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Processing of Cisplatin Interstrand crosslinks (ICLs) by DNA repair proteins

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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An Abstract of

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Interstrand crosslinks (ICLs) formed by cisplatin are unique lesions formed by the drug. They covalently crosslink both strands of DNA. Cisplatin induced cell death primarily occurs through the formation of various lesions on DNA. Hence, the persistence of the adducts is crucial for its cytotoxicity. DNA repair systems which ensure the integrity of the genome are mainly responsible for the development of resistance to the drug, due to their role in removing the damage on the DNA created by the drug. Repair of ICLs is thought to involve multiple repair pathways. Emerging evidence suggests that there is a replication dependent pathway which depends on stalling of replication forks, and a replication independent mechanism. The exact mechanistic details of cisplatin ICL repair remain poorly understood. Information from studies addressing the repair of ICLs formed by other agents cannot be directly applied to understand cisplatin ICL repair. Each lesion produces unique distortions, and as a result activates different DNA repair pathways. Using cell extracts and purified proteins, we propose a specific pathway for the processing of flanking DNA adjacent to cisplatin ICLs. This mechanism involves the action of two pathways, Base excision repair (BER) and Mismatch repair (MMR) which is activated due to conversion of cytosine adjacent to the cisplatin ICL. We demonstrate
that proteins involved in BER –Uracil DNA glycosylase, Apurinic endonuclease, and DNA Polymerase β directly process this uracil. Finally, we showed that action of these BER proteins leads to the activation of MMR downstream of BER. We propose a novel mechanism in which this common mechanistic pathway acts adjacent to cisplatin ICL, but does not influence repair of damage caused by other crosslinking agents. It influences the overall rate of repair by interfering with the repair processes that act to resolve the crosslink.
I would like to dedicate this work to my father; Mr. Jagannadha Rao and mother Mrs. Krishna Kumari whose constant encouragement, belief and faith have helped me achieve this goal. I would like to dedicate this work to my brother Ravi Shankar.
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List of Symbols

α.................................Alpha
β.................................Beta
γ.................................Gamma
δ.................................Delta
ε .........................Epsilon
κ.................................Kappa
μ .........................Micro
λ.................................Lambda
ε .........................Eta
δ .........................Zeta
Chapter 1

Lature Review

1.1 Introduction

Platinum based chemotherapy has been in use in the clinics for decades, and it is one of the most successful treatment protocols for a wide variety of cancers [1]. Cis-di-ammine di-chloro platinum (Cisplatin), discovered serendipitously, was the first generation platinum based drug that paved the way for the development of second and third generation platinum drugs carboplatin and oxaliplatin [2]. Cisplatin is a highly efficient treatment strategy for testicular cancer, with cure rates approaching a staggering 90% [3]. Various studies have provided significant insights into the mechanisms of the action of the drug, and the responses elicited by the cancer cells upon administration of the drug have largely remained enigmatic [4]. Significant progress has been made in understanding the mode of action of the drug. The clinical efficacy of the drug is mainly hampered by the development of resistance [5]. The development of resistance to the drug is multi factorial with entry of the drug across the cell membrane, interaction of the drug with various cellular proteins and removal of the adducts on DNA by various DNA repair pathways, all contributing to the reduced efficacy of the drug [6]. Understanding the precise mechanisms by which cisplatin adducts are formed on the DNA and the various strategies employed by the cancer cells to remove the lesions have important implications for the design of new agents which will evade these processes and overcome
resistance to the drug. DNA is considered to be the primary target of cisplatin [7]. Interstrand crosslinks (ICLs) are covalent lesions formed by the drug on both strands of DNA. In addition to ICLs, cisplatin forms adducts on the same strand of DNA which include both mono adducts and intra strand adducts [8]. Even though ICLs comprise a minor fraction of the total adducts formed by the drug, they are believed to be extremely important for the drug's cytotoxicity. ICLs block the fundamental cellular processes of DNA replication and transcription [9]. It is imperative to understand the mechanism by which these adducts pose blocks to cellular processes and how various DNA repair proteins act on the crosslinks and remove them. **The main objective of my work is to characterize the underlying mechanism by which proteins involved in Base Excision Repair (BER) and Mismatch repair (MMR) process the flanking DNA adjacent to cisplatin interstrand crosslinks.**

**Figure 1: Cisplatin and next generation Platinum analogues**

This figure is modified from Katherine S Lovejoy et al, September 2011 10(9) 1709-1719 Molecular Cancer therapeutics
I will summarize the overall organizational scheme of my thesis. In Chapter 1, I will briefly summarize the current state of knowledge on the mode of action of the drug and current treatment protocols where cisplatin is effectively used. The major emphasis of Lature survey will be on formation of interstrand crosslinks and various mechanisms cells use to repair them. I will highlight the existing Lature with respect to what is known about the molecular details of the proteins involved in BER and MMR and their connections to cisplatin chemotherapy. At the end of the section, I will direct my attention on the details which led us to formulate a hypothesis which suggests an overlapping role for BER and MMR proteins in a common mechanistic pathway for influencing the repair of cisplatin ICLs. In Chapter 2, I will describe all the experimental approaches employed in my work to address the important questions that I pose at the end of the Lature survey section. Most of the studies I conducted are biochemical in nature. In Chapter 3, I will present the data obtained which strengthened each step of our hypothesis. In chapter 4, I will provide a discussion which focuses on interpretation of the results followed by interesting questions that my work has raised. I will end my thesis with Chapter 5, which summarizes my work and discuss its significance.

1.2 Mechanism for the entry of the drug and its reaction with DNA

The most important step required for the action of cisplatin is its entry across the cellular membrane [10]. The efficiency of drug entry depends on the dose at which it is administered. Higher concentration of administration leads to higher percentage of the drug which gets taken up [11]. But a critical parameter that needs to be considered upon increasing the dose of the drug is higher toxicity to non cancer cells which leads to
plethora of side effects like neurotoxicity, nephrotoxicity etc. Cisplatin contains two ammines and two chlorine atoms in a cis configuration surrounding the central platinum atom. When administered intravenously, cisplatin circulates through the blood stream before making an entry into the cell membrane of cancer cells [11]. The positive charge of cisplatin is neutralized in the blood stream as a result of higher concentration of chlorine ions in the plasma (nearly 100 mM). The lipid bilayer membranes of cells pose an obstacle to charged molecules. Neutral molecular species are easily transported across the membrane by passive diffusion. It is widely accepted that cisplatin traverses across the cell membrane by passive diffusion. Once cisplatin crosses the membrane, it undergoes a series of aquation reactions due to the drastic reduction in the intra cellular concentration of chlorine (nearly 4 mM). This aquation reaction results in the replacement of chloride atoms by water molecules [6]. This aquated form of cisplatin is very effective in its reaction with various cellular molecules like lipids, proteins, RNA and DNA. DNA is considered to be the primary biological target of activated cisplatin. The details of the interaction of the drug with its targets, mechanism for the passage of the drug across the nucleus remain enigmatic due to the lack of techniques and approaches to address these issues. The passage of cisplatin across the membrane and its subsequent conversion to aquated form prevents escape of the drug from inside of the cell [1]. In addition to passive diffusion as a mode of entry into the cells, some membrane transporters have been shown to have a role in the uptake of cisplatin. Genetic screens conducted in resistant yeast cells have pointed to a role of copper transporter CTR1 as a major protein for uptake of cisplatin [12]. A recent study found that in a mouse model of cervical cancer, pretreatment with a copper chelator before administration of cisplatin
led to better response and survival of the mice [13]. This study, examining the relative expression of the major copper transporter CTR1 across a panel of human ovarian cancer cell lines, found that tumors which have higher expression of CTR1 displayed better response to platinum based chemotherapy. Other studies implicate the role of organic cationic transporters OCT1 in the uptake of the drug [14]. Apart from these studies indicating a role for the transporters in the influx of the drug, members of the ATP binding cassette family (ABC) transporters have been shown to contribute to the efflux of the drug [15]. These studies suggest that in addition to passive diffusion, cisplatin traverses the cell membrane by active transporter mechanisms. Further work needs to be done to elucidate the molecular mechanisms of transporter based entry of cisplatin. Once cisplatin enters the cells and it becomes aquated, it passes through the nuclear membrane and interacts with DNA due to the high nuclear concentration of nucleotides [1]. The reaction of aquated cisplatin with DNA is time dependent. Loss of one water from cisplatin facilitates the interaction with the N\textsuperscript{7} atom of a guanine or adenine resulting in the formation of a monoadduct with a half-life of nearly 10 min. It takes nearly 2 hr to form bifunctional adduct (either intra or ICL) which is a result of the loss of second water molecule. There is also a possibility that aquated cisplatin reacts with S-donor ligands such as glutathione and methionine resulting in inactivation of the drug [1].

1.3 Cancer treatment with cisplatin and development of resistance to the drug:

Cisplatin based chemotherapy is used in the treatment of a wide variety of cancers including lung, ovarian, testicular, head and neck and bladder cancer [16]. Testicular cancers respond particularly well to cisplatin based chemotherapy regimen. Ovarian cancers respond well initially, but eventually develop resistance to the drug [17]. The
cancers that have become refractory to cisplatin treatment also develop cross resistance to other chemotherapeutic drugs such as etoposide resulting in the poor prognosis and higher mortality rate. The development of resistance is a significant barrier to the efficacy of the drug. Hence, it is highly imperative to understand the mechanisms by which cancer cells develop resistance to the drug. Another challenge is to identify patients who are most likely to benefit from platinum based chemotherapy. The central challenge in understanding drug resistance lies in defining the molecular mechanisms adopted by cancer cells to survive upon treatment with the drug. Several mechanisms of resistance have been postulated to have a role in decreasing the efficacy of the drug. The mechanisms include decreased accumulation of the drug, increased efflux of the drug which is mediated by binding of the drug to certain cellular proteins like glutathione and decreased expression of membrane transporters like CTR1 [1]. These events will undoubtedly effect the initial accumulation of cisplatin in the cells upon intravenous administration. Once cisplatin enters the cells the major target of the drug is DNA. The reaction of cisplatin with DNA results in the formation of distorting lesions on DNA. The formation of these adducts on DNA is a significant step because this event culminates in the generation of a multitude of cellular responses which impact the overall cytotoxicity of the drug [18]. The cellular responses elicited upon treatment with cisplatin are typical of what is seen with agents that damage DNA like mitomycin C and UV damage suggesting a similarity in mode of action of the drug. Evidence in this direction came from the studies of DNA repair deficient cells which display a sensitive phenotype to cisplatin treatment. Apart from the data obtained in a vast number of repair deficient cell culture models [5], a recent study was conducted in a direction to identify the most
important mechanism for the development of resistance in a preclinical setting [19]. In KRas G12D conditional knockin mice which recapitulates human disease in the presence of an allele conferring a knockdown of p53, treatment with cisplatin resulted in an increase in DNA repair leading to enhanced removal of the adducts on DNA which turned out to be the predominant mechanism for the development of resistance to cisplatin. This study highlighted the central importance of DNA repair in the development of resistance to the drug in an \textit{in vivo} setting as opposed to cell culture. Increased DNA repair resulting in increased removal of the adducts from DNA is the earliest mechanism activated upon treatment with the drug and is the main pathway effecting the cytotoxicity of cisplatin. Important evidence in support of this notion is the observation of a strong correlation between the levels of platinum DNA adducts and cellular cisplatin cytotoxicity [20].

\subsection*{1.4 Structures of adducts formed by cisplatin}
Cisplatin DNA adducts cause significant distortion to the double helical structure of DNA. The degree of distortion depends on the nature of the adduct. Upon undergoing the aquation reaction, chloride is lost. The amine persists with water molecules coordinating around the central platinum atom. The reaction of cisplatin with DNA gives rise to two main classes of adducts. Since there are two coordinating water molecules the adducts are mainly bifunctional in nature. The intrastrand adducts result from the reaction of the drug with N\textsuperscript{7} atoms of adjoining adenine and guanine or a guanine and guanine. The 1,2 d(GpG) adduct is the predominant adduct formed by the drug and it accounts for nearly 65\% of the total adducts formed by the drug on DNA. 1,2 d(ApG) is the second most abundant adduct with nearly 20-25\% occurrence rate [1]. A minor percentage of intra
adducts are formed between two guanines separated by a different DNA base. Another important lesion formed by cisplatin is the interstrand crosslink, which is highly sequence dependent and forms between guanines at 5'GpC sites on the opposite strands of DNA. The excessive formation of intra adducts over the ICLs has attracted wider interest in understanding the mechanism of the repair of intra adducts and has led to the general belief that intra strand adducts are the determining lesions for cytotoxicity of cisplatin. This notion is further strengthened by the evidence that the clinically ineffective isoform of cisplatin, transplatin is unable to form 1, 2 intra adducts. The importance of ICLs in the cytotoxicity of ICLs cannot be undermined owing to consideration that interstrand crosslinking agents like mitomycin C and nitrogen mustards are effectively used for cancer chemotherapy [9]. I will discuss the importance of ICLs in platinum chemotherapy in subsequent sections.

The structure of all the adducts formed by cisplatin have been solved both by X-ray crystallography and NMR [21]. The main feature of these adducts is the distortion of the double helical structure of DNA. The 1, 2 intra adducts bend the DNA towards the major groove, causing a great degree of widening and unwinding of the duplex [22]. In contrast, the cisplatin ICL bends the DNA in the direction of the minor groove. These differences in distortion which are the result of the differential coordination of the N7 atom of guanines, seemingly affect the overall damage recognition and the rates of repair by various DNA repair proteins. In addition to structural distortion, the nature of the leaving group influences the differential recognition and repair as observed in the case of Oxaliplatin [23], a second generation platinum analogue with a different leaving group (diamminecyclo hexane as opposed to chlorine for cisplatin). Oxaliplatin is highly
effective in the treatment of cancers that have developed resistance to cisplatin indicating the central importance of the nature of distortion on the cytotoxicity of the drugs. These adducts are strong blocks to the replication and transcription processes [24]. By the use of several DNA repair mechanisms that guard the integrity of the genome [25], cancer cells employ strategies to restart stalled replication forks as well as remove the lesions present on transcribed strands for their survival.

