhaust and environmental pollutants. The response to DNA damage in eukaryotic cells can be described in three steps: recognition of the damage, assessment of the damage and response to the damage in terms of repair, genomic instability or cell death (Rich et al., 2000).

Crosslinking agents

Some DNA reactive agents are bifunctional, including mitomycin C (figure 1a), cisplatin (figure 1b), acetaldehyde (figure 1c) and psoralen (after light-induced activation) (figure 1d-f). In addition to generating monoadducts, these agents may induce a second bond to DNA or proteins, resulting in the formation of crosslinks. The formation of a second bond is thought to be slow for most drugs, and only a fraction of the induced monoadducts can form crosslinks (McHugh et al., 2001). This fact is exemplified by mitomy-
cin C, which requires several reaction steps to be able to generate DNA-DNA interstrand crosslinks (ICLs) (Seow et al., 2004). There are two types of DNA-DNA crosslinks: those that are in the same DNA strand (intrastrand crosslinks) and those that are between the complementary DNA strands (ICLs) (McHugh et al., 2001). Bifunctional agents can also give rise to DNA-protein crosslinks by covalently linking proteins to DNA.

A = Mitomycin C
B = Cisplatin
C = Acetaldehyde
D = Psoralen
E = 5-metoxy psoralen
F = 8-metoxy psoralen
G = Angelicin, Iso-psoralen

Figure 1. Chemical structure of crosslinking agents. The chemical structure of some ICL inducing agents and angelicin.
Due to their therapeutic efficiencies in comparison to other groups of cancer drugs, DNA crosslinking agents are among the most widely used anticancer agents (McHugh et al., 2001).

Bifunctional agents exhibit very different capacities for forming ICLs (table 1). As an example, mitomycin C mainly induces ICLs (Tomasz, 1994), but only 5-13% of the induced DNA adducts are ICLs (Warren et al., 1998). However, UVA-activated psoralen (PUVA) induces up to 40% ICLs (Dronkert and Kanaar, 2001), whereas cisplatin mainly induces intrastrand crosslinks (Brabec and Leng, 1993; Zamble and Lippard, 1995), giving rise to 90% intrastrand crosslinks and 5-8% ICLs (Dronkert and Kanaar, 2001; Jones et al., 1991; Noll et al., 2006). Nitrogen mustard is another crosslinking agent that induces up to 5% ICLs (Dronkert and Kanaar, 2001). Endogenous production of ICL-inducing agents has also been proposed, originating from unsaturated aldehydes (e.g., acrolein, crotonaldehyde or malondialdehyde) (Niedernhofer et al., 2003; Wilson et al., 1991).

<table>
<thead>
<tr>
<th>Interstrand crosslinks (ICL), %</th>
<th>DNA distortion</th>
<th>Helix bending</th>
<th>ICL half-life</th>
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<tr>
<td>Psoralen</td>
<td>&lt;~40</td>
<td>Minor</td>
<td>No</td>
</tr>
<tr>
<td>MMC</td>
<td>5-13</td>
<td>Minor</td>
<td>No</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5-8</td>
<td>Major</td>
<td>47°</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>&lt;&lt;10</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
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Table 1. ICL inducing agents effect on DNA. Selected ICL inducing agents ability to induce ICL and their effect on the DNA structure (Dronkert and Kanaar, 2001; Wang et al., 2000)
Most ICLs produce major distortions to the DNA structure and thereby prevent strand separation. Thus, ICLs may obstruct DNA replication and transcription, leading to catastrophic consequences, such as DNA DSBs, if left unrepaired (McHugh et al., 2001). ICLs formed by mitomycin C do not induce bending of the DNA helix (Rink et al., 1996) but lead to an expansion of the minor groove (Norman et al., 1990). Crosslinking agents exhibit the specific characteristics of being more clastogenic than mutagenic (Noll et al., 2006). Another feature of interest is that the stabilities of different ICLs are quite different. Nitrogen mustard-induced ICLs have a half-life of 2 h (Henriques et al., 1997), cisplatin-induced ICLs have a half-life of 29 h (Dronkert and Kanaar, 2001), and mitomycin C and psoralen are more stable (Dronkert and Kanaar, 2001). Preferential DNA-binding positions of these ICL agents and their binding structures are illustrated in figures 2 and 3.
Figure 2. Activation and ICL forming pathways for ICL inducing agents. (a) psoralen, (b) mitomycin C, (c) cisplatin and (d) acetaldehyde. Adapted from (Deans and West, 2011; Wang et al., 2000)
Furocoumarins

Furocoumarins are found in some vegetables and have long been used for medical purposes. The ancient Egyptians (~1500 B.C.) used this constituent in plant extracts for the treatment of skin diseases such as vitiligo (Gupta and Anderson, 1987). Today, PUVA is commonly used in the treatment of psoriasis (Derheimer et al., 2009). One side effect due to the usage of psoralen in medicine is that this treatment has been demonstrated to increase the risk of skin cancer.

Furocoumarins are a family of planar tricyclic compounds that are naturally occurring in edible plants. They can be absorbed by cells and have both toxic and mutagenic properties (Cimino et al., 1985; Ostertag et al., 2002). They are found in celery, carrots, parsley, parsnips, grapefruit, lemons and oranges (Ostertag et al., 2002). An estimation of the daily average furocoumarin intake in the US is approximately 1.3 mg per person, and the major source of intake is lemon- or lime-flavoured drinks (Ostertag et al., 2002). These compounds are nontoxic in the absence of light (Cimino et al., 1985). The best-studied member of this family of compounds is psoralen.

PUVA give rise to an antiproliferative effect due to the formation of monoadducts and ICLs (Derheimer et al., 2009). Psoralen-induced ICLs are more cytotoxic, mutagenic and recombinogenic than monoadducts (Dronkert and Kanaar, 2001). Replication bypass of pyrone-side psoralen T-adduct give rise to T→A and T→C substitutions and A-insertions, whereas furan-side adducts give rise to T→G substitutions and G insertions (Barre et al., 1999). Psoralen ICLs do not induce the bending of the DNA helix but do induce a 25° unwinding of the duplex (Haran and Crothers, 1988). The blocking of replication and transcription induced by psoralen adducts in S-phase cells is a strong signal for the cell to go into apoptosis (Derheimer et al., 2009).

Furocoumarins react selectively with thymine-rich DNA, thus targeting DNA poly-[dA-dT] regions (Serrano-Perez et al., 2008; Smith et al., 2004).
Psoralens intercalate more readily to the linker regions of DNA compared to core sequences in nucleosomes (Islas et al., 1991). Pyrone-side (right side of the structure in figure 1d) monoadducts are less prone than furan-side monoadducts to yield di-adducts (Serrano-Perez et al., 2008). If a furan-side (left side of the structure in figure 1d) monoadduct absorbs a second photon, this would yield a di-adduct (Serrano-Perez et al., 2008). After the first photon ab-
sorption by an intercalated psoralen, a monoadduct is formed. Following the generation of the monoadducts, a conformational change is thought to occur after a short period of 1 µsec; thereafter, absorption of a second photon may give rise to an ICL (Spielmann et al., 1995). Up to 40% of the DNA adducts formed by psoralen can be converted to ICLs (Brendel and Ruhland, 1984). Although psoralens react selectively with thymine, the majority of crosslinking agents react and form adducts with guanines (Harrington et al., 2010; Saris et al., 1996; Serrano-Perez et al., 2008; Smith et al., 2004).

Acetaldehyde

Acetaldehyde is a metabolite of ethyl alcohol that is formed after metabolic oxidation exerted by alcohol dehydrogenase (ADH) in the liver (Lorenti Garcia et al., 2009; Vaca et al., 1995). Acetaldehyde can also be produced as an intermediate of sugar metabolism (Lorenti Garcia et al., 2009) and is one of the most commonly produced aldehydes in car exhaust and tobacco smoke (Vaca et al., 1995). Acetaldehyde dehydrogenase (ALDH) catalyses the oxidation of acetaldehyde to acetate (Boffetta and Hashibe, 2006). Acetaldehyde is electrophilic and reacts with nucleophiles, such as DNA and proteins (Lorenti Garcia et al., 2009). It gives rise to several different types of monoadducts and DNA protein crosslinks. Acetaldehyde also induces DNA ICLs with a preference for G – G crosslinks in opposite strands that are two bases away from each other (Wang et al., 2000) (figure 3).