1.5 Repair of cisplatin intrastrand adducts by NER

NER is the predominant mechanism for the repair of bulky adducts caused on the DNA by agents like UV light, as well as bulky lesions formed by many DNA damaging chemotherapeutic agents [26]. It has an infinitely large substrate range, and can sometimes act as a backup repair pathway for other kinds of damages [27, 28]. Some of the proteins involved in NER have roles other than the basic NER process. NER reaction process occurs at rapid rates in physiological conditions when bulky lesions are formed on DNA. The main feature of NER is recognition of the distortion in the double helical structure of DNA followed by an increase in local concentration of proteins involved in the process by thermodynamic proofreading and cooperativity [29]. The steps of NER reaction are the recognition of damage, followed by the unwinding of the helix around the damage, excision on the damage containing strand both 5' and 3' side by structure specific endonucleases which results in the release of a strand containing 24-30 nucleotides and subsequent repair synthesis and ligation. Two subpathways of NER have been implicated which primarily differ in the damage recognition step [30]. The transcription coupled NER operates primarily on the transcribed strands and utilizes RNA polymerase II as the damage sensor. Stalling of RNA polymerase II results in recruitment
of Cockayne syndrome proteins (CSA and CSB) prior to the recruitment of downstream NER factors [31]. The Global genome NER pathway scans lesions throughout the genome utilizing XPC-HHR23B as the damage recognition protein. After initial damage recognition, both pathways operate with a core set of common NER proteins. The NER reaction has been reconstituted in vitro using purified proteins [32]. The proteins involved in NER are XPC-hHR23B, XPA, RPA, TFIIH, XPG and XPF-ERCC1. As shown in figure 2, XPA and RPA have roles in the damage recognition step of the process and they function cooperatively to increase the local concentration of these proteins and facilitate the subsequent downstream process of opening of the helix and incision. The helix is unwound by the action of XPB and XPD helicases (part of the TFIIH complex) followed by the recruitment of XPG and XPF-ERCC1 which make nicks on the 3’ and 5’ end, respectively. NER reaction has important disease significance with a specific disorder known as xeroderma pigmentosum. This disease is characterized by the extreme sensitivity to sunlight, which basically stems from the deficiency in the repair of damage caused by UV light [30]. Evidence for the role of NER in cisplatin cytotoxicity, comes from the observation that all the XP mutants ranging from XPA to XPF are highly sensitive to cisplatin. The remarkable response observed in testicular cancer following cisplatin treatment is generally believed to be due to the lower expression of NER proteins XPA and XPF-ERCC1 [33].
Additionally, ovarian cancers that have developed resistance to cisplatin have increased expression of the endonuclease XPF-ERCC1 [30]. Considering the nature of the bulky distortion produced by the cisplatin intra adducts, NER is the primary pathway for their removal.

1.5 Repair of Interstrand crosslinks

Interstrand crosslinks are covalent lesions formed on both strands of DNA [34]. They produce unique distortions on the double helical structure of DNA due to the contact made with both strands. This feature is responsible for their higher level of toxicity because many fundamental cellular processes such as DNA replication and transcription...
depend on the strand separation of DNA [35]. Many environmental and endogenous agents also form ICLs. The common use of ICL forming agents is in cancer chemotherapy where agents like nitrogen mustards, psoralen and mitomycin C are used. In addition to these standard chemotherapeutic agents, ICLs are sometimes generated as byproducts of lipid peroxidation and reactions of nitric oxide providing an evolutionary basis for the development of mechanisms for repairing ICLs [36]. The importance of ICL toxicity is further illustrated by the observation that it takes just a single ICL to kill repair deficient bacterial cells and nearly 40 ICLs to kill repair deficient mammalian cells [37].

The chemotherapeutic agents such as cisplatin and oxaliplatin form primarily intra adducts, but the toxicity resulting from minor percentage of ICLs has not been evaluated thoroughly. Repair of ICLs occurs at all phases of the cell cycle. The stalling of replication forks which occurs when cells enter S phase is believed to be a major activator of the repair process of ICLs [38]. When replication machinery stalls at the ICL, the replication fork stalls and activates a damage response, resulting in the recruitment of various proteins involved in DNA repair. As shown in **Figure 3** proteins involved in several DNA repair pathways are implicated in the repair of ICLs. Some of these include proteins of NER [36], MMR [39, 40], Homologous recombination (HR) [41], and Fanconi Anemia (FA) [42] and Translesion synthesis [43]. These observations suggest that a wide network of proteins participate in a coordinated fashion to remove the ICLs. Some proteins have roles in multiple DNA repair pathways making it difficult to conclusively ascribe a role for the involvement of one repair pathway acting on the ICL. Several genetic and biochemical studies carried out in *E. coli* and *S. Cerevisiae* have significantly contributed to an increased understanding of the common mechanistic pathways that are most likely
to act in the repair of ICLs in human cells. The nature of the unique distortion created by each drug, the sequence context surrounding the adduct and the phase of the cell cycle have an impact in the array of proteins that recognize ICLs and the rate at which they are repaired. A unifying mechanistic theme for the repair of all kinds of ICLs formed by drugs has clearly not been found. Numerous studies carried out using various cell systems and different ICL substrates will advance our understanding of differential aspects of ICL repair. In the sections that follow, I will describe the important proteins of each DNA repair pathway and what is known about them with respect to ICL repair. Then, I will center my attention on two DNA repair pathways, BER and MMR, and their role in cisplatin cytotoxicity. I will conclude the chapter, with a discussion of the hypothesis pointing to an overlapping role of BER and MMR in mediating cisplatin cytotoxicity, which is dependent on a unique structural distortion created by cisplatin ICLs.
1.6 Role of NER in ICL repair: Lessons from Bacterial and Human cell systems

One of the earliest studies in understanding the role of NER in ICL repair was performed by Cole and co-workers, in an effort to understand the incision of a substrate containing an ICL by the *E.coli* NER system [44]. The molecular players involved in this process are UVrA which is involved in damage recognition along with UVrB, followed by UVrB and
UVrC which act as endonucleases cleaving on 3' and 5' of the damaged strand. The length of the *E.coli* NER oligonucleotide differs from that of the one generated during mammalian NER systems which is approximately 12 nucleotides [45]. The early studies using *Ecoli* NER generated significant insights on the mechanism of action of NER. The main conclusion was that the NER reaction generates a nick on both sides of the crosslink resulting in unhooking of the ICL. This reaction provides an opportunity for homologous recombination which is primarily catalysed by RecA protein in *E.coli*. The topological density of the DNA around the crosslink has an effect on the efficiency of the incision reaction mediated by the UVrABC NER system. The knowledge obtained by the *E.coli* NER system has prompted future studies to address the role of mammalian NER in ICL repair. All the XP mutant cell lines are sensitive to ICL forming agents suggesting a role for NER in ICL repair [46]. The most profound phenotype was observed with cell lines defective in XPF-ERCC1 indicating a major role for this endonuclease in ICL repair [47]. The hypersensitive phenotype observed in XPF cell lines as compared with other XP cell lines also suggest an additional function of this complex in ICL repair apart from its role in NER reaction [48]. The sensitivity displayed by all the XP cell lines raises the possibility that NER has a definitive role in ICL repair with a much more prominent role for XPF-ERCC1. Two models of NER have been proposed. The first model proposes that majority of the ICLs are actively repaired during S-phase upon stalling of the replication forks when replicative DNA polymerases encounter ICLs. In addition to the replication dependent ICL repair model, some ICLs are repaired exclusively during the G0/G1 phase of the cell cycle when ICLs cause transcriptional inhibition by arresting RNA polymerase II[49]. The common theme of all these models is the participation of NER in some stage
of ICL repair regardless of the phase of the cell cycle. Studies were performed to assess
the relative contribution of each protein of NER in mediating ICL repair. Using comet
assay to measure the removal of ICLs at single cell resolution, Hartley and co-workers
observed that NER mutants are sensitive to cisplatin; with XPF cells being most sensitive
[50]. Studies conducted in our laboratory suggest that the single stranded binding protein
RPA recognized and bound to a synthetic oligonucleotide containing a single cisplatin
ICL with higher affinity compared to substrates containing cisplatin intra adduct and
undamaged DNA [51]. Because of the multiple roles for RPA during several cellular
processes involving DNA, it is difficult to ascertain whether the observed affinity is
primarily due to its role in NER repair of cisplatin ICL. A study conducted to assess the
repair of ICLs caused by nitrogen mustards, found that XPA cells are as sensitive to
nitrogen mustard treatment as XPF cells suggesting that the relative roles of NER factors
in the repair of ICL depends on the drug used for the treatment and the nature of the
distortion produced by the drug [52]. In addition to cell based studies, several
biochemical experiments were conducted to monitor the excision of NER on substrates
containing either psoralen ICL [53-55] or a specific chemically induced crosslinks such
as those generated by N^4C-ethyl-N^4C and observed incision surrounding the crosslink
indicative of a role for NER endonucleases. The subsequent steps after initial NER
reaction which might result in cleavage of the crosslink are not assessed completely.
Using plasmids containing a site specific cisplatin ICL fused to a luciferase construct
[56], a host cell reactivation study was conducted recently and the main observation from
the study is that the repair of cisplatin ICLs is mediated by the transcriptional coupled
arm of the NER reaction with contributions from CSB, XPA, XPB and XPF. When this
plasmid containing cisplatin ICL was transfected into cells deficient in XPC, no
difference in the expression of luciferase was observed compared to wild type cells
complemented with XPC suggesting that cisplatin ICLs are not repaired via global
genomic NER. This study highlighted the importance of the NER reaction in cisplatin
ICL repair outside of S phase. The main summary of this section is to suggest that NER
has a definite role in the repair of ICLs including cisplatin ICLs, but the precise
mechanism of the steps in the processing of ICLs and the relative roles of each protein of
NER in ICL processing and repair remains to be elucidated.

1.7 Bypass of ICLs by translesion polymerases:
Replication of the genome involves several enzymatic activities that require DNA
polymerases for synthesis. The repertoire of proteins that carry out DNA replication is
increasing. At present, there are nearly 15 DNA polymerases in human cells [57]. This
raises an interesting question of why cells need so many DNA polymerases. Elegant work
from the lab of Thomas Kunkel has conclusively ascribed DNA polymerase δ and ε as
the major replicative polymerases for leading and lagging strand synthesis [58]. These
replicative polymerases have high fidelity and also have 3'-5' exonuclease activity
making them efficient enzymes to carry out DNA replication. A major obstacle for DNA
replication using these enzymes is that dividing cells accumulate certain amount of
sporadic damage during every round of DNA replication. The replicative polymerases
lack the ability to incorporate nucleotides past the damage and they stall whenever they
encounter a damage site. To deal with this problem, specific translesion polymerases
belonging to the Y family of DNA polymerases have evolved to carry out strand
synthesis past the damage [59]. These polymerases accommodate nucleotides past the
damage but have reduced fidelity leading to increased mutagenesis. Translesion synthesis involves the following steps: 1) Stalling of the replicative DNA polymerase at the site of the lesion; 2) recruitment of the translesion polymerase; and 3) incorporation and extension of nucleotides past the site of the damage [60]. The last step involves the dissociation of the translesion DNA polymerase and replacement with the replicative DNA polymerase. The monoubiquitination of PCNA, the clamp that encircles the DNA and increases the processivity of the replicative DNA polymerase acts as a molecular switch which increases the relative concentration of specialized translesion DNA polymerases at the site of the lesion and dissociation of the DNA replicative polymerase [61]. The translesion polymerases which were identified in human cells include the B-family polymerase Polδ, a heterodimer consisting of Rev3 and Rev7, and other Y-family polymerase which include Polε, Polη, Polκ and Rev1 [62]. The specific polymerase recruited at the site of the lesion depends on parameters such as the nature of the damage and the relative concentration of the specialized polymerase present at the site of the damage. Biochemical studies using cisplatin adducts demonstrate that all the translesion polymerases including DNA polymerases ι, κ and ε can bypass cisplatin GG and GXG intrastrand adducts leading to adduct tolerance [63, 64]. Tolerance of the adducts, provides an opportunity for the dividing cancer cells to cope with DNA damage and carry out replication leading to the development of drug resistance [62]. Two studies conducted in mice models of lung cancer and lymphoma demonstrated that, downregulation of Polδ led to dramatic reduction in tumor burden, better response to cisplatin chemotherapy and a significant increase in the survival [65, 66]. These studies highlighted the importance of translesion synthesis in adduct tolerance and drug resistance to cisplatin treatment. These
studies suggest that administration of a small molecule inhibitor of DNA polymerase δ along with cisplatin chemotherapy as a potential option for the treatment of cancers. A recent study also found that ICLs formed by both cisplatin and Nitrogen mustards can be efficiently bypassed by a number of translesion polymerases including ε, ι, δ and κ [67]. The efficiency of bypass varied depending on the polymerase, the degree of distortion induced by the ICL (cisplatin ICL induces significant distortion as opposed to Nitrogen mustard ICL) and the double stranded region surrounding the crosslink. This report is the first demonstration that even cisplatin ICL can be bypassed by translesion polymerases. Functional studies are needed in cell lines deficient in these polymerases to monitor the rate of repair of the ICLs due to the increased tolerance of the crosslinks.

1.8 Fanconi Anemia pathway and its role in ICL repair

Fanconi anemia (FA) is a pathway characterized by extreme sensitivity to crosslinking agents [68]. This disease is manifested in the clinic with severe hematological and congenital abnormalities. Mutations in nearly 13 different genes have been shown to have a role in this pathway. All the 13 gene products primarily function in a common core pathway with important roles in DNA damage signaling. The proteins involved in the FA pathway are FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, and FANCN [69]. The main event in the pathway is the monoubiquitination of FANCD2 and FANCI which occurs upon damage incurred on the DNA. Monoubiquitination is caused by the FA core complex consisting of the upstream proteins FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM which possess ubiquitin ligase activity [70]. As shown in figure 4, FA pathway is involved in various steps of processing of ICLs. Apart from their roles in
crosslink repair, FA pathway has also been shown to have roles in homologous recombination (HR) as well as non homologous end joining (NHEJ) pathways. Proteins involved in DNA damage singnaling ATR, ATM and CHK1 also have been shown to interact with core proteins of the FA pathway [42] highlighting the connection between FA and DNA damage signaling pathways. The extreme sensitivity of the mutations in FA proteins to any crosslinking agents illustrated the central importance of this pathway. As shown in figure 4 monoubiquitination of FANCD2 serves an important role in the activation of the pathway.

Figure 4

Schematic displaying all the Proteins involved in FA pathway

Monoubiquitination of FANCD2

This figure is modified and adapted from Younghoon Kee and Alan D. D’Andrea Genes & Dev. 2010. 24. 1680-1694
1.9 BER and its role in cisplatin sensitivity

The inherent instability of the DNA poses a threat to the integrity of the genome. There are several potential endogenous threats that can damage DNA [71]. One possible mechanism by which DNA bases are damaged is through hydrolysis. In addition to spontaneous hydrolysis, reactive oxygen species (ROS) produced as byproducts of cellular metabolism cause significant damage to DNA. These stresses cause damage to the DNA by modifying the bases in DNA resulting in base damages like oxidation and alkylation. BER is a specialized repair pathway that has evolved to deal with any base damage incurred on the DNA. Nearly $10^4$ base damages occur per cell daily in the human body. The vast number of the base damages highlights the paramount importance of the BER pathway [72]. Hydrolytic deamination of cytosines results in the generation of uracils. Nearly 70-200 uracils are formed per cell daily. Even though, the number of uracils produced due to the deamination reaction appear small compared to the number of base damages generated, they are extremely mutagenic because the DNA polymerases cannot distinguish between thymine and uracil in a template. This lack of discrimination results in G: C to A: T transition mutations in the context of G: U mispairs. In addition to hydrolytic deamination, hydrolytic depurination reactions occur at nearly 10,000 lesions per cell per day [71]. In addition to endogenous sources of damage, chemotherapeutic agents like temozolomide produce base damages. As shown in figure 5 the general mechanism of the BER reaction includes 1) recognition of the damaged nucleotide by a specific DNA glycosylase; 2) removal of the damaged base by cleaving the glycosidic bond between the base and the phosphodiester backbone and formation of an abasic site, 3) cleavage at the abasic site; 4) generation of a single nucleotide gap, synthesis and
BER reaction culminates in the removal of the damaged base [73]. In this section, I will focus on our current understanding of various proteins involved in BER in separate subsections for each protein, followed by what has been known about the involvement of BER in cisplatin sensitivity.

**Figure 5: Schematic showing the BER pathway**

This figure is modified from Hegde ML, Hazra TK and Mitra S Cell Research 2008 Jan 18(1) 27-47

**Deamination of cytosine and formation of uracil**

Rate of conversion of cytosine to uracil has been gauged by various biochemical as well as genetic methods. The current estimate of deamination events per cell per day is nearly 70-200 [73]. Another interesting feature observed in the case of deamination reactions is that the relative rates are higher for single stranded DNA compared to double stranded duplex DNA. Single stranded DNA is generated during every cell cycle, especially in S-
phase and during transcription. Every process generating single stranded DNA increases
the probability of deamination of cytosine [74]. Exogenous agents such as gamma-
radiation and elevated temperatures also increase the frequency of spontaneous
deamination events. Recently, members of the APOBEC/ AID family of cytidine
deaminases which are involved in class switch recombination in B-cells, have been
demonstrated to carry out protein induced cytosine deamination resulting in the formation
of uracil in DNA [75].

**DNA glycosylases**

DNA glycosylases catalyse the cleavage of the glycosidic bond between the damaged
base and the backbone of DNA [76]. Fifteen different glycosylases have been discovered
and characterized extensively. Each glycosylase has unique substrate specificity and
reaction mechanisms. The glycosylases recognize and remove the damaged base. Certain
glycosylases remove not only the damaged bases, but also conduct cleavage at the abasic
site by using AP lyase activity. The glycosylases which carry out the removal of uracil,
alkyl bases and mismatched bases lack the AP lyase activity. Uracil DNA glycosylase
(UNG) is the glycosylase which removes uracil from the deamination site. Three other
DNA glycosylases catalyse the remove of uracil: single stranded mono functional DNA
glycosylase (SMUG1), T/U mismatch DNA glycosylase (TDG) and methyl CpG binding
glycosylase (MBD4) [77].

**Uracil DNA glycosylase (UNG):** UNG is a highly conserved protein across all species
and is considered to be the primary glycosylase responsible for removing uracil. It
actively removes uracils from both single stranded as well as duplex DNA [78]. But it
exhibits a slightly higher preference for uracil on single-stranded DNA over duplex
DNA. It has a high turnover rate and has been shown to directly interact with proliferating cell nuclear antigen (PCNA) linking it directly to DNA replication [79]. The robust activity of UNG makes it a highly suitable candidate for removing uracils which occur in genome through oxidative deamination or misincorporation of dUMP by replication polymerases. SMUG1 is considered to be an efficient backup enzyme for most of the uracils generated in the genome. The expression of all glycosylases is cell cycle regulated and the sequence surrounding the target uracil has an impact on the selection of a specific glycosylase [76]. The mechanism by which SMUG1 makes contact and interacts with uracil is different from UNG, suggesting a differential catalytic rate of uracil removal mediated by these two glycosylases.

**Single stranded mono functional DNA glycosylase (SMUG1):** SMUG1 has initially been characterized to have a role in removing uracils on single-stranded DNA. However, some studies have also detected uracil removal activity for SMUG1 on double stranded DNA. Studies conducted with mouse cells which were depleted of both UNG and SMUG1 have indicated that both have non-redundant functions in the cells. The mechanism by which UNG and SMUG1 coordinate with other steps of BER is also distinct[80]. SMUG1 has been proposed to have function in non-proliferating cells and it binds tightly to apurinic sites after uracil removal and inhibits cleavage by APE1.

**T/U mismatch DNA glycosylase (TDG):** Eventhough the name suggests a preference for T/U mismatches, TDG exhibits higher preference for uracil as opposed to thymine [81]. It exhibits a similarity in the mode of action in which it binds tightly to apurinic sites after removal of the damaged base. The catalytic activity of TDG appears to be
stimulated by APE1. TDG appears to have an important role outside of S phase where UNG has robust activity.

**DNA glycosylase MBD4:** MBD4 exhibits high selectivity in removing uracils which are present in 5’ CpG context [82]. It has a high preference for binding to methylated DNA. MBD 4 also has been shown to interact with MLH1 suggesting a role in mismatch repair. However, the importance of MBD4 in removing uracils when UNG is expressed at high levels is not completely understood. Apart from this uracil specific glycosylases, there are numerous other glycosylases which recognize distinct base damages. MYH is a mismatch specific DNA glycosylase which recognizes A from A: 8-oxoguanine and A: G mispairs. Oxidized bases are removed primarily by bifunctional glycosylases which include OGG, NTH1 and NEIL family of glycosylases. The AP lyase activity of these bifunctional glycosylases cleave at the abasic site resulting in formation of 3’ blocking groups which are further processed by other enzymes [72].

**Repair of abasic sites**

The action of glycosylase generates abasic sites which need to be processed further by subsequent downstream members of the pathway. Apurinic Endonuclease 1 (APE1) cleaves at an abasic site resulting in the formation of a gap with 3’OH and 5’dRP residue. The process of APE1 recruitment is tightly regulated with sequential handoff from monofunctional glycosylases. Bifunctional glycosylases bypass the requirement for APE1 cleavage. Downregulation of APE1 triggered apoptosis in several cancer cell lines [83]. Also, APE1 has been demonstrated to have distinct roles in the transcriptional regulation of important transcription factors like C-Jun and p53 [84]. These studies have established two independent roles for APE1, as an endonuclease cleaving the abasic sites.
generated due to glycosylase mediated removal or spontaneous formation and its function as transcriptional regulator. APE1 has also been shown to regulate the expression of PTEN providing some explanation for the apoptosis in cancer cells [85]. The multifaceted role of APE1 may provide an explanation for the embryonic lethality observed in APE1 null mice [86]. We are far from understanding the vast number of interacting proteins of APE1 as well as the multitude of posttranslational modifications which might impact the selection for one or the other function. The best documented role of APE1 is in BER, which is crucial for protection against damage incurred on the DNA bases [72].

**DNA Polymerase β: Short patch and Long patch BER**

The result of cleavage of the abasic site by APE1 is a single nucleotide gap, which has 3'OH and 5' dRP residue. The dRP residue is processed to make the gap amenable to synthesis by a DNA polymerase. DNA polymerase β (Polβ) carries out synthesis across short gaps of 3-6 nucleotides [87]. It has a molecular weight of 39KDa and has a domain which can carry out AP lyase function. It lacks 3'-5' exonuclease activity and other endonuclease activities. Considering the small size of the enzyme, as well as its strong conservation across all species, it provided a useful model system to study the functions of polymerases. Its polymerase activity is contained in the 31 KDa polymerase domain. Due to its ability to carry out single nucleotide incorporation, several studies have ascertained the fundamental importance of Polβ in BER. Crystallographic structures of Polβ have been obtained, and provide molecular insights into the mechanism of nucleotide insertion [88]. The relative efficiency of incorporation of Polβ is dependent on various parameters including the sequence context, the nature of the distortion produced
by the adduct and the length of nucleotides that need to be incorporated. Polβ null mice display embryonic lethal phenotype demonstrating the importance of the enzyme in participating in BER during development [89]. Low fidelity of Polβ has been mainly determined to stem from its lower efficiency in incorporating correct nucleotides rather than incorrect nucleotides. Polβ null fibroblasts display hypersensitivity to alkylating and oxidative damaging agents demonstrating the importance of the enzyme in repairing the damage caused by those agents [90]. Nearly 30% of all human cancer display imbalances in the expression of Polβ [91]. The lyase activity Polβ efficiently catalyzes the removal of 5’dRP residues, followed by single nucleotide incorporation. This pathway of dRP removal by Polβ is termed short patch BER [92]. If the lyase activity is inhibited, the 31 KDa polymerase domain carries, out strand displacement synthesis resulting in the generation of a flap containing 5-6 nucleotides. The flap is removed by a downstream endonuclease called Flap endonuclease 1 (FEN1); Polβ incorporates the missing nucleotides. This pathway is known as long patch BER [93]. Polβ has roles in both sub pathways of BER indicating its central importance in BER. Some studies indicated that BER also proceeds without the involvement of Polβ, using backup polymerases like DNA polymerase λ or the replicative polymerases [94]. Our increase in knowledge of BER illuminated the redundancy present in repairing the vast amount of endogenous base damage created every day. There are uncertainties regarding the choice of each enzyme, the coordination between different proteins in the pathway and the backup functions provided by other repair pathways like NER confounding the interpretation of the results in various cell systems and different kinds of damages. Once, Polβ carries out synthesis in the gaps, the nicks are sealed by the action of DNA ligase I or the complex of DNA
ligase III and XRCC1. The BER reaction is generally accepted to proceed through sequential handoff with the product of one step becoming a substrate for the next step in a tightly coordinated process [95].

**BER and connection to cisplatin sensitivity**

Conflicting results have been obtained concerning the role of BER in cisplatin sensitivity. A small molecule inhibitor of Polβ sensitized cisplatin resistant ovarian cancer cells indicating a possible role for the involvement of Polβ in the repair of cisplatin adducts [96]. However, Polβ null cells displayed a cisplatin resistant phenotype [97]. Biochemical studies using purified Polβ showed that the enzyme carries out translesion synthesis past cisplatin GG adducts [98]. The profile of incorporation is different from that of the oxaliplatin GG adduct suggesting that it can lead to adduct tolerance and help maintain the sensitivity of the cells [98]. The misincorporation efficiency is higher for cisplatin GG adduct compared to oxaliplatin GG adduct resulting in an increased mutation spectrum which might impact drug resistance. A study conducted to assess the sensitivity of Polβ to oxaliplatin in human colorectal cancer cell lines displayed hypersensitivity indicating a role for Polβ in the repair of oxaliplatin adducts [99]. The differential bypass properties and incorporation properties of Polβ coupled with distinct phenotypes in Polβ deficient cells to cisplatin and oxaliplatin suggest that we still need to gain a better understanding of the role of BER in impacting the repair of different structural distortions created by each drug. Further studies are clearly needed to define the precise molecular mechanism of BER in cisplatin cytotoxicity.
1.10 MMR and cisplatin cytotoxicity:

MMR is a post replicative repair process whose primary function is dealing with mismatches arising during DNA replication. The human MMR system was reconstituted in vitro by using purified proteins [100, 101]. The components of the system include, MutSα or MutSβ, MutLα, Exonuclease 1(EXO1), Replication factor C (RFC), Proliferating cell nuclear antigen (PCNA), Replication protein A (RPA), polymerase δ and DNA ligase I. Biochemical studies using plasmids containing a mismatch indicated that presence of a nick on the newly synthesized strand present as much as 1kb away from the mismatch serves as strand discrimination signal and directs mismatch repair apparatus [102, 103]. The mismatch repair is bidirectional, operating when the nick is present on the 5’ or 3’ side of the mismatch [103]. As shown in figure 6 the broad steps of mismatch repair include damage recognition of the mismatch which is carried out primarily by the protein MutSα. If 2-3 base pair insertion/deletion loops are produced, those are bound by MutSβ. Binding of MutSα to a mismatch converts the protein into a sliding clamp which brings an exchange of ADP for ATP. MutLα is recruited to the site forming a ternary complex between MutSα, MutLα and DNA [104]. This complex translocates along the DNA until it encounters the PCNA loaded by RFC on the 3’ nick [105]. If the nick is present on the 3’ side of the mismatch, the latent cryptic endonucleolytic activity of MutLα is activated, generating additional nicks surrounding the mismatch [106]. The degradation step of the mismatch repair strand includes loading of EXO1 which degrades up to 150 nucleotides past the mismatch generating a large gap which is resynthesized by DNA polymerase δ and sealed by DNA ligase I. MMR proteins
have diverse roles in cells including triplet nucleotide expansion, antibody maturation and DNA damage signaling [102].