DNA repair

Human DNA repair is complex, and the different repair pathways involve approximately 150 proteins (Wood et al., 2005). The mammalian genome has been estimated to be subject to approximately 60,000 – 100,000 DNA modifications in each cell every day (Friedberg et al., 1995; Lindahl and Barnes, 2000). Of these, there are approximately 50,000 SSBs, 10,000 depurinations, 5,000 alkylating lesions, 2,000 oxidative lesions, 600 depyrimidations, 10 DSBs and 10 ICLs (Lindahl and Barnes, 2000). If the DNA damage is not repaired or if it is repaired incorrectly, mutations or DNA rearrangements may result. When DNA damage is severe and the level of un repaired lesions
is high, the cell cycle checkpoint mechanisms are activated, and cellular apoptosis occurs (Hoeijmakers, 2001). The repair pathways are activated by specific lesions in DNA. For example, nucleotide excision repair (NER) repairs many helix-distorting adducts (Gillet and Scharer, 2006; Sugasawa, 2006), while base excision repair (BER) takes care of more subtle damage, such as oxidative lesions, alkylated bases and SSBs (Barnes and Lindahl, 2004; Caldecott, 2008; Slupphaug et al., 2003). Homologous recombination (HR) and non-homologous end-joining (NHEJ) repair DSBs; NHEJ accomplishes this by ligating the two ends together, whereas HR uses a sister chromatid or a homologous chromosome as a template.

Nucleotide excision repair (NER)

Because nucleotide excision repair (NER) senses the distortion of DNA caused by a lesion, it is extremely versatile with respect to the DNA lesion it can process. There are two sub-pathways of NER: global genome repair (GGR) (figure 4) and transcription-coupled repair (TCR). GGR performs NER in the non-transcribed part of DNA and in the non-transcribed strand in transcribed genes. More than 30 proteins are involved in GGR in a stepwise process (Clement et al., 2009). The DNA damage sensing in NER of higher eukaryotes is exerted by a complex composed of XPC, HR23B and centrin 2 (Araki et al., 2001; Sugasawa et al., 1998; Volker et al., 2001). In this complex, XPC is the main sensing protein, whereas the other proteins have accessory functions (Clement et al., 2009). The binding of XPC to DNA, both damaged and undamaged, induces a 39-49° bend in the DNA backbone (Janicijevic et al., 2003). XPC recognises DNA distortions rather than specific base modifications and exhibits preference for damage that lowers the DNA melting temperature (Clement et al., 2009; Gunz et al., 1996). In addition to destabilisation of the DNA base pairing, NER recognition/activation also requires covalent DNA modifications (Dip et al., 2004; Hess et al., 1997). After damage recognition, XPC acts as a platform for the recruitment of the TFIHH-complex and guides it to the damage site by interaction with the XPB and p62 subunits of TFIHH (Clement et al., 2009; Yokoi et al., 2000). TFIHH consists of several distinct subunits: XPB, XPD, p62, p52, p44, p34, cdk7, cyclin H and MAT1 (Drapkin and Reinberg, 1994). TFIHH has been proposed to act
as a verifier for the existence of damage, ensuring that there is a bulky lesion at the damaged site and not only DNA distortions (Dip et al., 2004). The unwinding of the DNA by TFIIH generates a bubble structure of approximately 15-30 nucleotides (Clement et al., 2009; Dip et al., 2004). This helix unwinding is not symmetric and is mostly localised to the 5’ side (unwinding 15-25 nucleotides) with only 3-9 nucleotides unwinded on the 3’ side (Dip et al., 2004). At the 5’ end of the bubble, the XPF-ERCC1 endonuclease cuts one of the DNA strands, this is followed by XPG incision at the 3’ end (Staresincic et al., 2009), although the latter appears to be recruited first (Dip et al., 2004). After the initial incision by XPF-ERCC1, DNA polymerase (Pol) δ/ε can restore the DNA duplex using the undamaged DNA strand as a template starting in the 5’ direction, thus avoiding exposure of single-stranded DNA and creating a flap. XPG cleaves this flap in a second incision, possibly initiated by the block of the polymerase. Thereafter, the polymerisation can proceed to completion (Ogi and Lehmann, 2006; Shivji et al., 1995; Staresincic et al., 2009), followed by ligation by Ligase III in conjunction with XRCC1. Ligase I is only recruited to sites of UV damage in proliferating cells (Biggerstaff et al., 1993; Moser et al., 2007). Alternatively, when not giving rise to strong DNA distortion, DNA damage in the absence of direct XPC activation can be recognised by the DDB complex (Payne and Chu, 1994). Binding of DDB to a damaged strand leads to a bending of the DNA helix by 55° (Fujiwara et al., 1999). This DDB-induced bending of the helix allows the recruitment of XPC (Tang and Chu, 2002).

In transcription-coupled repair, lesions are detected by the stalling of RNA polymerase II at the damaged site (Tornaletti, 2009). When the RNA polymerase is arrested at the site of a lesion, there is a strong pro-apoptotic signal (Tornaletti, 2009). A stalled RNA polymerase at the site of a lesion is believed to recruit the repair enzymes CSA, CSB, TFIIH and XPG (Dip et al., 2004; Mellon et al., 1986), followed by the process of GGR.

For NER, there is a need for an undamaged DNA strand to act as a template in lesion repair (Noll et al., 2006). In the case of an ICL, both DNA strands are damaged. Thus, no template is easily available on the complementary strand but may be available on the other chromosome or in other parts of the genome.
Figure 4. A simplified model of NER. The bulky and DNA distorting adduct is recognized by XPC or the DDB complex. TFIIH is recruited to the lesion and unwinds the DNA followed by XPA that verifies the damage and RPA that covers the ssDNA. ERCC1-XPF cuts the DNA on the 5’ side of the lesion and polymerases start replacing the damaged DNA. The incision on the 3’ side is done by XPG and final ligation is performed by ligase III and XRCC1.
Base excision repair (BER)

The primary repair pathway for oxidative, alkylating and depurinated damage is the base excision repair (BER) pathway (Robertson et al., 2009). There are two types of BER pathways: short-patch BER, which excises only the damaged base, and long-patch BER, which replaces up to 12 bases (Robertson et al., 2009). The first step of BER is the recognition and removal of the damaged base from the DNA backbone by a DNA glycosylase, generating an apurinic (AP) site (Baute and Depicker, 2008; Robertson et al., 2009). APE1, a bi-functional glycosylase, then cleaves the DNA backbone, generating a single nucleotide gap (Baute and Depicker, 2008; Doetsch and Cunningham, 1990; Robertson et al., 2009). In short-patch BER, one nucleotide is incorporated into the gap by Pol β (Singhal et al., 1995; Sobol et al., 1996), followed by ligation with ligase III/XRCC1 or Ligase I (Cappelli et al., 1997; Fortini et al., 1998; Nash et al., 1997). In long-patch BER, Pol β incorporates the first nucleotide, and additional polymerisation is performed by either pol δ or pol ε (Fortini et al., 1998; Stucki et al., 1998). Finally, the endonuclease FEN1 removes the generated flap (Baute and Depicker, 2008; Robertson et al., 2009), followed by ligation by ligase I (Levin et al., 1997). Other proteins involved in BER include XRCC1 and PARP1, which act as sensors for SSB (Hegde et al., 2008). Furthermore, XRCC1 can also interact with and activate several BER proteins (Hegde et al., 2008).

Homologous recombination (HR)

Both endogenous and exogenous sources that induce DSBs exist, but DSBs can also arise from replication failures. It is essential that these DSBs are properly repaired to maintain genomic stability (Pardo et al., 2009). One-ended DSBs may arise from replication fork collapse, which occurs when a fork collides with a single-strand gap (Pardo et al., 2009). These one-ended DSBs are repaired by break-induced replication (Morrow et al., 1997; Voelkel-Meiman and Roeder, 1990). DSBs are primarily detected by the MRN complex, consisting of Mre11, Rad50 and Xrs2, through direct interaction with free DNA ends (Chen et al., 2001; Hopfner et al., 2002) (figure 5). A single unrepaired
Figure 5. A simplified model of NEHJ and HR. Repair of DSB can be performed both by HR and NHEJ, upon recognition of the DSB, 53BP1, MRN complex, KU70/80 and DNA-PK is loaded. 53BP1 will inhibit end resection and thereby prevent HR. NHEJ brings the two sides of the DSB together and ligate them giving small deletions. If 53BP1 is removed by BRCA1 this allows access for restriction nucleases to the DNA ends. The free single stranded DNA generated by the end resection is covered initially by RPA by is later on replaced by Rad51, Rad52, Rad54 and Rad55/57, thereby stimulating strand invasion of the homolog DNA. Using the sister chromatid as a template allows HR to be performed in an error free manner. Adapted from (Pardo et al., 2009).
DSB can induce cell death; therefore, DSBs are considered to be the most harmful lesions (Pardo et al., 2009). The repair of DSBs is divided in two pathways: HR and NHEJ (figure 5) (Chen et al., 2001; Pardo et al., 2009). HR is an error-free repair pathway (Hopfner et al., 2002) wherein the template used is identical to the damaged DNA; otherwise, mutations or genomic rearrangements can occur (Pardo et al., 2009). MRN complexes on each side of the DSB are thought to connect the two DNA ends by Rad50 dimerisation prior to repair (Hopfner et al., 2002; Moreno-Herrero et al., 2005; Williams et al., 2008). After DNA end processing, RPA quickly binds the single-stranded DNA (Sung and Klein, 2006). Thereafter, a complex of BRCA1/BARD1, BRCA2/DSS1 and likely PALB2 replaces RPA with RAD51 (Pellegrini et al., 2002; Sharan et al., 1997; Sy et al., 2009; Wong et al., 1997; Zhang et al., 2009b). Rad51 then searches for homology by random collisions between rad51 and DNA molecules (Bianco et al., 1998). When a homologous sequence is found, the invading strand generates a d-loop, which is followed by DNA synthesis using the homologous sequence. Cleavage of the Holiday junction can lead to gene conversion.

Non-homologous end-joining (NHEJ)

Non-homologous end-joining (NHEJ) repairs damage by re-ligating the two DNA ends without the requirement for a homologous sequence (Pardo et al., 2009). Vertebrates seem to use NHEJ more frequently than lower organisms; it is possible that their complex genomes make the homology search of HR ineffective (Pardo et al., 2009). In fact, NHEJ is the major DSB repair pathway in mammals and functions regardless of the cell cycle phase (Helleday et al., 2007; Peterson and Cote, 2004; Shrivastav et al., 2008). NHEJ has been observed to generate small deletions or insertions and has therefore been considered an error-prone repair pathway (Moore and Haber, 1996; Wilson et al., 1997). However, at blunt or compatible DSBs, the error rate of NHEJ is approximately 1 per 1000 breaks (Moore and Haber, 1996; Wilson and Lieber, 1999). In human cells, Ku, a heterodimer of Ku70 and Ku80, acts as a sensor for DSBs and can promote the alignment of the DSB
ends (Cary et al., 1997; Downs and Jackson, 2004; Walker et al., 2001). Ku recruits DNA-PK, which, together with the Artemis protein, stimulates processing of the DNA ends (Ma et al., 2002; Yaneva et al., 1997). DNA-PKcs bound to Ku are not required for the recruitment of XRCC4 but are required for the stabilisation of XRCC4 (Dvir et al., 1992; Dynan and Yoo, 1998; Gottlieb and Jackson, 1993; Yano et al., 2009). XRCC4 and ligase IV catalyse the rejoining of the two compatible DNA ends. For the ligation of incompatible or mismatched DNA ends, XLF interacts with Ku. The stimulatory effect on XRCC4/Ligase IV appears to bridge DSB sensing and the ligation step (Gu et al., 2007a; Gu et al., 2007b; Hentges et al., 2006; Tsai et al., 2007).

Cells deficient in either of the NHEJ proteins Ku70/80, DNA-PKcs, XRCC4 or DNA ligase IV do not exhibit increased sensitivity to ICL-inducing agents (Collins, 1993; Frankenberg-Schwager et al., 2005), suggesting that NHEJ does not play any major role in mammalian ICL repair, as has previously been shown in yeast (Biedermann et al., 1991; McHugh et al., 2000). Nonetheless, NHEJ may play minor roles in processing intermediates of ICL repair (Dronkert and Kanaar, 2001) and at sites where the generation of two DSBs from collapsed converging replication forks has occurred.

Mismatch repair

Mismatch repair (MMR) is responsible for repairing DNA base pair mismatches. MutSα, a heterodimer of MSH2-MSH6, preferentially recognises base-base mismatches and insertions and deletions where one of the DNA strands contains 1-2 unpaired nucleotides. MutSα has also been shown to have endonuclease activity and is able to cleave DNA on both sides of a mismatch (Drummond et al., 1995; Genschel et al., 1998; Kadyrov et al., 2006; Modrich, 2006; Palombo et al., 1995; Palombo et al., 1996). MutSβ, an MSH2-MSH3 heterodimer, recognises insertions and deletions with 2-10 mismatched nucleotides (Genschel et al., 1998; Palombo et al., 1995). There are three MutL complexes in mammalian cells: MutLα (MLH1 and PMS2), which supports MutSα- or MutSβ-initiated repair, and the other complexes, MutLβ (MLH1 and PMS1) and MutLγ (MLH1-MLH3), which have no or
only modest involvement in MMR (Cannavo et al., 2005; Li and Modrich, 1995; Raschle et al., 1999). Exo1 excises the DNA that is thereafter coated with RPA (Modrich, 2006). Finally, polymerase δ fills in the missing bases, and ligase I seals the nick.

Fanconi Anemia

On a cellular level, Fanconi Anemia (FA) cells have a characteristic hypersensitivity to crosslinking agents, such as cisplatin and mitomycin C. FA cells also have elevated levels of chromosomal aberrations (Moldovan and D'Andrea, 2009). The FA repair pathway consists of three types of member proteins: the FA core complex, the FA ID complex and downstream members. The FA core complex is composed of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FA NCL, and FANCM together with FAAP20, FAAP24 and FAAP100 (Gurtan and D'Andrea, 2006; Kee and D'Andrea, 2010; Kennedy and D'Andrea, 2005; Leung et al., 2012; Wang, 2007). Upon DNA damage, the core complex acts as an E3 ligase and, together with ATM/ATR, mono-ubiquitinates the FA ID complex, which consists of FANCD2 and FANCI (Dorsman et al., 2007; Friedel et al., 2009; García-Higuera et al., 2001; Montes de Oca et al., 2005; Sims et al., 2007; Smogorzewska et al., 2007). Ubiquitination and phosphorylation of the ID complex results in the translocation of these proteins to the chromatin and their subsequent formation of foci. The activated ID complex in these foci interacts with the downstream members of the FA pathway (FANCD1/BRCA2, FANCN/PALB2, FANCI/BACH1, FANCO/RAD51C and FANCP/SLX4) and other proteins connected to DNA repair, such as REV1 and PCNA (figure 6) (Geng et al., 2010; Guo et al., 2006; Howlett et al., 2009; Hussain et al., 2004; Long et al., 2011; Niedzwiedz et al., 2004; Nojima et al., 2005; Sy et al., 2009; Wang et al., 2004; Xia et al., 2006; Zhang et al., 2009a). FANC, FANCC and FANCG appear to be important for the incision activity of ERCC1-XPF to unhook ICLs (Deakyne and Mazin, 2011; Kumaresan et al., 2007).
Figure 6. A simplified model of the Fanconi Anemia signalling pathway. When the replication fork encounters a DNA lesion and progression is blocked, FANCM recruits the FA core complex that via FANCL monoubiquitinate the ID complex. The activated ID complex interacts with FAN1 as well as FA downstream members and probably also recruits TLS polymerases to the stalled replication fork. Adapted from (Kee and D'Andrea, 2010).
FANCM, the FANCM-associated histone-fold-containing protein complex (MHF1 and MHF2), FAAP24 and FAAP100 appear to play distinct roles outside the core complex and are required for the recognition of stalled replication forks and the subsequent recruitment of the core complex (Ciccia et al., 2007; Kim et al., 2008; Singh et al., 2010; Yan et al., 2010). FANCM is a helicase that can translocate along the DNA and has been shown to promote fork regression (Deakyne and Mazin, 2011; Gari et al., 2008).

Although hypersensitivity to ICL-inducing agents is the definition of FA cells, they have also been shown to be hypersensitive to other agents, including gamma- and UV-irradiation, bleomycin and MMS (Duckworth-Rysiecki and Taylor, 1985; Tebbs et al., 2005). Therefore, FA may be a more general pathway to handle replication-blocking lesions (Thompson et al., 2005).