Figure 6: Schematic showing the MMR pathway

Basic steps of Mismatch Repair

(a) Mismatch recognition

Presence of a nick directs Mismatch damage recognition Proteins to the newly synthesized strand

(b) Recruitment of MLH1–PMS2

(c) Excision, resynthesis and ligation

This figure is adapted from Wei K, Kucherlapati R and Edelmann W Trends in Molecular Medicine 2002 July 8(7) 346-53

MMR proteins are involved in a crosstalk with proteins of several pathways including those involved in interstrand crosslink repair like FA proteins, and proteins involved in
DNA replication and DNA damage signaling [107]. Loss of MMR proteins has shown to result in resistance to several other DNA damaging agents like temozolomide, MNNG and methyl methane sulfonate (MMS) [108]. Two different models have been proposed to explain the phenotype observed with these damaging agents. The first model posits that damage recognition proteins of MMR, MutSα and MutSβ make repeated attempts to remove the damage [109]. These futile attempts eventually lead to apoptosis. The second model posits that the MMR damage recognition proteins act as damage sensors and recruit signal transducers of DNA damage response ATR-ATRIP resulting in ATR mediated checkpoint signaling [110]. Another recently uncovered aspect of MMR involves interaction of MutSα with chromatin assembly factor (CAF1), which participates in nucleosome loading and remodeling [111].

The most widely accepted notion in the involvement of MMR in pathophysiology of human disease, is the realization of the fact that nearly 30% of people with germline mutations in MMR proteins develop hereditary non polyposis colon cancer (HNPCC) [112]. This disease is characterized by an extremely high mutation spectrum in satellite DNA sequences. Recent studies indicated that in S. cerevisiae, MutSα is coupled with the replisome where as the downstream MutLα is not coupled to replication indicating additional layers of complexity in the coordination of MMR proteins during mismatch repair [113]. The connection between the MMR pathway and cisplatin cytotoxicity is well studied[114]. Cancer cells deficient in MMR are resistant to treatment with cisplatin [115]. Biochemical evidence supports a model in which MMR damage recognition protein hMSH2-hMSH6 (MutSα) recognizes and binds to cisplatin intra adducts with higher affinity compared to undamaged DNA [109]. The study proposes a model in which the
MMR pathway acts by making futile attempts to remove the damage but fails in this process. The repeated attempts by MMR result in lethal strand breaks and cells die due to this overwhelming production of breaks. DNA damage signaling aspect of MMR which leads to release of cytochrome c into the cytosol and activation of caspase 3 and caspase 9 have also been proposed to explain the cisplatin resistant phenotype upon MMR deficiency [115]. MSH2 is overexpressed in some tumors which are responsive to cisplatin including testicular and ovarian cancers. Alternative MMR protein binding may shield the adduct from productive DNA repair mechanisms. Even though, several models have been proposed to explain MMR resistance to cisplatin none of the models have not been conclusive [116]. A recent study found that MutSβ recognizes cisplatin ICLs [117]. MMR proteins cooperate with NER proteins in recognizing psoralen ICLs indicating that they have a role in processing crosslinks [40]. Participation of MMR protein in cisplatin repair by adduct binding (both intra adduct and ICL) or a damage signaling role have both been invoked to explain the resistance phenotype. An explanation that will clearly define the actual role of MMR in cisplatin resistance is lacking and attempts need to be made to understand more about the molecular mechanism for cisplatin resistance.

1.11 Main objective:

The crystal structure and solution structure of cisplatin demonstrates significant distortion in the double helical structure. The formation of cisplatin ICL is sequence specific and it is formed at the 5'GpC sites. An interesting feature displayed by cisplatin ICL is, the cytosines adjacent to crosslinked guanines are forced out from the double helix resulting in exposure of the flipped out bases to cellular environment. The DNA is unwound by 70-80° and bent towards the minor groove due to the presence of the crosslink. Cisplatin ICL
produces significant distortion of double helical DNA compared to other crosslinking agents such as nitrogen mustards and Mitomycin C. The flipped out nature of cytosines is unique to cisplatin ICL alone. This flipped out nature of cytosine made us speculate about the possibility of deamination events occurring on the cytosines adjacent to crosslinked guanines. Because mismatched bases are more prone to oxidative deamination in cells, we hypothesized that cytosines get converted to uracils and this conversion event might direct BER at this site. As described in the earlier section, at least 4 different glycosylases can remove uracils that occur in DNA. If highly coordinated BER acts at the site, glycosylase mediated uracil removal will lead to the generation of an abasic site which will be cleaved by APE1, followed by subsequent gap filling by Pol β. Considering the low fidelity nature of Pol β, if misincorporation occurs at that site, it will result in the generation of a mismatch and subsequent activation of MMR repair pathway. Cells deficient in BER and MMR display resistance to cisplatin [118]. BER and MMR pathways act in the vicinity of the crosslink by targeting the uracil, which is formed as a consequence of the flipped out cytosines. The experimental data from our laboratory indicated that cells deficient in Ung and Pol β displayed resistance to cisplatin, and this resistance was not observed with other crosslinking agents like mitomycin C and oxaliplatin [118]. Moreover, cisplatin treatment led to rapid induction of abasic sites in these cells and not observed to the same extent with other crosslinking agents further supporting our hypothesis. This resistance phenotype observed upon BER and MMR downregulation is only specific to cisplatin and not seen with other damaging agents. As seen in figure 7, we speculate that futile processing of uracil by both BER and downstream MMR pathways and the physical presence of the proteins involved in these
pathways at the ICL site may hamper the effectiveness of the crosslink repair resulting in persistent crosslinks. Based on this hypothesis, the questions which I wished to answer in my thesis are:

1) What is the mechanism by which proteins involved in BER act in removing the uracil adjacent to crosslink? Will the presence of the crosslink influence the choice of a specific protein over the other protein in the BER pathway?

2) Can MMR proteins recognize mismatches due to the low fidelity of DNA polymerase β adjacent to cisplatin ICL?
Figure 7

Cisplatin ICL

Extra helical cytosine

Deamination

Spontaneous or Protein Induced?

Uracil

Recruitment of Uracil DNA glycosylase

Which Glycosylase?

UDG? SMUG1? MBD 4? TDG?

Removal of uracil

How does abasic site gets cleaved?

Is APE1 required?

APE1 recruitment and incision at abasic site

Polβ recruitment and extension

Incorporation of the correct nucleotide

BER Processing Short patch/Long Patch

Incorporation of the correct nucleotide

Is misincorporation by Pol β required for MMR activation?

Activation of Mismatch Repair

Correct Repair?

Mutagenic Repair?

Persistent crosslinks and Cisplatin Sensitivity

Polβ misincorporation

Persistent crosslinks and Cisplatin Sensitivity

Is misincorporation by Pol β required for MMR activation?
Chapter 2

Materials and Methods

2.1 Materials

Oligonucleotides used in these studies were from IDT DNA technologies. Cisplatin was obtained from Sigma-Aldrich. Radiolabelled nucleotides [α-\(^{32}\)P] dATP, [α-\(^{32}\)P] dCTP, [α-\(^{32}\)P] dTTP and [γ-\(^{32}\)P] ATP were from MP Biomedicals. DNA Polymerase β (specific activity 5u/ul) was from chimerx. Sequenase, used for 3’ labelling of nucleotides was purchased from USB. T4 Poly nucleotide kinase, Uracil DNA glycosylase from *E.Coli* (UDG) 2000U/ml, and human Apurinic Endonuclease (APE1) 10,000 U/ml, Uracil Glycosylase inhibitor (UGI) 2000U/ml and all restriction endonucleases were from New England Biolabs. Chromatorgraphy resins used for protein purification were from standard commercial suppliers. Supplies for electrophoresis were from Biorad. Other chemicals and reagents were obtained from various commercial sources. Methoxyamine hydrochloride, an inhibitor of APE1 cleavage reaction was from Sigma. Streptavidin beads used for biotin pull down experiments were from Dynal Biotech. The magnetic separation strand was from Promega.
2.2 Preparation of oligonucleotides containing site specific cisplatin adducts:

Oligonucleotides with cisplatin adducts were designed using a protocol optimized to generate single site specific defined lesions [119]. The oligonucleotides were either left unprocessed to create an undamaged substrate or treated with cisplatin to contain a single site-specific cisplatin intrastrand (GG) adduct or a cisplatin interstrand crosslink (ICL). The oligonucleotide containing a Hae III restriction site was used for the preparation of a GG intrastrand adduct. The recognition site for Hae III is 5' GGCC 3'. The presence of cisplatin adduct makes the substrate refractory to cleavage by the enzyme to ensure that the final product is fully platinated and is the oligonucleotide containing the 1, 2 GG adduct. The oligo substrate used for the preparation of the ICL substrate was designed to contain a single G in the top strand to ensure the formation of a mono adduct. The length of oligonucleotides used for different experiments varied according to the design of the experiment. Platination reactions were carried out in 10mM sodium phosphate (pH 7.5) and 30 mM NaCl at a drug to nucleotide ratio of 5:1 for GG adduct and 15:1 for ICL substrates in the dark for 12-14 hr to ensure the adduct formation. Unreacted platinum was removed from the preparation by passing the reaction mixture through a tip 20 column (Qiagen). The substrates from the column were ethanol precipitated and annealed to the respective complementary oligonucleotides to generate duplex substrates containing site specific lesions. The GG adduct duplex substrate is further restriction digested with HaeIII and loaded on a native gel to separate the GG adduct duplex from unannealed and unplatinated mixture. For the preparation of ICL substrate, the annealed duplex was dialysed overnight at 37⁰ C in 100mM sodium perchlorate and 10mM Tris-HCl (pH 7.5) to increase the efficiency of the crosslink. The substrate was recovered from dialysis,
ethanol precipitated and loaded onto a sequencing gel. The presence of the crosslink retards the separation of the duplex oligo in denaturing conditions and runs as a smeared product on the gel. This smeared band is excised from the gel, DNA is eluted and the eluate is ethanol precipitated to obtain the defined cisplatin ICL substrate. Several variations of the substrates which can be obtained from changing the sequence around the central G as well as different end labelling protocols were used throughout the studies. The variations, if any will be discussed during the explanation of the results for each experiment. The following is a table to indicate the list of various substrates and the sequences used during the course of my studies. All the substrates used were annealed to the complementary oligonucleotides to generate duplexes.

**Table I: DNA oligonucleotides used in our studies**

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>(5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>42 GG</td>
<td>CTCTTCCCCCCTCTCCTTCCTTGGCCCTTTCCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 ICL</td>
<td>CTCTTCCCCCCTCTCCTTCCTTGGCCCTTTCCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 ICL</td>
<td>CTCTTCCCCCCTCTCCTTCCTTGG/CCTCTTTCCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 ICL</td>
<td>CTCTTCCCCCCTCTCCTTCTTG/abasic site/CCTCTTCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 G/T</td>
<td>CTCTTCCCCATCTCCTTTCCGTCCTTTCCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 ICL</td>
<td>CTCTTCCCCATCTCCTTTCCCGCCTTTCCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 ICL G/T</td>
<td>CTCTTCCCCATCTCCTTTCCGTCTCTTTCCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 ICL U (MMR experiment)</td>
<td>CTCTTCCCCATCTCCTTTGUGCCTCTCCCTCCCTCCCT</td>
</tr>
<tr>
<td>42 ICL U (BER experiments)</td>
<td>CTCTTCCCCCCTCTCCTTTGUGCCTCTCCCTCCCTCCCT</td>
</tr>
</tbody>
</table>
2.3 Purification of wild type, D256A (polymerase dead) and K72A (lyase dead) versions of DNA Pol β

Bacterial glycerol stocks of wild type and mutants carrying mutations in the polymerase and lyase domain of DNA Pol β were kindly provided by Dr. Samuel Wilson NIEHS. Dr. Wilson also provided a detailed protocol for the purification of Pol β [120]. Briefly, *E.coli* expressing Pol β were grown by inoculating frozen glycerol stock into terrific broth containing ampicillin (100 ug/ml). Cells from 2.5 L cultures were used for the purification of all 3 proteins. Bacterial cells were lysed and harvested in 0.5 M NaCl. Protease inhibitors (pepstatin, leupeptin, PMSF and benzamidine) were added to the buffers used for the purification. Using a combination of DEAE cellulose, phosphocellulose, SS DNA cellulose and Mono S FPLC columns, Pol β was purified and the aliquots were stored at -80°C. The mutants were created to have mutations in the active site with no effect on the DNA binding. Hence, we were able to employ the same purification scheme for the purification of all the mutants. We were able to obtain highly purified preparations of these enzymes in milligram quantities.