Replication bypass mechanisms

When the replication fork encounters a lesion in the template DNA that stalls it, two strategies exist to circumvent a replication fork arrest. One is an essentially error-free pathway involving HR; the other involves use of translesion synthesis (TLS) polymerases to bypass the lesion.

If the lesion is on the leading strand, replication might not be restarted downstream of the damage, as would be the case for the lagging strand. Instead, the lagging strand can continue for some distance; it is possible that the lagging strand may regress, and nascent strand re-annealing may give rise to a chicken-foot structure. This structure offers the possibility of replication beyond the lesion without the use of either TLS polymerases or using the lesion as a template. Furthermore, the formation of DSBs at or close to the stalled replication fork, which can be repaired using HR, is an option for resolving replication fork arrest. This formation of DSBs can be the result of a collapse of the replication fork or can be caused by specific endonucleases, such as mammalian Mus81-Eme1, which cleaves the DNA at the replication fork (Budzowska and Kanaar, 2009).

Bypassing DNA base damage by replicative TLS polymerases occurs in all life forms (Friedberg et al., 2005). TLS is likely the major bypass mechanism in mammalian cells (Lehmann et al., 2007).
TLS polymerases have a more open structure, which gives them a reduced fidelity compared to the replicative polymerases (Boudsocq et al., 2002). Additionally, TLS polymerases lack 3’-5’ proofreading activity (Goodman, 2002). Due to the low fidelity of the TLS polymerases, it is important that they are quickly replaced with the replicative polymerases to avoid the generation of mutations (Ohashi et al., 2004). The main TLS polymerases in eukaryotes are Pol eta (η), Pol iota (ι), Pol kappa (κ) and REV1, all belonging to the Y-family, along with Pol zeta (ξ), which belongs to the B-family and consists of the REV3 catalytic subunit and the REV7 non-catalytic subunit (Barkley et al., 2007; Lehmann et al., 2007). Other polymerases, such as pol θ, λ, μ, and ν, may have a role in TLS, but this possibility has not yet been confirmed. The error rate of pol δ and pol ε is 1 error per $10^5$ bases synthesised; the proofreading associated with these enzymes improves their fidelity 100-fold (McCulloch and Kunkel, 2008). The error rates for the X- and Y-families of TLS polymerases are approximately 1 per $10^4$ and 1 per $10^5$ bases, respectively (Kunz et al., 2009). Upon stalling of the replication fork after DNA damage, PCNA is mono-ubiquitinated to mediate a polymerase switch from the replicative polymerases to a TLS polymerase (Barkley et al., 2007). Following mono-ubiquitination, the affinities of PCNA for pol η, pol ι, pol κ and REV1 are increased (Lehmann et al., 2007).

TLS proceeds according to a two-step model in which the first polymerase incorporates 1 or 2 nucleotides opposite the lesion, and a second polymerase extends the polymerisation to a position downstream of the lesion where the replicative polymerase with its proofreading mechanism can continue replication (Goodman, 2002). REV1 may regulate the two polymerases for this binding, first binding one, and then releasing this first polymerase to bind the second extension polymerase (Pages and Fuchs, 2002). REV1 a dCMP transferase, not a Pol, and therefore inserts dCMP opposite an abasic site or a G (Nelson et al., 1996a). The requirement of REV1 for error-prone TLS by Pol ζ is not dependent on the dCMP transferase activity of REV1 (Baynton et al., 1999). TLS polymerases can bind to REV1 in a competing manner (Guo et al., 2003). REV1 can physically associate with pol κ and can interact with REV7, Pol ι and pol η (Guo et al., 2003). REV1 may be able to facilitate the exchange between Pol ι and Pol ζ (Barkley et al., 2007). REV1 may also help eliminate inappropriately bound TLS enzymes from Ub-PCNA (Barkley et al., 2007). Pol κ, pol η, pol ι and REV 7 have been found to interact with...
REV1 (Ohashi et al., 2004). REV1 also has a BRCT domain required for binding to PCNA (Guo et al., 2006). REV3 exhibits a high degree of similarity with the catalytic subunit of pol δ (Nelson et al., 1996b). Pol η can bypass cyclobutane pyrimidine dimers with the same accuracy and efficiency as undamaged DNA, but it cannot bypass 6-4 photoproducts by itself (Prakash and Prakash, 2002). Pol ι is not able to extend synthesis from the first base it inserts opposite a damage template (Lehmann et al., 2007).

REV1 and REV3 are recruited to stalled replication forks by their interactions with PCNA and are required for crosslink resistance (Niedzwiedz et al., 2004; Simpson and Sale, 2003). REV1 can incorporate a cytosine opposite the unhooked ICL (Hlavin et al., 2010; Masuda et al., 2003).

Repair of DNA interstrand crosslinks

It has been known for many years that excision repair and recombination pathways are necessary for crosslink repair in bacteria. In bacteria, ICLs are repaired by NER; incisions are made on both sides of the ICLs in one DNA strand, removing one of the damaged templates, thereafter bypass of the remaining lesions is performed by HR (error-free) or TLS (error-prone). Following the repair of the first strand, NER is able to repair the remaining part on the opposite strand (Smeaton et al., 2008). How ICLs are repaired in mammalian cells is still unknown, but it is fully established that NER proteins are involved (Noll et al., 2006). All mammalian repair pathways have been suggested to be involved in ICLs in various studies; the interactions between the different repair pathways for ICL repair are illustrated in figure 7.

ICLs are considered to be a challenge to NER because there is no DNA template for replication; therefore, the repair must be a combination of NER and either TLS or HR (Kuraoka et al., 2000). Furthermore, even though XPE can recognise UVC and cisplatin lesions, it has been demonstrated that it cannot distinguish between undamaged DNA and psoralen-induced monoadduct lesions (Reardon et al., 1993).
Figure 7. Known interaction between pathways suggested in mammalian ICL repair. Fanconi anemia (yellow dots), NER (green), BER (blue), MMR (light blue), HR (red), NHEJ, (dark red). Only selected interactions are shown, based on bioGRID 3.1.
Intrastrand crosslinks and monoadducts are both recognised as bulky DNA adducts (Nouspikel, 2009). In bacteria, NER appears to be the major pathway for removal of psoralen-induced monoadducts (Couve-Privat et al., 2007; Shen et al., 2006). Monoadducts can be found by the XPE complex (DDB1 and DDB2), the XPC complex (XPC and HR23B), or by transcription/replication blockage (Muniandy et al., 2009). Furthermore, psoralen monoadducts have been suggested to be excised by the human DNA glycosylase NEIL1 as in the start of BER (Couve-Privat et al., 2007). Normally, when NER begins the repair of a DNA adduct, the DNA strands are separated around the lesion; in the case of an ICL, this separation cannot occur (Smeaton et al., 2008). Following the unhooking of the ICL in the first round of repair, the accumulation of XPE at ICL sites acts a marker for the initiation of the second round of repair (Muniandy et al., 2009). The accumulation of XPC at ICLs is fast, and the repair of ICLs in G1 is dependent on XPC (Muniandy et al., 2009). XPA-deficient cells are sensitised to UV lesions but not to ICL-inducing agents (Niedernhofer et al., 2006). These data suggest that the full NER complex is not needed for ICL repair.

MMR has also been suggested to be involved in the error-free repair of ICLs in human cells (Wu et al., 2005). MutSβ (MSH2-MSH3), an MMR-factor, has been proposed to recognise ICL adducts in cell extracts with the stimulation of PCNA (Zhang et al., 2002). However, MutSβ is not required for psoralen-induced ICL repair in G1 (Muniandy et al., 2009).

DSBs are normally considered a necessary intermediate during ICL repair, and many studies have found that ICL-inducing agents give rise to DSBs. The formation of DSBs may be an enzymatic process because the accumulation of RAD51 at the site of the ICL precedes the formation of a DSB (Long et al., 2011). If DSBs occur on both sides of ICL adducts, this will immediately remove the ICL (Dronkert and Kanaar, 2001); this DSB would then likely be repaired by NHEJ.

ICL repair has been proposed to perform both in a replication-dependent manner and in the non-replicative stages of the cell cycle (Akkari et al., 2000; McHugh and Sarkar, 2006), but several studies have demonstrated that ICLs are more efficiently repaired in actively transcribed genes than in the rest of the DNA (Islas et al., 1991; Misra and Vos, 1993). Replication appears to play an important role in the repair rate of ICLs (Mladenova and...
Russev, 2006), as indicated by the finding that cells in S phase repair more ICLs than G1 cells.