2.4 Activity assays for DNA Pol β

Activity assays were carried out as described [92]. Primer extension assays were conducted in a reaction containing 50mM Tris-HCl (pH 7.4), 100mM KCl, and 10mM MnCl₂. Poly dA₄₂ annealed to dT₁₆ was used as a template. The reaction contained 200 nM DNA substrate, 3.2 nM DNA Pol β and 15 μM dTTP in a reaction volume of 10 μl. The reaction was quenched by the addition of sequencing dye containing formamide and heated to 95°C and loaded onto a 15% sequencing gel. The gel was dried and exposed to X-ray film (Fuji film) for autoradiography.
2.5 Deamination assay for the conversion of cytosine to uracil:

This assay was performed according to the protocol described in the EZ methylation kit from Zymo Research. Briefly, substrates (100fmol) each were treated with sodium bisulfite (CT conversion reagent). Following treatment, desulphonation was conducted and DNA samples were loaded onto Zymo-spin IC columns. DNA bound to the columns was eluted with high salt buffer. The eluted DNA substrates were treated with UDG for 10 min at 37°C at an amount of 2 U of the enzyme. The DNA substrate was treated with 10 U of APE1 for 30 min at 37°C. The reaction was stopped by the addition of the sequencing dye containing formamide, loaded onto a 12% sequencing gel, dried and exposed to X-ray. The substrates used in this experiment were labeled at the 3’ end by using sequenase.

2.6 Preparation of HeLa whole cell extracts:

HeLa whole cell extracts were prepared by using a protocol developed in the laboratory of Richard Wood that typically gives 10-15mg/ml protein. HeLa cell pellet from 1 L of exponentially growing cells was incubated in 4 ml of hypotonic condition in 10mM Tris-HCl (pH 8.0), 1mM EDTA, 5mM DTT, 0.5mM PMSF, 1 µg/ml each of leupeptin and pepstatin. Cell pellets were incubated in the hypotonic buffer for 20 min at 0°C. The cells were homogenized with a Dounce homogenizer in 50mM Tris-HCl (pH 8.0), 10mM MgCl₂, 2mM DTT, and 25% sucrose; 50% glycerol was added to the homogenized cell pellet mixture. One ml of 4.1M saturated ammonium sulfate solution was added and the solution was stirred for 30 min. The solution was transferred to polycarbonate tubes (Beckman) and was centrifuged at 42000 rpm for 3 hr. The supernatant was collected and a 0.33g/ml ammonium sulfate was added. The pellet was resuspended in 25mM HEPES-
NaOH (pH 7.9), 0.1 M KCl, 12mM MgCl₂, 1mM EDTA, 2mM DTT and 17% glycerol and dialysed overnight in the same buffer. Buffer was exchanged three times. After dialysis, insoluble material was removed by centrifugation at 5000 rpm for 10 min. The concentration of the extract was measured by Bradford assay and the solution was aliquoted and frozen at -80°C.

2.7 Preparation of whole cell extracts from mouse embryonic fibroblasts and breast cancer cell line MDA MB 231:

Whole cell extracts were prepared from the following cell lines using the procedure described below: Mouse embryonic fibroblasts (MEFs), Polβ knock out MEFs, Polβ knock out MEFs complemented with cDNA encoding D256A (polymerase dead version of Polβ), Polβ knock out MEFs complemented with cDNA encoding K72A (lyase dead version of Polβ). These MEFs were kindly provided by Dr. Samuel Wilson, NIEHS. Extracts from human breast cancer cell line MDA MB 231 were also prepared. Human cell lines for extract preparation were wildtype MDA MB 231 cells, Polβ knock down cells, and Polβ knock down cells complemented with cDNA encoding D256A mutation. The cell pellets were resuspended in 3X the volume of the pellet in 10mM Tris pH8.0, 120mM NaCl, 0.5% NP-40, and 1mM EDTA in the presence of the protease inhibitors and kept on ice for 30 min vortexing every 10 min. The pellets were centrifuged at 10,000 rpm for 15-20 min. The supernatant was used to determine protein concentration by Bradford assay.

2.8 In vitro glycosylase assay with HeLa whole cell extracts:

The whole cell extracts prepared by the above stated protocol were used for all the experiments. All the reactions were conducted by incubation of the samples at 37°C
The reactions were carried out in 50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA and 10% (v/v) glycerol. DNA substrates containing either uracil or uracil adjacent to cisplatin ICL substrates were incubated with HeLa whole cell extracts in the glycosylase reaction buffer for the indicated times. Samples were processed by the addition of NaOH to 0.2M and heated at 70°C for 15 min to cleave the abasic site during the glycosylase reaction. The reactions were quenched by adding sequencing dye containing formamide and were heated for 5 min at 95°C and resolved on a 15% sequencing gel. The gels were dried and exposed to X-ray film for autoradiography. For UDG inhibition, UGI was preincubated with HeLa extract before the addition of DNA substrate and the reactions were carried out as described above. Whole cell extracts from MEFs as well as MDA MB 231 were used for certain glycosylase experiments.

2.9 APE1 inhibition assay with methoxyamine:

APE1 inhibition assay was conducted using the same reaction conditions as the glycosylase assay. HeLa whole cell extracts were incubated with methoxyamine at 200 mM and were used for the APE1 inhibition experiments [122]. DNA substrates containing a uracil or a uracil adjacent to cisplatin ICL were incubated with HeLa whole cell extract preincubated with methoxyamine for 60 min at 37°C. Samples were heated at 70°C for 15 min and loaded onto a 15% sequencing gel, dried and exposed to X-ray film for autoradiography. Whole cell extracts from MEFs and MDAMB231 were used for some glycosylase experiments.
2.10 Primer extension assays using wild type, D256A (polymerase dead) and K72A (lyase dead) versions of DNA Pol β:

DNA oligos containing a uracil adjacent to cisplatin ICL were treated with UDG (2 U) and APE1 (2 U) to remove the uracil and subsequent cleavage generating a primer and template product. The primer extension reaction product needs to be incorporated from the site of uracil on the DNA substrate. After UDG and APE1 treatment, the oligos in the reaction mixture were obtained by treatment with proteinase K which digests UDG and APE1. DNA was ethanol precipitated and was used for primer extension reactions. Primer extension reactions were performed as described in 10 µl of 50mM Tris- HCl (pH8.0), 10mM MgCl₂, 2mM DTT, 20mM NaCl, 0.2 µg BSA, 2.5% glycerol and 0.28mM each dNTP. The reactions contained 10 nM DNA, and 15 fmol of wild type and mutant versions of Polβ.

2.11 In vitro BER assay conducted with whole cell extracts:

BER reactions were reconstituted in vitro in 50mM Tris-HCl (pH7.5), 100mM NaCl, 5mM MgCl₂, 4mM ATP and 25 µM each dNTP. DNA substrates containing uracil adjacent to cisplatin ICL were used for the reconstitution reaction. The reaction was carried out with 10nM of the substrate, incubated in the reaction buffer with whole cell extract from various cell lines as described in the results for 1 hr at 37⁰C. The reaction was quenched by the addition of sequencing dye containing formamide and EDTA and loaded onto a 18% sequencing gel, dried and exposed to X-ray film for autoradiography.

2.12 Purification of mismatch damage recognition protein MutSα:

300 ml of SF9 cells were infected with recombinant baculovirus containing hMSH2-hMSH6 at MOI of 5:1. 3 days after the infection, the cells were harvested and the cell
pellet with modification to the scheme described in a previous report [123]. Briefly, the insect cell pellet was resuspended in 3X packed cell volume of lysis buffer containing 25mM HEPES-NaOH (pH 8.0, 1mM EDTA, 1mM benzamidine, 2mM mercaptoethanol, 0.5mM spermidine and 0.1mM spermine. Buffers used in the purification procedure contained protease inhibitors. The cell suspension was freeze thawed thrice in a dry ice ethanol bath and water bath at 37°C. Afterwards, the solution was passed through a 18½ gauge syringe with filter and dounce homogenized 15-20 times. The solution was collected and poured into a 50ml glass beaker, and was spun at low speed (< 300 rpm). Saturated ammonium sulfate solution (11ml/100ml) and one packed cell volume of glycerol was added to the solution. The solution was allowed to stand at 4°C for 30 min. Then, it was transferred to beckman polycarbonate tubes and was centrifuged at 60,000 rpm for 90 min in an ultracentrifuge. The supernatant was collected and dialysed against 25 mM HEPES (pH7.5), 1mM DTT, 0.1mM EDTA, 200 mM NaCl and 20% glycerol. It was washed twice before leaving it overnight for the third time. The dialysed extract was clarified by centrifugation at 10,000 rpm for 10 min and the supernatant was collected. The concentration of the NaCl was brought down from 200 mM to 150 mM. It was loaded onto a phosphocellulose column equilibrated at 150mM NaCl. The column was washed with 200mM NaCl and the protein was eluted with 1M NaCl. The fractions were assayed and peak fractions were pooled and loaded onto a 5 ml PBE 94 column at 250mM NaCl. The protein was eluted from PBE 94 with 800mM NaCl and the fractions were collected, assayed and pooled. The eluate was loaded onto 2 ml heparin column equilibrated with 250mM NaCl and the bound MSH2-MSH6 protein was eluted at 800mM NaCl. The salt concentration was brought down to 150mM NaCl and loaded
onto a mono S column and a linear gradient of 150mM NaCl-500mM NaCl was utilized to purify MSH2-MSH6. The buffer used for the indicated purification is the same as the dialysis buffer with the salt concentration varied as indicated. The fractions containing pure protein were pooled and dialyzed against storage buffer 25mM HEPES (pH 7.5), 1mM DTT, 0.1mM EDTA, 200mM NaCl and 20% glycerol. The concentration of the purified protein was measured using Bradford assay and aliquots were frozen at -80°C.

2.13 Biotin-DNA pull downs to assess the binding of MSH2-MSH6 to cisplatin damaged substrates:

Biotinylated duplex DNAs either undamaged or containing a single mismatch, a single cisplatin ICL or a cisplatin ICL with a mismatch were synthesized using the protocol as described previously. The substrates were bound to streptavidin magnetic beads (Dynabeads, Dynal Biotech) in 20 mM HEPES, pH 7.8, 2 mM DTT, 0.001% NP-40, 100 mM NaCl and 2mM MgCl₂ at 4 °C for 30 min. The beads were washed 3 times with binding buffer to remove unbound DNA substrates. 5 µg hMSH2-hMSH6 baculovirus infected SF-9 insect cell extract was added with 30 fold excess of poly dI-dC competitor DNA. The tubes were rotated for 1 hr at 4 °C. Tubes were kept in a magnetic separation stand (Promega). The tubes were then washed 3 times in wash buffer (binding buffer with 200 mM NaCl) followed by elution using 1M NaCl. The tubes were left to rotate for 30 min and placed in a magnetic separation strand and the supernatants were collected and TCA precipitated. The pellets were resuspended in binding buffer and applied to an 8% SDS gel, transferred to PVDF membrane and probed with MSH2 (Calbiochem) antibody. A similar protocol was followed for the cisplatin ICL substrate containing a uracil adjacent to the crosslink except that it was treated with UDG, APE1 and Polβ in the
presence of all the dNTPs. This BER processed DNA generates a substrate with either a
correct base or incorrect base adjacent to the cisplatin ICL due to the low fidelity nature
of pol β. This cisplatin ICL DNA substrate was subsequently recovered by ethanol
precipitation and used for the biotin pull downs.

2.14 Electrophoretic mobility shift (EMSA) analysis of MSH2/6 binding synthetic
oligonucleotides

100 fmol of 42 mer radiolabelled DNA substrates (undamaged, undamaged containing a
mismatch, a single cisplatin ICL, a single cisplatin ICL with a mismatch) were used for
EMSA experiments. These substrates were labeled at the 5’ terminus with $\gamma^{32}\text{P}$ ATP
using T4 polynucleotide kinase. The mobility shifts were conducted according to a
published report [89]. DNA substrates were incubated in the presence of binding buffer
(25mM HEPES pH 7.8, 5mM MgCl$_2$, 80 mM KCl, 1mM EDTA, 1mM DTT, 10%
glycerol, 1 mg/ml BSA) with 100 nM purified MSH2-MSH6 complex at room
temperature for 30 min with poly dI-dC competitor DNA (20 fold excess). Reactions
were resolved on a 4% polyacrylamide gel at 4 °C, dried and exposed with X-ray film for
autoradiography.
Chapter 3