**Replication-independent ICL repair**

Mammalian cells have a replication-independent method of handling the repair of crosslinking in the G1 and G0 phases of the cell cycle. This pathway is dependent upon the NER proteins XPC and XPA but does not require the involvement of HR like replication-dependent repair does (figure 8) (Collins, 1993; De Silva et al., 2000; Muniandy et al., 2009; Murray and Meyn, 1986; Rothfuss and Grompe, 2004; Wang et al., 2001; Zheng et al., 2003). Both angelicin and psoralen DNA adducts induced by localised laser emission can be repaired in G1 in a NER-dependent pathway (Muniandy et al., 2009). The repair of ICL in G1 is dependent upon XPC (Muniandy et al., 2009; Zheng et al., 2003). Studies in yeast have revealed that mono-ubiquitination of PCNA is required for the recruitment of TLS polymerases to bypass the unhooked ICLs (Li et al., 1999; Sarkar et al., 2006; Shen et al., 2006; Zhang et al., 2002).

**Replication-dependent ICL repair**

**One-fork model**

When the replication fork encounters an ICL, the replication is stalled. This signals FANCM and ATR to begin repair of the ICL (Niedernhofer, 2007), leading to the recruitment of Fanconi-associated Nuclease 1 (FAN1) by activated FANCD2 (MacKay et al., 2010). In addition, the SLX4 ‘super complex’ consisting of SLX4-SLX1, together with MUS81-EME1, and XPF-ERCC1, are recruited to the site of damage (Kim et al., 2011; Munoz et al., 2009). Following dual incisions and unhooking of the ICLs, mono-ubiquitination of PCNA recruits TLS polymerases that can bypass the DNA lesion (figure 9) (Barkley et al., 2007; Shen et al., 2006). A second round of repair aimed at removing the remaining monoadducts can be performed by
Figure 8. A simplified model of the non-replicative model for ICL repair. When an ICL is recognized, dual incisions unhook the ICL from one DNA strand and flips it out. Thereafter TLS polymerases can bypass the unhooked ICL. A second round of NER incisions removes the ICL and now DNA polymerases may fill the gap.
NER (Cole, 1973; Evans et al., 1997; Kuraoka et al., 2000) or by the DNA glycosylase NEIL1 (Bandaru et al., 2002). The fork restoration is likely accomplished by HR followed by NER removing the unhooked lesion.

**Converging-fork model**

Using *Xenopus* egg extracts and plasmids with defined crosslinks, significant knowledge has been gained regarding what processes occur when two replication forks converge at an ICL. To begin, the leading strand of each replication fork arrests 20-24 nucleotides from the crosslink (figure 10) (Raschle et al., 2008). Thereafter, one of the leading strands approaches the ICL up to 1 nucleotide before the lesion. This is then followed by incisions on both sides of the ICL in one of the DNA strands, unhooking the lesion (Raschle et al., 2008). The incision step when two replication forks converge at an ICL has been proposed to be accomplished by several endonucleases, such as XPF-ERCC1 (Bhagwat et al., 2009), MUS81-EME1 (Hanada et al., 2006) and FAN1 (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). Once the ICL is unhooked, TLS polymerases, such as REV1, pol ζ or pol η, may incorporate new nucleotides opposite the unhooked lesion (de Groote et al., 2011; Waters et al., 2009). This is followed by the repair of the remaining adduct, which involves NEIL1 (Bandaru et al., 2002) or NER (Cole, 1973; Evans et al., 1997; Kuraoka et al., 2000) together with HR (Raschle et al., 2008). This pathway of ICL repair requires FA because in the absence of the FA-ID complex, no incorporation of new nucleotides opposite the ICL occurs (Knipscheer et al., 2009).
Figure 9. A simplified model of the replicative model for ICL repair. When an ICL is encountered the replication forks is stalled at the lesion. Either the fork collapse together with once side incision or incisions on both sides of the ICL unhook the lesion. Using TLS polymerases the unhooked ICL is bypassed, thereafter the other DNA strand containing the DSB does strand invasion and also bypass the unhooked ICL. The DSB repair is finalised by ligation and the replication fork is restored. A second round of incisions removes the remaining unhooked ICL and once again DNA polymerases fill in the gap before ligation. Red lines represent newly synthesised DNA.
Figure 10. A simplified model of the converging fork model for ICL repair. When an ICL is encountered, two replication forks stall at the lesion 20-40 nucleotides before the ICL, this is then followed by the approach of the leading strand from one of the replication forks up to 1 nucleotide before the ICL. Dual incisions on the opposite DNA strand unhook the ICL allowing TLS polymerases to bypass the adduct and continue the replication. The DSB is repaired by HR using the unhooked ICL as template for TLS polymerases. A second round of incisions and DNA polymerisation finalize the ICL removal. Red lines represent newly synthesised DNA. Adapted from (Long et al., 2011).
Interstrand crosslink incisions

In bacteria, ICL unhooking is performed by NER, but in mammalian cells, deficiency in different NER enzymes only leads to moderate sensitivity to ICL-inducing agents. Several different enzymes have been proposed to handle the incision step necessary for unhooking ICLs in mammalian cells. One of the main candidates is XPF, which works with ERCC1 to perform the 5‘ incisions of NER. XPF-ERCC1 has an endonuclease activity with specificity for incisions at the junction of single-strand DNA and double-strand DNA similar to the structure of a stalled replication fork (Ciccia et al., 2008; de Laat et al., 1998; Fisher et al., 2008; Raschle et al., 2008; Sijbers et al., 1996). Although XPF-ERCC1 normally makes the 5’ incisions at the junction of single-strand DNA and double-strand DNA, XPF-ERCC1 has been shown to be able to make incisions on both sides of an ICL (Fisher et al., 2008; Kuraoka et al., 2000) and also in linear DNA (Kumaresan et al., 1995; Kumaresan et al., 2002; Kumaresan and Lambert, 2000). Studies of cell lines from XPF patients have demonstrated efficient processing of ICLs concurrent with an inability to repair monoadducts (Zhang et al., 2000). Following treatment with HN2, XPF has been shown to be required for the unhooking of the ICL (Clingen et al., 2007). XPF-ERCC1 may not only play a role in the unhooking of an ICL; it has also been proposed to play a role in HR in mammalian cells, participating in the resolution of Holiday junctions (Bergstralh and Sekelsky, 2008; Yildiz et al., 2002). In cells deficient in XPF-ERCC1, the γH2AX (phosphorylated H2AX) marker for DNA damage persists for a longer amount of time, indicating delayed repair of the lesions (Arora et al., 2010). CHO cells deficient in XPF-ERCC1 are extremely sensitive to cisplatin (Damia et al., 1996; Hoy et al., 1985).

Another candidate that may be responsible for ICL unhooking is FAN1. This protein is recruited to chromatin via its interaction with ubiquitinated FANCD2 and has been shown to have 5’ flap endonuclease activity and 5’-3’ exonuclease activity (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). FAN1 has been suggested to be active in later steps of the ICL repair than the initial incisions, possibly in the DSB resection step (Kratz et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010).

In addition, MUS81-EME1 has been implicated in ICL repair due to its endonuclease activity and observations that deficiency in these proteins leads
to hypersensitivity to crosslinking agents (Dendouga et al., 2005; Hanada et al., 2006; McPherson et al., 2004; Nomura et al., 2007). MUS81-EME1 has been shown to be required for ICL-induced DSB formation (Hanada et al., 2006).

A novel enzyme suggested in ICL unhooking is SLX4 (FANCP), which forms a functional complex with the endonuclease SLX1 and may also act as a scaffold for other endonucleases, such as XPF-ERCC1 and MUS81-EME1, thereby potentiating them (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). XPF-ERCC1, MUS81-EME1 and SLX1-SLX4 can form a ‘super complex’ in cells (figure 11) (Svendsen et al., 2009). It is possible that all of these endonucleases function in ICL repair but may repair specialised types of ICLs. For example, ICLs may or may not be DNA distorting (Deans and West, 2011). SLX4 has also been shown to have several other interacting partners: MSH2-MSH3 (MutSβ), TRF2, RAP1 and PLK1 (Garner and Smogorzewska, 2011). In the G1 phase of the cell cycle, the mismatch repair complex MutSβ can recruit SLX4 to ICLs that cause distortions of the DNA (Fink et al., 1996; Svendsen et al., 2009; Zhao et al., 2009). Cells deficient in SLX4 are hypersensitive to ICL agents, but cells deficient in SLX1 have wild-type sensitivity (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Stoepker et al., 2011; Svendsen et al., 2009). This finding indicates that the main function of the SLX1-SLX4 complex in ICL repair is not as an endonuclease but rather as a scaffold for other proteins. Although SLX4 is thought to coordinate the incisions at ICLs, SLX4-deficient cells accumulate γH2AX foci (Wang et al., 2011), possibly indicating that the cells still incise the crosslink and form DSB and that SLX4 acts late in the repair process.