Results

3.1 Monitoring the conversion of cytosine to uracil by utilizing sodium bisulfite:

Our central hypothesis is that BER participates in the processing of cisplatin ICL due to the conversion of cytosine to uracil by deamination. We were limited by the lack of approaches to establish that the cytosine adjacent to the crosslink is deaminated due to structural distortion of the ICL. To address this issue, we constructed three synthetic duplex substrates using protocols described in the materials and methods section. We also constructed 42 nucleotide duplex undamaged substrates and cisplatin GG intra adduct substrates. Our rationale for this experiment is to monitor the relative efficiency of conversion of cytosine adjacent to the crosslink with those cytosines which exist in perfectly duplex confirmation as seen in undamaged DNA substrate, as well as cytosines which will not be flipped out of duplex as observed in the case of GG adduct. To this end, we incubated all three substrates with sodium bisulfite which converts cytosines to uracil. To monitor the conversion products we incubated the samples of the reaction with UDG and APE1 and observed the excision products on a sequencing gel. UDG removes uracil generated during the reaction with sodium bisulfite, and APE1 cleaves at the abasic site generated resulting in the release of products. The oligos contained cytosines at defined positions adjacent to GG intra adduct or ICL. All the substrates were labelled at the 3' site
by using α p^{32} dCTP. The cytosines were present at base number 19, 20 and 21 in the oligos. So the products of the cleavage reaction will be 19, 20 and 21 mer in length if deamination occurs. As shown in figure 8, lanes 1-4 indicate the reactions carried out with undamaged substrate, lanes 5-8 with GG substrate and 9-12 with cisplatin ICL substrate. We observed the clear appearance of products of length 19, 20 and 21 mer for cisplatin ICL substrate incubated with sodium bisulfite (15 min), followed by treatment with UDG and APE1 as seen in lane 12. We observed products of similar length upon shorter incubation time (5 min) as observed in lane 11. These products demonstrate that 3 cytosines adjacent to cisplatin ICL underwent conversion to uracil upon incubation with sodium bisulfite, and were processed by UDG and APE1. No products of similar length were observed for undamaged and cisplatin GG intra adduct (lanes 3 and 4 for undamaged and 7 and 8 for GG) suggesting that longer periods of incubation with sodium bisulfite is required for conversion to uracil. No products were observed without incubating the samples with sodium bisulfite even though UDG and APE1 were added (lanes 1 and 2 -undamaged, lanes 5 and 6 -GG adduct and lanes 9 and 10 for ICL) in the reaction mixtures. These reactions demonstrate the requirement for uracil for cleavage reactions to occur. These results suggested that cytosines adjacent to ICL which are flipped extrahelical, are more prone to undergo conversion to uracil compared to cytosines in an undamaged duplex substrate and those adjacent to intra adducts. The presence of the crosslink impedes the separation of the strands of the duplex and is generally visualized as a smear on a sequencing gel.
**Figure 8**

Cytosine deamination assay

<table>
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<th>Cisplatin ICL</th>
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</thead>
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<td>+ + + + - +</td>
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<td>+ + + + - +</td>
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<tr>
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<td>0 0 5 15</td>
</tr>
</tbody>
</table>

Crosslink

42

21

20

19

1 2 3 4 5 6 7 8 9 10 11 12

**Figure 8: Conversion of cytosines to uracils.** Lanes 1-4 represent reactions of undamaged DNA substrate. Lanes 5-8 reactions of cisplatin GG substrates. Lanes 9-12 reactions of cisplatin ICL substrates. The reactions were carried out at 37°C. The combinations of reaction conditions used for each lane were indicated above the figure. This figure has been published in the Journal of Biological Chemistry April 2011 286(16) 14564-74.
3.2 Determining the glycosylase responsible for the removal of uracil on an undamaged DNA substrate:

The evidence obtained in the previous experiment established the possibility of conversion of cytosine to uracil. Removal of uracils in cells is mediated by various glycosylases. UNG is the enzyme responsible for the removal of uracils in most of the spontaneous deamination events that occur in cells. We attempted to determine if the glycosylase responsible for removing uracil, that might arise adjacent to crosslink is indeed UNG. To do so, first we carried out experiments on undamaged DNA substrates containing uracil at a defined location. Our strategy for this experiment is to incubate the undamaged uracil containing synthetic oligonucleotide with HeLa whole cell extract in reaction conditions which favor glycosylase activity. The reactions were conducted in the absence of magnesium, as glycosylases do not need magnesium for catalysis. Glycosylases present in the HeLa whole cell extract remove uracils. To determine if the uracil is removed by UNG, we used a highly specific inhibitor of UNG (UGI) which has a general ability to inhibit UNG family of glycosylases in all species and gauged the relative disappearance of the product. We prepared synthetic oligonucleotide which was 42 base pairs in length. Uracil is placed at position 19 in the top strand of the duplex. This uracil containing strand was labelled at the 5' end using γ\(^{32}\)P ATP. We incubated the substrate with UDG and APE1 and observed the formation of a 19 mer product as seen on lane 2 of figure 9. This product served as a control for the reaction catalysed by glycosylases in the extract. Next, we incubated UDG with UGI (inhibitor) and observed that the 19 mer product was not detected upon incubation with UGI (lane 3). We therefore decided to proceed by incubating the uracil containing undamaged DNA...
substrate with HeLa extract and then quenched the reaction in the presence of either formamide or NaOH. As we were able to observe that APE1 was able to cleave at the abasic sites generated upon removal of uracil in these reaction conditions, we believe that APE1 activity is not completely inhibited. Typical glycosylase reactions involve carrying out the reaction and quenching it with an alkali and heat to cause cleavage at the abasic sites. Upon incubation of the undamaged DNA substrates with HeLa extracts, we observed the 19 mer product in formamide quenched reactions indicating an activity of either a bifunctional glycosylase or an endonuclease (lane 4). We observed the 19 mer product in reaction quenched with NaOH and heat, indicating the activity of glycosylase in catalysing the removal of uracil (lane 5). As observed in figure 9, upon incubation of the HeLa extract with increasing concentrations of UGI, the 19 mer product formation was clearly observed (lanes 6, 7 and 8) supporting the conclusion that UNG is the not the only glycosylase capable of processing uracil on an undamaged substrate.
Figure 9

*In vitro* glycosylase assay for Undamaged DNA

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</tbody>
</table>

**Figure 9:** UNG is not the primary enzyme responsible for removal of uracil in an undamaged DNA substrate. Undamaged DNA (10nM) was incubated with 5 micrograms of HeLa extract. Lane 1, DNA alone. Lane 2, DNA treated with UDG and APE1 to generate a 19 mer product. Lane 3, reaction of the DNA substrate treated with UDG, APE1 and 1 unit of UGI. Lanes 4 and 5, DNA incubated with HeLa extract; lanes 6, 7 and 8 reactions in which HeLa extract was preincubated with the indicated concentrations of UGI.
3.3 Determining the glycosylase responsible for the removal of uracil adjacent to cisplatin ICL substrate:

We intended to determine the glycosylase responsible for the removal of uracil from a cisplatin ICL substrate containing a uracil adjacent to the crosslink. Previous experiments indicated that the uracil on an undamaged DNA substrate could be removed by alternative uracil removing glycosylases. The presence of uracil in the vicinity of the crosslink can influence the choice of a glycosylase. A specific glycosylase might accommodate the flipped out base in its catalytic site. To determine whether UNG is the primary glycosylase, we prepared cisplatin ICL synthetic oligonucleotide substrate containing uracil adjacent to the crosslink. Due to the distortion, uracil will be flipped extrahelical. As shown in Figure 10, when we incubated this substrate with UDG and APE1, we observed a 19 mer product which served as a control (Figure 9 lane 2). UGI inhibits UDG activity on a cisplatin ICL substrate suggesting the high specificity of the inhibitor (Figure 9, lane 3). Incubation of the substrate with HeLa extract resulted in a 19 mer product (Figure 9) and this product was inhibited upon incubation with UGI (lane 4 and 5). Generation of this product indicates the glycosylase activity is present in in HeLa extract. Quenching the reaction with NaOH yielded a 19 mer product (lane 6). When the DNA substrate was incubated with HeLa extract which was pre incubated with increasing concentration of UGI (lanes 7, 8 and 9) the product was not detected indicating a highly specific requirement for UNG in the removal of uracil adjacent to cisplatin ICL. Product formation was not inhibited for an undamaged DNA substrate. The data suggest that UNG is the primary glycosylase responsible for the removal of uracil adjacent to a cisplatin ICL.
Figure 10: UNG is the primary glycosylase responsible for removing uracil adjacent to cisplatin ICL. Reactions contain cisplatin ICL substrate (10nM) containing uracil adjacent to ICL. Lane 1, DNA which was not processed. Lane 2 and 3, reaction where the DNA substrate is incubated with purified UDG, APE1 and UDG, APE1 as well as UGI respectively. Lanes 4 and 5, DNA incubated with HeLa extract and quenched with formamide and incubated with UGI. Lane 6, DNA incubated with HeLa extract and quenched with NaOH. Lanes 7, 8 and 9, DNA substrate was incubated with HeLa extract which was pre incubated with increasing concentrations of UGI. The position of the 19 mer product is indicated. The substrate was $^{32}$P labelled at the 5' end.
3.4 Determining the glycosylase responsible for removing uracil adjacent to cisplatin ICL using extracts from MEFs:

The availability of MEFs obtained from Ung<sup>−/−</sup> mice and matched control prompted us to determine whether we could recapitulate the results obtained in the previous experiment. We prepared extracts from wildtype MEFs as well as Ung<sup>−/−</sup> MEFs as described in the Materials and Methods. We performed similar glycosylase assays in which undamaged and ICL uracil containing substrates were incubated with extracts and product release was monitored. Undamaged uracil substrate was incubated with wildtype MEF extract and the 19 mer product was detected indicating cleavage at the position of uracil. This suggests that MEF extracts contain glycosylases which catalyze removal of uracil (Figure 11A, lane 3). 19 mer product, was detected when wildtype MEF extract was incubated with UGI, consistent with the result obtained with HeLa extracts (Figure 11A, lane 4). The 19 mer product was detected when the undamaged substrate was incubated with Ung<sup>−/−</sup> extract (Figure 11A, lane 5) which strengthens the notion that other back up glycosylases can catalyze the removal of uracil from an undamaged duplex DNA substrate. When Ung<sup>−/−</sup> extract was supplemented with purified UDG, there was no increase in product formation (Figure 11A, lane 6) suggesting that the efficiency of removal of uracil from undamaged duplex DNA by back-up glycosylases is as efficient as the abundant UNG. To identify the specific requirement of UNG for removing uracil adjacent to cisplatin ICLs, we conducted similar experiments with the ICL DNA substrate. When ICL uracil substrate was incubated with extract from wildtype MEF extract, a 19 mer product (Figure 11 B, lane 3) was generated. The product disappeared upon the addition of UGI consistent with the result obtained with HeLa extract (Figure
11B, lane 4). We did not observe product with extracts from Ung-/- MEFs (Figure 11B, lane 5). Product formation was restored when the Ung-/- extracts were supplemented with purified bacterial UDG (figure 11B, lane 6) suggesting a specific requirement for UNG in removing uracil adjacent to crosslink.
**Figure 11A**

*In vitro* glycosylase assay for undamaged substrate using MEF extracts

<table>
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</table>

Undamaged 42

19

1 2 3 4 5 6

**Figure 11A Glycosylase assay using extracts from MEFs.** Sequencing gels containing reactions for undamaged DNA and cisplatin ICL substrates containing uracil, respectively. The substrates were $^{32}$P labeled at the 5' end and the 19 mer product is indicated. Lane 1, DNA alone. Lane 2, DNA treated with UDG and APE1. Lane 3, DNA incubated with wildtype MEF extracts. Lane 4, DNA incubated with wildtype MEF extract which was preincubated with UGI. Lane 5, DNA substrate incubated with Ung$^{-/-}$ extract. Lane 6, DNA incubated with Ung$^{-/-}$ extract supplemented with purified UDG. Lanes 3-6 reactions were quenched with NaOH.
Figure 11B

*In vitro* glycosylase for ICL substrate using MEF extracts

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Figure 11B Glycosylase assay using extracts from MEFs for ICL substrate. Sequencing gels containing reactions for undamaged DNA and cisplatin ICL substrates containing uracil, respectively. The substrates were <sup>32</sup>P labeled at the 5' end and the 19 mer product is indicated. Lane 1, DNA alone. Lane 2, DNA treated with UDG and APE1. Lane 3, DNA incubated with wildtype MEF extracts. Lane 4, DNA incubated with wildtype MEF extract which was preincubated with UGI. Lane 5, DNA substrate incubated with Ung<sup>-/-</sup> extract. Lane 6, DNA incubated with Ung<sup>-/-</sup> extract supplemented with purified UDG. Lanes 3-6 reactions were quenched with NaOH.

58
3.5 Determining the influence of quenching the reaction with formamide or NaOH using both HeLa and Ung<sup>-/-</sup> extracts:

As observed in the experiment with HeLa extract, we were able to observe the product formation when the reaction was quenched in the presence of either formamide or NaOH. Quenching the reaction with formamide in principle should not display product formation, because the cleavage of abasic sites occurs only upon treatment with NaOH and heat. The reactions were conducted in the presence of 2mM EDTA which should chelate the Magnesium ions required for the endonuclease activity. Hence, we wished to further examine the reaction quenching in the presence of formamide and NaOH, as well as increased concentration of EDTA. We used the same cisplatin ICL uracil substrate as was used in previous experiments and incubated the substrate with HeLa and Ung<sup>-/-</sup> extracts as well as quenching them with formamide and NaOH. As shown in figure 12, we were able to observe the 19 mer product in reactions incubated with HeLa and quenched with either formamide (lane 4) indicating that abasic sites generated were getting cleaved. This product was lost upon the addition of UGI which clearly indicated that UNG is removing uracil (lane 5). Had the product formation persisted, it would have raised a possibility for the involvement of a bifunctional glycosylase. When the substrate was incubated with Ung null extracts, no product was observed whether the reaction was quenched in the presence of formamide or NaOH (lanes 8 and 9). This observation suggests that the removal of uracil adjacent to crosslink is primarily mediated by UNG. When the reaction was conducted in the presence of 10mM EDTA, the product formation persisted (lane 6) indicating that UNG is removing uracil generating an abasic site, which is further processed by an endonuclease rather than a bifunctional glycosylase.
Figure 12: *In vitro* glycosylase assay with ICL substrate

Figure 12: Cleavage of abasic sites mediated primarily by an endonuclease rather than a bifunctional glycosylase: Cisplatin ICL substrate was P$^{32}$ labeled at the 5' end and containing uracil at position 19 on the same strand is used. Lane 1, DNA alone. Lane 2, DNA incubated with UDG and APE1. Lane 3, DNA incubated with UDG, APE1 and UGI. Lane 4, DNA substrate incubated with HeLa extract and quenched with formamide. Lane 5, the lane 4 reaction except that UGI was added to the reaction. Lane 6, DNA substrate incubated with HeLa extract in the presence of 10mM EDTA. Lanes 7 and 8, reactions containing DNA substrate incubated with Ung$^{-/-}$ extract and quenched with NaOH and formamide respectively. Lanes 9 and 10, reactions incubated with HeLa extracts and with and without UGI.
3.6 Formation of abasic sites and cleavage by APE1, after the removal of uracil:

The experiments conducted thus far provide evidence that UNG removes uracil adjacent to cisplatin ICL and the removal of uracil on an undamaged substrate can be mediated by other back-up uracil glycosylases. We attempted to assess the downstream processing events that occur upon the removal of uracil. The data suggested that APE1 cleaves at the abasic sites in the presence of EDTA. We wished to determine whether uracil removal which results in the formation of abasic site was indeed generated by further cleavage by APE1. To monitor the abasic site formation upon uracil removal, we used an inhibitor of BER, known as methoxyamine. Methoxyamine binds to the abasic sites and blocks the cleavage reaction. We incubated the undamaged DNA substrate with purified UDG and APE1 and detected 19 mer product (figure 13A, lane 2). Addition of methoxyamine inhibited the cleavage at the abasic site catalyzed by APE1 indicating that methoxyamine is effectively binding at the abasic sites (figure 13A, lane 3). When undamaged DNA substrate was incubated with HeLa extract, the 19 mer product was detected (figure 13A, lane 4). When the HeLa extract and methoxyamine were added simultaneously to the DNA substrate, the 19 mer product was not detected (figure 13A, lane 5) suggesting that abasic sites generated upon uracil removal became refractory to cleavage. This experiment validated the use of methoxyamine as a tool to the gauge the formation of abasic sites and inhibiting them from further cleavage. We used methoxyamine to probe formation of abasic sites upon uracil removal adjacent to cisplatin ICL substrate. We incubated the cisplatin ICL substrate containing uracil with purified UDG and APE1 in the presence of increasing concentrations of EDTA in the reaction buffer (figure 13 B, lanes 2,3 and 4). The 19 mer product formation was not inhibited even at 20mM EDTA.
raising the possibility that APE1 activity might not require magnesium or that purified APE1 obtained from commercial vendor might have magnesium present with it. When the DNA substrate was incubated with boiled HeLa extract, the product formation is lost clearly indicating that the observed reaction products were due to the processing of substrates by proteins present in the extract (figure 13B, lane 6). Importantly, the product formation was lost when methoxyamine and HeLa extract were simultaneously added to the DNA substrate indicating that upon removal of uracil, an abasic site is being generated, which gets tightly bound by methoxyamine and subsequent incision reaction gets inhibited. This experiment provides evidence that proteins involved in BER participate adjacent to the cisplatin ICL and this processing mechanism mirrors the classical BER.
Figure 13A

Undamaged DNA - APE1 inhibition assay

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Figure 13A: Abasic sites generated upon uracil removal were blocked by methoxyamine. Undamaged DNA substrate was $^{32}$P labeled at the 5' end containing uracil at position 19. Lane 1, DNA alone. Lane 2, DNA incubated with UDG and APE1. Lane 3, DNA incubated with UDG, APE1 and UGI. Lane 4, DNA incubated with HeLa extract. Lane 5, DNA incubated with HeLa extract and methoxyamine.
**Figure 13 B**

*In vitro* glycosylase assay for ICL substrate with EDTA and methoxyamine

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Crosslink

![Image of gel electrophoresis](image)

**Figure 13B: Methoxyamine blocks the abasic sites generated during BER processing.** Lane 1, ICL DNA substrate alone. Lanes 2, 3 and 4 substrate incubated with purified UDG, APE1 in the presence of increasing concentrations of EDTA. Lane 5, DNA incubated with UDG, APE1 and UGI. Lane 6, DNA incubated with boiled (B) HeLa extract. Lanes 7 and 8, DNA incubated with HeLa extract with and without UGI and quenched with formamide. Lanes 9 and 10 are similar to 7 and 8 except that the reactions were quenched with NaOH. Lane 11, incubation with HeLa extract with 10mM EDTA. Lanes 12 and 13, DNA substrate incubated with HeLa extract and 200mM methoxyamine.
3.7 Cleavage at the abasic site is catalyzed by APE1:

Methoxyamine blocks BER by binding to the abasic sites and inhibiting their subsequent cleavage. We were interested in determining whether APE1 was the primary activity responsible for excision at abasic sites generated by uracil removal. We used a specific small molecule inhibitor of APE1, CRT0044876 which inhibits APE1 at low micromolar concentrations. As shown in figure 14, CRT0044876 inhibited the production of 19 mer when the cisplatin ICL uracil was incubated with HeLa extract in the presence of the inhibitor. CRT0044876 inhibited the activity of purified APE1 validating its use to probe for the requirement for APE1 activity. These experiments indicate that the abasic sites generated by uracil removal adjacent to a crosslink were cleaved by APE1 present in the HeLa extract. These reactions were conducted in the absence of magnesium ions.
Figure 14

APE1 inhibition assay

Figure 14: APE1 cleaves at abasic sites generated by uracil removal. The reactions are identified at the top of the lane. As clearly observed the product formation is lost upon incubating the HeLa extract with small molecule inhibitor of APE1. Note that inhibition of APE1 activity was observed.
3.8 Polymerases in HeLa extract can insert nucleotides adjacent to cisplatin ICL

The repair of uracil adjacent to cisplatin ICL uses the canonical BER pathway, with removal of uracil by UNG, generation of an abasic site and cleavage on the 5’ side of the abasic site by APE1. We investigated the synthesis reaction, mediated by a DNA polymerase. Cleavage by APE1 at an abasic site generates a single nucleotide nick, bordered by 3’OH and 5’ dRP residue. The removal of dRP residue requires a lyase activity which is associated with DNA Polβ. We determined if polymerases in the extracts incorporate nucleotides past cisplatin ICLs. Using the cisplatin ICL DNA substrate containing uracil, HeLa extract and cisplatin ICLs were incubated with magnesium as well as dNTPs. As shown in Figure 15, we detected the 19 mer product resulting from removal of uracil and cleavage at abasic site by APE1. Due to the presence of magnesium and dNTPs in the reaction, there were polymerase extension products observed up to 42 bases in length (lane 4). These polymerase extension products were not observed in the absence of dNTPs suggesting that they are the result of DNA synthesis (lane 3). In control reaction containing DNA substrate with purified UDG, APE1 and Polβ, we observed similar extension products from position 19 (lane 2) indicating that polymerases in the HeLa extract insert nucleotides past cisplatin ICLs.
Figure 15: Synthesis past cisplatin ICLs by DNA polymerases in HeLa extracts:

DNA substrate was labeled at the 5' end. 10nM of DNA substrate was incubated with HeLa extracts in the presence of the reaction buffer containing dNTPs. Lane 1, DNA alone. Lane 2, DNA incubated with UDG, APE1 and Pol β. Lane 3, DNA incubated with HeLa extract in the absence of dNTPs. Lane 4, DNA incubated with HeLa extracts in the presence of all dNTPs.
3.9 Identity of the DNA polymerase which incorporates nucleotides past cisplatin ICL substrate:

Previous experiments indicated that DNA polymerases in HeLa extracts incorporate nucleotides adjacent to the cisplatin ICL following processing by UNG and APE1. To identify the polymerase, we considered DNA Polβ the most likely candidate. Several lines of evidence made us consider this possibility. First, Polβ can incorporate single nucleotides. Second, its role in the BER reaction is generally as the predominant enzyme for nucleotide synthesis in BER. Third, when the ICL uracil substrate was incubated with purified Polβ, DNA synthesis was observed. Mouse embryonic fibroblasts obtained from a Polβ knock out animal has helped us to conduct similar experiments as the previous experiment. We prepared whole cell extracts from wild type and Polβ knock out extracts and carried out DNA synthesis reactions by incubating the DNA substrate with these extracts (Figure 16). We detected the 19 mer product and extension past the product (lane 4). In similar reactions with Polβ knock out extract, we were able to observe the 19 mer product but no extension (lane 5). This result greatly supports the idea that the polymerase carrying out synthesis at a nick adjacent to cisplatin ICL is Polβ. Some nuclease degradation of the 19 mer product was observed due to nuclease activity in the extract.
Figure 16

Primer extension assay with WT and Polβ null MEF extracts

Figure 16: Pol β incorporates nucleotides past cisplatin ICL. Lane 1, DNA alone. Lane 2, DNA incubated with UDG and APE1. Lane 3, DNA incubated with UDG, APE1 and pol β. Lane 4, DNA incubated with wildtype MEF extract. Lane 5, DNA incubated with pol β knock out extract.
3.10 Purification and characterization of wild type, polymerase dead (D256A) and lyase dead (K72A) Pol β:

Having established that Pol β can insert nucleotides past cisplatin ICLs, we wished to purify the enzyme for further studies. Pol β is a 39 KDa polypeptide and it has two main domains. One domain is the 31 KDa polymerase domain and the other domain is 8KDa lyase domain. The lyase domain is responsible for cleaving the dRP residue after incision of the abasic site by APE1. We obtained bacterial glycerol stocks of wildtype, as well as mutants carrying mutations in either polymerase domain (D256A) or lyase domain (K72A) from Dr. Samuel Wilson, NIEHS. As shown in figure 17A, we were able to achieve a purity of nearly 95% as judged by staining on a coomassie gel. As indicated in the figure the wildtype as well mutants ran at similar molecular weight of 39Kda on the SDS gel which was stained with coomassie. The expression and purification strategy yielded nearly milligram quantities of protein for each version of Polβ. Having obtained large quantities of wildtype and mutants, we conducted primer extension assays with a rationale that wildtype and lyase dead mutants of Polβ should be able to extend primer substrates attached to template. The polymerase mutant (D256A) should not be able to extend the primer. Henceforth, we prepared primer (dT-16) and template substrate (dA 42) and incubated with 3.2 nM of all the 3 types of enzymes. As shown in figure 17B, both wild type and lyase mutant were able to extend the primer up to 42 bases as expected (lanes 2 and 4). The polymerase mutant was not proficient in extending the primer (lane 3).
Figure 17A

Purification of Wild type, polymerase dead (D256A) and lyase dead (K72A) Polymerase β

Figure 17 A. Purification of Polβ. A photograph of a coomassie stained SDS polyacrylamide gel is displayed. Lanes 1, 2, and 3 contain 8 µg of wildtype, D256A and K72A Polβ, respectively. The position of 39 KDa is clearly indicated. High and Low molecular weight markers are displayed on the gel.
Figure 17B: Primer extension assay with purified wildtype and mutants of Polβ.

Primer-template DNA substrate (dA42)- (dT 16) (200nM) was labeled at the 5' end and incubated with 3.2nM Polβ in the presence of dNTPs.
3.11 Time course of primer extension on an ICL uracil substrate:

Having obtained active purified proteins, we wished to assess whether Polβ conducts extensive synthesis reaction past cisplatin ICL containing uracil DNA substrates. Therefore, we conducted a time course experiment in which cisplatin ICL uracil substrate was first incubated with UDG and APE1 to generate a primer substrate and used this for primer extension analysis. When we incubated the UDG, APE1 treated substrate we were able to observe a 19 mer product (figure 18). We observe a 20 mer product (lane 2) which could result from the presence of heterogeneous mixture of 41 and 42 mer DNA substrates during the preparation and purification of cisplatin ICL uracil substrate. Next we incubated the ICL uracil primer template substrate with 40 fmol of Polβ for increasing time periods from 15 min to 120 min. We found that upon increased incubation, there was an increase in the extension products (lanes 3, 4, 5 and 6). This experiment suggests that Polβ can conduct processive extension synthesis adjacent to cisplatin ICL.
**Figure 18**

Primer extension assay on ICL uracil substrate

| DNA | + | + | + | + | + | + |
| UDG | - | - | + | + | + | + |
| APE1 | - | - | + | + | + | + |
| Pol ß | - | - | + | + | + | + |
| Time (min) | 60 | 60 | 15 | 30 | 60 | 120 |

**Figure 18: Primer extension activity of Pol ß on a ICL uracil substrate**

ICL uracil substrate either left untreated (lane 1) or treated with UDG+APE1 and incubated with 40 fmol of Polß. 100 fmol of DNA substrate was used for the reaction (lanes 2, 3, 4, 5 and 6). Lane 2, DNA treated with UDG and APE1. Lanes 3, 4, 5 and 6 time course experiment in which Polß was added to the reaction mixture for 15 min, 30 min, 60 min and 120 min.
3.12 Polβ demonstrates lack of fidelity adjacent to cisplatin ICL:

After demonstrating that Polβ inserts nucleotides adjacent to cisplatin ICLs at the site of uracil removal, we wished to assess the fidelity of Polβ on the DNA sequence flanking the crosslink. We prepared both undamaged and cisplatin ICL substrates containing uracil at position 21. We labeled the substrates at the 3' end by [α-32p] dCTP to generate 42mers. To clearly observe the incorporation at uracil site adjacent to ICL, we utilized radiolabelled dNTPs which will help us monitor the extension from the 5' end due to the incorporation of radioactivity in the substrate. First we conducted experiments with undamaged DNA uracil substrate. As shown in figure 19, we observed the formation of 21 mer reaction product upon incubation with UDG and APE1 (lane 1). Next we incubated UDG and APE1 treated substrate with Polβ and α-dCTP. We were able to observe incorporation up to 3 bases when the labeled dCTP was used suggesting the incorporation of correct nucleotide (lane 2). We observed incorporation of labeled dATP and dTTP suggesting misincorporation (lanes 3 and 4). To perform the experiment in more physiological conditions, wherein there is an equal opportunity for the incorporation of nucleotides, we repeated the experiment with a pool of dNTPs. The presence of a single labeled dNTP in the presence of all the pool of dNTPs will help us monitoring the relative extent of incorporation. We observed both the correct nucleotide dCTP, as well as the incorrect nucleotide dATP and dTTP were getting incorporated on an undamaged substrate when there was equal opportunity for incorporation of every nucleotide by pol β (lanes 5, 6 and 7). Similar experiments conducted in the presence of individual nucleotides (lanes 9, 10 and 11) as well as pool of dNTPs (lanes 12, 13, 14) for ICL uracil substrate demonstrated incorporation in all the cases. The incorrect nucleotides
dATP and dTTP were incorporated more for an ICL uracil substrate (lanes 13 and 14 compared to lanes 6 and 7) indicating that Polβ lacks fidelity at the vicinity of cisplatin ICL.