Finally, the MMR protein MutSβ has been suggested to be involved in ICL unhooking due to the findings that it is necessary for incisions at ICLs induced by psoralen and that it binds psoralen-induced ICLs (Zhang et al., 2002).
Interstrand crosslink bypass by TLS polymerases

When the ICL lesion is unhooked in one of the DNA strands, the ICL can flip outward to facilitate incorporation of new nucleotides past the lesion. REV1 can incorporate a nucleotide opposite a guanine crosslink but cannot continue past the crosslink (Minko et al., 2008). Polζ consists of REV3 and REV7 and appears to be the predominant translesion polymerase responsible for lesion bypass in recombination-independent ICL repair; pol η may play a secondary role in the bypass of these lesions (Shen et al., 2006), continuing the synthesis after REV1.

Pol κ is suggested to perform synthesis past guanine N2 adducts and N2-N2 Guanine ICLs, where it also can progress past the lesion (Lehmann et al., 2007; Minko et al., 2008). Pol η-defective human cells (XPV) are sensitive to cisplatin treatment, suggesting an involvement of pol η in the normal bypass of these lesions (Lehmann et al., 2007).
ICL repair rate

In repair-deficient mammalian cells, 20-40 ICLs are believed to be lethal for cells; ~2500 ICLs have been estimated to be lethal for repair-proficient cells (Akkari et al., 2000; Lawley and Phillips, 1996; Murnane and Byfield, 1981). The estimated repair rates of ICLs in cells vary between 11 ICLs per genome per hour to 16-25 ICLs per Mb per hour (Akkari et al., 2000; Mladenova and Russev, 2006).
The present investigation

Methodology

Alkaline DNA unwinding

The Alkaline DNA Unwinding (ADU) technique was developed to measure single-strand interruptions in cellular DNA. The method is based on the fact that strand separation of DNA in an alkaline solution requires strand breaks (figure 12). A replication fork consists of a pair of DNA single-strand ends from which unwinding in alkali will initiate. Depending on the protocol, the ADU technique can be used to analyse different repair pathways in mammalian cells. Comparing BER- or NER-deficient cell lines to wild type, it is possible to measure their involvement in the repair of different adducts. Furthermore, by using inhibitors for NER (Hydroxyurea and AraC) that inhibit DNA polymerisation or inhibitors for BER (PARP inhibitor 1,8-napthalimide) that inhibit DNA ligation (Loseva et al., 2010), it is possible to measure the accumulation of repair sites for these pathways. Because the papers included in this thesis focussed mainly on the ICL-induced effects on replication fork elongation and the detection of ICLs in genomic DNA, these assays are described in more detail below.

Replication fork elongation assay

The rate of replication fork elongation was measured here as previously described (Johansson et al., 2004). If DNA in the fork is pulse-labelled, it is possible to follow the rate of fork elongation because DNA only unwinds a
fixed distance from the single-strand ends (Figure 13). Thus, replication forks were pulse-labelled with $^3$H-TdR and allowed to progress from the labelled area for different lengths of time. Initially, the labelled region is closed to the fork and, consequently, will become single-stranded after unwinding. If the fork is blocked from elongation, the labelled DNA will stay single-stranded, but with continuous elongation, this will generate increasing amounts of labelled double-stranded DNA (figure 13). Before treatment, $10^5$ cells were seeded in each well and incubated for 24 h. Subsequently, the cells were labelled for 30 min (1 µl $^3$H-TdR/ml DMEM), and the cells were treated with psoralen or angelicin for 5 min at 37°C and 5% CO$_2$ followed by UVA radiation for 11 min (80 kJ/m$^2$, dose rate 122 J/s/m$^2$, at room temperature using a Osram UltraMed 400W as described (Biverstal et al., 2008)). Control cells were treated with 80 kJ UVA if not stated otherwise. After treatment, the cells were washed with HBSS$^{+}$, fresh media were added and the cells were incubated at 37°C. At each time point, the wells were
washed with ice-cold 0.15 M NaCl, and an unwinding solution consisting of 0.5 ml of ice-cold 0.03 M NaOH in 0.15 M NaCl was added to the wells and left for 30 minutes on ice in a dark box. The DNA unwinding was stopped with a forceful injection of 1 ml of 0.02 M NaH₂PO₄, and the DNA was sheared by sonication for 15 seconds. After the addition of 50 μl 7.5% sodium dodecyl sulphate (SDS), the samples were stored in a freezer until elution. Thawed samples were separated into single-stranded and double-stranded DNA fractions by use of hydroxyl apatite chromatography as previously described (Erixon and Ahnstrom, 1979).

Figure 13. A schematic illustration of the replication fork assay. DNA is radioactively pulse-labelled with ³H-TdR for 30 min. The replication fork is allowed to continue progressing away from the labelling. Addition of alkali will start unwinding the DNA from the free ends at the replication fork, and the relative distance the replication fork has progressed from the pulse-labelling can be measured as the amount of labelled DNA that is unwounded. Following neutralisation the DNA is fragmented by sonication and subsequently separated into a double and single stranded fraction by hydroxyl apatite elution and measured for radioactive content.
ICL assay

When DNA in an alkaline solution starts unwinding from strand breaks and encounters an ICL, the unwinding will be stopped, and DNA that otherwise would have been unwound will remain double-stranded (figure 14). This block of DNA unwinding can be used to measure the induction and unhooking of ICLs in genomic DNA in intact mammalian cells.

Cells were seeded in 24-well plates (40 000 cells/well) in F12 media and incubated overnight. Thereafter, the media was exchanged for DMEM containing ³H-TdR (0.1 µl/ml). On day 3, the labelling media was removed, and fresh media were added to the cells for 60 minutes. The cells were then washed with HBSS++ before the addition of 500 µl of HBSS++ with or without furocoumarin. The cells were incubated for 5 minutes prior to UVA activation (80 KJ at room temperature from an Osram UltraMed 400 W in Bahco-armature with 4.5 mm Sekuritglas Heatfilter + Blueglassfilter (type UG1) 2 mm). Following treatment, the cells were washed twice with ice-cold 0.15 M NaCl and placed on ice for gamma irradiation. After gamma irradiation, the cells were once again washed with 0.15 M NaCl. Thereafter, 0.5 ml of ice-cold 0.03 M NaOH (unwinding solution) in 1 M NaCl was added to the wells for 30 minutes in a dark box at room temperature. The DNA unwinding was abruptly stopped with the addition of 1 ml of 0.02 M NaH₂PO₄, and the DNA was cleaved by sonication for 15 seconds. Following sonication, 50 µl 7% SDS was added, and the samples were stored in a freezer until elution.

With the use of hydroxyl apatite chromatography, the thawed samples were separated into single-stranded and double-stranded DNA fractions. Columns containing hydroxyl apatite gel were mounted in a thermo-stated aluminium block (60 +/- 2°C) (obtained from Erixon Scientific Consulting). The columns were first washed with 0.5 M KP to eliminate traces of earlier samples, followed by washing with 0.01 M NaP. The samples where then added to the columns together with 0.01 M NaP and washed once with 0.01 M NaP. The single-stranded DNA was thereafter eluted using 0.1 M KP, and the samples were collected in scintillation vials. The double-stranded DNA was eluted using 0.25 M KP, and the elutions were collected in new scintillation vials. The columns were thereafter washed with 0.5 M
Figure 14. A schematic illustration of the ICL method. (a) Cells only irradiated with gamma), (b) Cells treated with ICLs and irradiated with gamma. DNA is radioactively labelled with $^3$H-TdR, when the DNA is broken by SSB or DSB and alkali is added the DNA will start winding at the breaks. The length of the unwinding can be regulated by temperature, salt concentration and time. If the unwinding encounters a ICL it will be stopped. Following DNA unwinding and neutralisation the DNA is fragmented by sonication and subsequently separated into a double and single stranded fraction by hydroxyl apatite elution and measured for radioactive content. The amount of ICL is in relation to the amount of DNA that is protected from unwinding.
KP and left to set with 0.01 M NaP. Last, 10 ml of Ultima gold XR scintillation cocktail was added to the scintillation vials, and the radioactive decay was measured using a scintillation counter.
DNA interstrand crosslinks induce a potent replication block followed by the formation and repair of double-strand breaks in intact mammalian cells.

Aim
The aim of this study was to differentiate the biological effects and repair pathways of monoadducts and ICLs.

Results and discussion
Using the ADU technique, we found that ICLs induced by psoralen block replication fork elongation, but monoadducts from psoralen and angelicin do not block elongation (figure 1, paper I). We also found that ICL-induced block of the replication fork cannot be bypassed (figure 2b, 2c, paper I), although UVC-induced lesions can be bypassed, leaving a gap at the lesion and continuing replication past the lesion. This was further confirmed when we determined that the overall replication was diminished by psoralen but not by angelicin, indicating that both replication elongation and firing of new origins of replication were inhibited by ICLs (figure 2c, paper I). For the majority of ICL repair models, DSBs are formed as an intermediate repair step. One of the most common makers of DSB is $\gamma$H2AX, and we found that both psoralen and angelicin give rise to increased numbers of $\gamma$H2AX foci-positive cells (>10 foci per cell) (figure 3a, paper I). This would indicate that both psoralen and angelicin result in DSB formation; however, $\gamma$H2AX has also been found to form foci upon areas of single-stranded DNA, as observed at stalled replication forks and during NER. To validate that these $\gamma$H2AX foci were indeed DSBs, we used the marker 53BP1 to indicate ongoing repair of DSBs. When using 53BP1, we observed only foci-positive cells following psoralen treatment, confirming that the $\gamma$H2AX foci noted after angelicin treatment were not DSBs. Furthermore, when measuring the recombination frequency, we found that only psoralen gave rise to an increased recombination rate at this dose. Angelicin-induced monoadducts were 200 times less recombinogenic compared to the psoralen-induced ICLs.
(figure 4a, paper I). In agreement with this, we also determined that psoralen was approximately 300 times more cytotoxic than angelicin (figure 4b, paper I). We also performed a screen for cell lines sensitive to ICLs and monoadducts (figure 5, paper I). Although all tested cell lines were sensitive to monoadducts, only ERCC1, FANCD2/BRCA1 and XRCC2/3 exhibited increased sensitivity toward ICLs. This does not necessarily prove that other proteins are not involved in ICL repair but rather indicates that they may be required for later steps in ICL removal, likely during a second round of incisions, which has little effect on cytotoxicity.

Significant findings in paper I:

- Psoralen blocks replication fork elongation in mammalian cells.
- Replication forks cannot bypass psoralen-induced ICLs.
- Although monoadducts are less potent than ICLs, they need to be taken into consideration for many crosslinking agents.
- Cells deficient in NER are hyper-sensitive to monoadducts, and deficiencies in ERCC1, FANCD1/BRCA2, and XRCC2/3 deficiencies promote hypersensitivities to both ICLs and monoadducts.
Paper II

Quantification and repair of psoralen-induced interstrand crosslinks in mammalian cells.

Aim

The aim of this investigation was to develop a method capable of quantifying the level of ICLs in genomic DNA in intact human cells to study induction and unhooking of ICLs induced by interstrand crosslinking agents.

Results and discussion

To date, most knowledge of ICL repair originates from work performed in cell extracts or on plasmids in cells. To test whether these findings are valid on genomic DNA in intact mammalian cells, we utilised the ADU technique to monitor the formation and removal of ICLs. As shown in figure 3a and 3b in paper II, we were able to induce measurable ICLs from psoralen in both VH10 and HAEB cells. As expected, angelicin did not form any ICLs. We were also able to measure the ICL induction by inducing background strand breaks with two different methods, both using gamma irradiation, creating direct strand breaks evenly distributed all over the genome or using UVC irradiation followed by strand break accumulation with NER inhibitors (Figure 4, paper II). Furthermore, we showed that ICL formation was dependent not only on the concentration of psoralen but also, as expected, on the amount of UVA activation. Using the induction curves for psoralen in VH10 cells and computer simulations predicting the amount of ICLs needed to inhibit DNA unwinding (figure 2, paper II), we were able to quantify the amount of ICLs for each psoralen and UVA dose (figure 5, 6, paper II). We were unable to find any previous quantification of psoralen in the literature against which to compare our data, but both 5-metoxypsoralen and 8-metoxypsoralen had previously been quantified, with various results (table 1, paper II). Therefore, we measured the induction of ICLs for these agents (figure 7, 8, paper II). To test the unhooking capacity in wild type human cells, we treated cells with one dose of psoralen and measured the percentages of remaining lesions at different time points. We found that both VH10 and HAEB exhibited the same unhooking capacity and that both were able to
unhook 50% of the induced lesions within 3 hours of exposure, resulting in an unhooking capacity of approximately 3 000 ICLs per hour (figure 9a, paper II). Unsurprisingly, we observed that VH10 cells were more sensitive to ICLs than were HAEB cells (figure 9b, paper II).

**Significant findings in paper II:**

- We have developed a method for quantification of the induction and removal of ICLs in genomic DNA in intact cells.
- Human cells can unhook up to 3000 ICL per hour, although ~400 ICLs is a lethal dose.
- A significant difference was found when quantifying ICLs by our biological assay compared to chemical analysis.
Paper III

**XPA plays a role in the unhooking and DSB formation steps of ICL repair.**

**Aim**

The aim of this study was to shed more light on the role of NER enzymes in the unhooking step of ICL repair.

**Results and discussion**

NER proteins have been shown to be hypersensitive to ICL-inducing agents and have been proposed to play roles in ICL unhooking and in the second round of ICL repair. Therefore, we used the ADU assay and measured the NER incisions of psoralen and angelicin. We were able to measure a low incision rate for angelicin compared to NER incisions of UVC (figure 1, paper III). Following psoralen treatment, no incisions could be observed, possibly because ICLs block unwinding for NER incisions (figure 1, paper III). To test the involvement of NER in ICL repair, we tested both XPA- and XPE-deficient cells for sensitivity to psoralen and angelicin. We did not observe any enhanced sensitivity for the tested deficiencies towards psoralen (figure 2a, paper III), although XPA-deficient cells had an elevated sensitivity for angelicin-induced monoadducts (figure 2b, paper III). Using the method developed in paper II, we analysed the ICL unhooking efficiency in wild type and XPA- and XPE-deficient cells. Cells deficient in XPA were found to have a reduced unhooking capacity (50% of wild type) (figure 3c, paper III). Although the major deficiency was observed in XPA-deficient cells, no effect was observed on the blocking of replication fork elongation following psoralen treatment in either XPA- or XPE-deficient cells (figure 4, paper III). Monoadducts induced by angelicin were only found to induce blocked replication fork elongation in XPE-deficient cells (figure 4c, paper III). Using 53BP1 as a marker for DSB repair, we observed a significant increase in 53BP1 foci-positive cells (>10 foci per cell) following psoralen treatment in wild type and XPE-deficient cells (figure 5, paper III). In wild type cells,
UVA alone was found to significantly increase the number of 53BP1 foci-positive cells (figure 5a, paper III). In XPE-deficient cells, angelicin gave rise to a significant increase in the number of positive cells (figure 5c, paper III), possibly corresponding to the increased block of replication fork elongation in these cells (figure 4c, paper III). In XPA-deficient cells, no significant increase in DSBs was observed, although increased numbers of DSBs following psoralen and UVA treatment were identified (figure 5b, paper III).

Significant findings in paper III:

- NER is incapable of incision in psoralen-induced ICLs but is able to make incisions of monoadducts.
- XPA-deficient cells have only 50% unhooking capacity compared to wild type cells.
- XPA-deficient cells have an increased background level of DSBs, and ICLs did not lead to any enhancement of DSBs.
- XPE-deficient cells have an enhanced replication fork elongation delay and form DSBs upon monoadduct treatment.
Paper IV

Genotoxicity of alcohol is linked to DNA replication-associated damage and homologous recombination repair.

Aim

The aim of this investigation was to evaluate the mechanisms behind the DNA damage response of alcohol, e.g., how alcohol-related DNA damage response is linked to DNA replication-associated repair of monoadducts or ICLs.

Results and discussion

Rats drinking 10% ethanol for 4 weeks exhibited increased genomic instability, as demonstrated by increased numbers of cells containing micronuclei (figure 1, paper IV). Acetaldehyde, the main metabolite formed by ethanol metabolism, were investigated to understand the mechanism behind the induced genomic instability. Treating cells with acetaldehyde delayed exit from the G2/M phase of the cell cycle (figure 2, paper IV). Acetaldehyde also blocked replication fork elongation in a dose-dependent manner (figure 3a, paper IV). Most cytotoxic agents exhibit unchanged or reduced toxicity when the dose rate is reduced. Here, we observed increased cytotoxicity after the same dose of acetaldehyde treatment for 24 h, 4 h or 1 h (figure 3b, paper IV). Blocked replication may result in a collapse of the replication fork, giving rise to DSB formation. To investigate this possibility, we monitored foci formation of two commonly used markers for DSBs, RAD51 and γH2AX. Both markers indicated increased levels of foci following acetaldehyde treatment (figure 4a, paper IV). Furthermore, cells deficient in HR were hypersensitive to acetaldehyde (figure 4b, paper IV) and acetaldehyde were observed to be recombinogenic (figure 4c, paper IV). The formation of γH2AX did not occur directly after exposure to acetaldehyde but took between 1 and 4 hours to appear (figure 5a, paper IV). This delay in foci formation could be due to the instability of the replication forks. To test this, we added the replication blocker aphidicolin, which specifically inhibits Pol α (Ikegami et al., 1978). Although aphidicolin by itself increased the γH2AX foci formation, it clearly reduced the acetaldehyde-induced γH2AX foci.
(figure 5b, paper IV). 53BP1 accumulates at DSBs as an early step of DSB repair both for HR and non-homologous end-joining, and we observed an increased number of 53BP1-positive cells 4 hours after acetaldehyde exposure. Both these foci and the RAD51 foci co-localised with γH2AX (figure 6, paper IV). Acetaldehyde increased genomic instability, leading to elevated levels of micronuclei and catastrophic mitosis (figure 5d, paper IV) corresponding to the effects observed in rats drinking ethanol (figure 1, paper IV).

Significant findings in paper IV:

- Acetaldehyde blocks replication fork elongation in mammalian cells.
- Acetaldehyde is more cytotoxic at lower dose rate.
- Replication is necessary for acetaldehyde-induced DSBs.
Paper V

The ERCC1/XPF endonuclease is required for completion of homologous recombination at DNA replication forks stalled by inter-strand cross-links.

Aim

The aim of this investigation was to investigate the role of ERCC1/XPF in replication fork elongation when stalled by ICLs.

Results and discussion

HR is a key element of several models of mammalian ICL repair; it has been shown that recombination-deficient cell lines are hypersensitive to crosslinking agents (Caldecott and Jeggo, 1991; Jones et al., 1987). ERCC1, which stabilises XPF, has also been shown to be hypersensitive to crosslinking agents and to have a role in ICL repair distinct from its role in NER (Bergstrahl and Sekelsky, 2008; De Silva et al., 2000; Thompson et al., 1985). The recombination frequencies in cells deficient in RAD51 were 11- to 17-fold decreased compared to wild type (figure 1b, paper V), demonstrating that in mammalian cells, RAD51 is required for HR. We confirmed that cells deficient in ERCC1, BRCA2 or RAD51 are hypersensitive to crosslinking agents (figure 2, paper V), but the degree of sensitivity differs for the different substances, indicating that there is a variation in the pathway being used for the repair. We found that the formation of DSB following mitomycin C treatment was independent of ERCC1/XPF and of XRCC3 (figure 3, paper V). Furthermore, we determined that this DSB formation was replication-dependent (figure 4, paper V). This replication-dependent formation of DSBs indicates that when elongating replication forks encounter mitomycin C-induced crosslinks, the fork is stalled and later collapses into a DSB. To verify this possibility, we measured the replication fork elongation and observed a dose-dependent delay in replication fork elongation (figure 5, paper V). There are two possible ways for ICL repair to be initiated in non-transcribed DNA: NER or replication fork blocking. To distinguish between these two mechanisms, we measured the NER incision rate following treatment with crosslinking agents. We could not observe any increased NER
activity following treatment with either mitomycin C or cisplatin (figure 6, paper V). RAD51 foci formation is independent of ERCC1 because cells deficient in ERCC1 have an equal induction of RAD51 foci following mitomycin C or cisplatin treatment compared to wild type cells (figure 7, paper V), although ERCC1 is necessary for HR completion (figure 8, paper V). To test whether ERCC1-XPF functions in later steps of HR, we used siRNA to deplete XRCC3, ERCC1 or both XRCC3 and ERCC1 (figure 9a, paper V). Depletion of either ERCC1 or XRCC3 promoted elevated sensitivity to cisplatin, but the double knockdown of both ERCC1 and XRCC3 did not exhibit any additional increased sensitivity. This finding indicates that ERCC1-XPF and XRCC3 function in the same pathway (figure 9b, paper V).

**Significant findings in paper V:**

- Mitomycin C blocks replication fork elongation in mammalian cells.
- NER is incapable of incising ICLs.
- Replication is necessary for mitomycin C-induced DSBs.
- Mitomycin C induces DSBs independently of ERCC1/XPF and HR.
- ERCC1/XPF is not necessary for HR initiation but is necessary for HR completion.
- ERCC1 and XRCC3 function in the same ICL repair pathway.
- Different crosslinking agents employ differential uses of repair proteins for lesion repair.
Conclusions and future perspectives

DNA crosslinking agents are commonly used in chemotherapy and are naturally present in fruits and vegetables. Nonetheless, knowledge of the biological effects and repair of their lesions is still unclear in human cells. How ICLs are repaired in bacteria is known, and major progress towards understanding the repair process in human cells has been achieved due to work performed in cell extracts or on plasmids, although this work needs to be verified in intact cells to draw any clear conclusions. Although the agents that induce ICLs can also induce other types of DNA lesions, e.g., monoadducts, ICLs are the more lethal lesion because they block replication and transcription due to hindered strand separation. Therefore, many studies investigating crosslinking agents only consider ICLs as the cause of the response. Using angelicin, which only forms monoadducts when irradiated with UVA, and psoralen, which induces both ICLs and monoadducts, we were able to distinguish the source of the responses upon psoralen treatment to either the monoadduct or the ICL. Although ICLs were more cytotoxic and recombinogenic compared to the monoadduct and blocked the replication fork elongation, the effects from the monoadducts must be considered for crosslinking agents because the monoadducts were also recombinogenic and promoted a strong γH2AX response. Another interesting observation was that ERCC1 was more important for tolerance to monoadducts than for ICLs, and BRCA2 and XRCC2/3 were as important for monoadducts as for ICLs.

To compare the biological effects of any DNA damaging agent, it is necessary to know not only the dose but also the actual number of DNA lesions. We have been able to develop the ADU method to quantitatively measure the induction and unhooking of ICLs on genomic DNA in intact mammalian cells. When comparing different furocoumarins, large variation was observed in the number of induced ICLs at equal doses. Therefore, it would be very interesting to further compare the biological responses to these and
other crosslinking agents and to correlate such data to the actual numbers of induced ICLs.

Crosslinking agents block replication fork elongation by preventing strand separation. Here, we demonstrate that psoralen, mitomycin C and acetaldehyde block replication fork elongation in intact mammalian DNA and that the replication fork cannot bypass these lesions; 6 hours after encountering the damage, the cells remained unable to restore replication.

From studies on cell extracts and in plasmids, several proteins have been proposed to be involved in ICL repair. Therefore, it would be interesting to investigate all of these proteins now that we have a method for quantifying the ICL unhooking rate in genomic DNA in intact human cells. Another approach would be to investigate the repair kinetics of other crosslinking agents and compare these to the psoralen unhooking rate.

Furthermore, by synchronising cells in different stages of the cell cycle, it should be possible to measure the unhooking efficiency of non-replication- and replication-dependent ICL repair.

Cells deficient in XPA but not XPE were found to have an impaired unhooking capacity of psoralen-induced ICLs without changes in lethality. In addition, we observed that human fibroblasts can unhook approximately 2,500 ICL/h, although 400 ICL/cell is lethal to 50% of the cells. This suggests that unhooking may not to be the most critical step in ICL repair, although further studies are needed to confirm this observation.
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