**Figure 19**

**D Polβ Lack of Fidelity**

![Diagram showing Polβ extension and APE1 incision](image)

Figure 19: **Pol β incorporates incorrect nucleotides even in the presence of correct nucleotides.**

Lanes 1-7, undamaged DNA substrate. Lanes 8-14, cisplatin ICL uracil substrate. This figure has been published in the Journal of Biological Chemistry April 2011 286(16) 14564-74.
3.13 In vitro reconstitution of BER reaction on cisplatin ICL uracil substrate:

After deciphering all the proteins that play a role in processing the uracil adjacent to cisplatin ICLs, we reconstituted the entire BER reaction in vitro using HeLa cell extracts as well as MDA MB231 extracts in reactions containing magnesium, ATP and dNTPs. We incubated the ICL uracil substrate with HeLa extracts and observed products consistent to those observed with standard BER reaction. As shown in figure 20A, we observed that upon incubation of the substrate with HeLa extract in the presence of UGI or methoxyamine, the product formation was lost suggesting that the classical BER reaction is occurring on the uracil (lanes 7 and 8). The appearance of the 19 mer product and extension of the product is consistent with the activities of UDG, APE1 and Pol β (lanes 1, 2 and 3). An interesting observation in this experiment was when we incubated the HeLa extract with Aphidicolin, there appears to be increased synthesis past the 19 mer (lane 10). Replicative polymerases δ and ε are inhibited by aphidicolin but pol β is refractory to inhibition. This could indicate a scenario in the cells wherein multiple polymerases can access the single nucleotide gap generated during the BER reaction. But Polβ with its unique ability to incorporate single nucleotides will prevail. The presence of multiple polymerases at the vicinity of the crosslink might indeed create competition between the polymerases. Aphidicolin mode of action involves competition with incoming dCTP binding to replicative DNA polymerases. As a result, more dCTP gets accumulated in the nucleotide pool [124]. This might indeed result in the incorporation of multiple nucleotides by Polβ. Apart from this, as observed earlier wildtype MEF extract displayed extension and Polβ extract did not (lanes 11 and 12). As mentioned earlier, HeLa extracts are greatly enriched for BER proteins, and they demonstrate great BER
activity in general. We wanted to test whether we obtain similar results with other human cell lines. Therefore, we selected breast cancer human MDAMB231 cell line and prepared extracts. As displayed in figure 20B, when we incubated cisplatin ICL uracil substrate with MDAMB extract, we were able to observe 19 mer product as well as some extension (lane 1). This 19 mer product was lost upon incubation with UGI (lane 2) or methoxyamine (lane 3) indicating that the observed results of classical BER reaction on a ICL uracil substrate does not pertain to HeLa extract alone. All the data obtained so far have clearly defined the requirement of BER proteins in processing the flanking DNA adjacent to cisplatin ICL.
Figure 20A

**In vitro** BER reconstitution on ICL uracil substrate

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**Crosslink**

**19**

**42**

**Figure 20 A: Reconstitution of *in vitro* BER reaction on a cisplatin ICL uracil substrate**: A photograph of a sequencing gel for cisplatin ICL uracil substrate labeled at the 5'end and uracil at position 19. The various combination of reactions used for the experiment were clearly indicated in a box over the table. Lane 4 contains cisplatin ICL uracil DNA substrate incubated with HeLa extracts in the absence of dNTPs. Lane 5 contains boiled HeLa extracts. Lane 6 contains HeLa extract in the presence of all the dNTPs.
Figure 20B

*In vitro* BER using MDAMB231 extracts

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**Crosslink**

**Polymerase extension**

19 42

**Figure 20 B:** A photograph of a sequencing gel showing cisplatin ICL uracil substrate incubated with HeLa extract. Lane 1 represents DNA substrate incubated with extract from MDAMB231 in the presence of all the dNTPs. Lanes 2 and 3 contain DNA incubated with MDAMB231 extract in the presence of UGI and methoxyamine, respectively.
3.14 Involvement of MMR in processing the mismatches generated due to the low fidelity nature of Polβ:

One interesting and important observation from the previous studies on BER is the demonstration that Polβ can be error prone and incorporate incorrect nucleotides at the ICL site. This misincorporation event by Polβ might activate the MMR pathway at this site. The primary damage recognition protein of MMR is MSH2-MSH6. As a next step of experiments, we were interested in addressing whether MMR damage recognition proteins can bind to the ICL directly, or will they absolutely require the presence of a mismatch to bind at the vicinity of the crosslink. Therefore, in an attempt to assess this issue, we prepared 42-mer biotinylated synthetic oligonucleotides containing a single cisplatin ICL and a cisplatin ICL containing a mismatch adjacent to the crosslink. We used substrates containing a mismatch (G/T) and undamaged duplexes as controls. We conducted biotinylated-streptavidin pull down experiments as described in the Materials and Methods section. This experiment allows us to monitor the relative retention of MSH2 on each substrate following incubation of the substrates with insect cell extract over expressing hMSH2-hMSH6. As observed in the figure 21A, MSH2 is preferentially retained on the DNA substrate containing a cisplatin ICL with a mismatch (lane 5). Very little retention of MSH2 is observed on the cisplatin ICL substrate (lane 4) suggesting that MMR binding and subsequent activation can occur only when a mismatch is created during the processing of cisplatin ICLs. These data are further supported by the fact that MSH2 retention is clearly observed on a G/T mismatched substrate (lane 3) and no retention on undamaged DNA substrate (lane 2). These data demonstrate that the presence of a mismatch might be absolutely required for the damage recognition proteins
of MMR to bind. To further demonstrate that this binding of MSH2-MSH6 is downstream of aberrant processing by BER, due to misincorporation of nucleotides by Polβ, we used cisplatin ICL uracil substrate and incubated it with UDG, APE, and Polβ in the presence of all the dNTPs. As earlier results suggested that Polβ incorporates incorrect nucleotides even in the presence of correct nucleotides, this reaction in principle should generate mismatches. We used this BER protein processed substrate and carried out the biotinylated IP as described. As shown in figure 21B, there was more retention of MSH2 on ICL uracil substrate compared with undamaged uracil substrate strongly supporting our earlier observation that frequency of mismatch generation on an ICL substrate is greater than on an undamaged uracil substrate. Having observed retention of MSH2 on an ICL substrate containing a mismatch but not on a ICL substrate without a mismatch, we became interested in directly showing this using purified MSH2-MSH6. Hence, we used similar substrates and carried out gel mobility shifts (figure 21C) to demonstrate the binding of purified MSH2-MSH6. We observed similar results in which, MSH2-MSH6 clearly bound to cisplatin ICL substrate with a mismatch (lane 8) and just mismatch alone (lane 4). We did not observe any retarded mobility in the lanes containing undamaged and cisplatin ICL substrate (lanes 2 and 6) when incubated with purified MSH2-MSH6. Overall, these findings bring the MMR pathway into the picture in which MMR acts downstream of BER and MMR activation is clearly dependent on the incorporation of incorrect nucleotides by Polβ.
**Figure 21 A and B**

Biotin IP to monitor the interaction of MSH2 with various substrates

**A**

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**B**

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<th>ICLU unprocessed</th>
<th>ICLU processed</th>
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**Msh2**

1 2 3 4 5

1 2

**Figure 21 A and B. Interaction of MSH2 with Cisplatin ICL containing a mismatch**

(A) Duplex 42 mer biotinylated DNA substrates, either left undamaged or containing a mismatch, a single cisplatin ICL or cisplatin ICL with a mismatch were bound to streptavidin magnetic beads. Equal amounts of overexpressed hMSH2-hMSH6 insect cell extract were added and the proteins bound to the DNA beads were eluted with high salt, TCA precipitated and immunoblotted with the antibody against MSH2. Lane 1 represents beads alone. Lane 2 represents MSH2 bound to undamaged DNA substrate. Lanes 3, 4
and 5 represent MSH2 bound to G/T mismatch, cisplatin ICL and cisplatin ICL with a mismatch respectively.  

(B) Duplex biotinylated 42mers containing a uracil adjacent to cisplatin ICL were left without any treatment or processed with UDG, Ape1, and Polβ before carrying out similar pull down experiments as described in A. Lane 1 represents MSH2 bound to non-processed cisplatin ICL uracil containing DNA substrate. Lane 2 represents MSH2 bound to cisplatin ICL uracil processed DNA substrate.

Figure 21C

EMSA

Figure 21 C: Electrophoretic mobility shift assay of MSH2-MSH6 binding to cisplatin ICL uracil substrate. Duplex 42mer substrates labeled at the 5’ terminus by γATP$^{32}$ and T4 polynucleotide kinase. Lanes 1, 3, 5 and 7 represent undamaged duplex
DNA, G/T mismatch, cisplatin ICL and cisplatin ICL G/T substrates respectively. Lanes 2, 4, 6 and 8 represent the substrates incubated with 100 nM hMSH2-hMSH6.
Chapter 4

Discussion

Cisplatin ICL produces a significant structural distortion with a unique feature of flipping out adjacent cytosines. Several biochemical and genetic studies have been performed to understand the repair of ICLs. The ramifications of the flipped out nature of cytosines adjacent to cisplatin crosslink repair remain largely unexplored. The experiments discussed in the results section are an attempt to test some key points of the hypothesis suggesting an overlapping role for BER and MMR in the repair of cisplatin ICLs. As observed, our results strongly support some key features of the proposed hypothesis. Several caveats and significant questions still remain. In this section, I will briefly discuss each result and elaborate on some aspects of the proposed hypothesis that need to be further addressed.

Deamination of cytosine generates uracil adjacent to cisplatin ICL:

Cytosine deamination is an extremely rapid process. We currently lack approaches to trace deamination events. Our findings indicating a specific role of BER and MMR in the repair of cisplatin ICL repair are completely reliant on the conversion of cytosines to uracil by undergoing deamination. Our in vitro data using sodium bisulfite provided evidence that indeed cytosines to ICL have more propensity to undergo conversion to uracil compared to cisplatin GG adduct and undamaged DNA. This result clearly highlights the fact that the degree of distortion on the cytosine is a major determinant of its conversion to uracil. Our result is in clear agreement with previous reports which suggested that mismatched bases are more prone to undergo deamination events [125].
As discussed in the introduction section, cytosine deamination can occur through spontaneous mode or can be induced by specific proteins. The major difficulty in proving our hypothesis lies in the fact that cisplatin does not only produce ICLs but intra adducts with higher frequency compared to ICLs. This makes it really difficult to ascertain with confidence that deamination events are ICL specific. In a recent report published from our laboratory [118] we have garnered indirect evidence, by measuring the downstream step of formation of abasic sites with cisplatin. Using an assay which quantitatively measures abasic sites in cell, we observed that upon treatment with cisplatin there was an increase in the number of abasic sites. This increase was not observed with other crosslink agents like mitomycin C or bulky adduct damage inducing agents like UV. These data made us hypothesize that the observed induction of abasic sites upon cisplatin treatment was due to the deamination of cytosines adjacent to the ICLs, as induction of abasic sites was not observed with UV damage which mimics intra adducts formed by cisplatin. In living cells, the sequence context and fundamental cellular processes like transcription, replication and repair will provide specific determining events by which deamination events can occur. Another dimension of complexity not addressed in our studies, is the presence of chromatin which will greatly determine the relative rates of recruitment of repair proteins as well as deaminases if cytosine deamination has to occur by a protein induced mode. Studies still need to address the effect of sequence context on naked DNA cisplatin ICL substrates as well. More systematic studies are needed to clearly point that uracils are being formed on both strands of DNA. A major difficulty in achieving this goal lies in finding an approach that will trap the newly formed uracil without letting it be amenable to subsequent repair. The physiological rates of
deaminations events are extremely rapid and the development of quantitative approaches that could capture these events is required.

**Choice of a specific glycosylase for the removal of uracil:**

After having established that the cytosines adjacent to cisplatin ICLs has more propensity for conversion to uracil, we directed our next set of experiments in addressing the mechanistic details of how this uracil might be processed. Uracil is a non normal DNA base, and BER is the most important and evolutionary conserved pathway for dealing with these kinds of base damages. A cell always has backup pathways which might contribute to the repair of classical BER substrates. The determining factor for the choice of a pathway remains enigmatic. But the specific choice or selection of the pathway is primarily dependent on the efficiency of the enzymatic reactions of each protein present in a specific pathway with the substrate. The importance of BER in uracil processing is well established. The presence of 4 glycosylases with an ability to carry out removal of uracil suggests the paramount importance of BER. UNG, SMUG1, MBD4 and TDG all have been shown to process uracils by the classical BER mechanism. Moreover, the monofunctional nature of these glycosylases suggests that the formation of abasic sites upon removal of uracil is a common endpoint for all these uracil glycosylases. UNG is highly abundant in cells leading to the notion that it is the primary glycosylase responsible for the removal of uracil which might occur due to spontaneous deamination events or due to misincorporation of dUMP during DNA replication. We also considered that cisplatin ICLs produce a unique distortion on the double helix of DNA and if uracils are produced as a random chance event, specific proteins with high specificity might be required to process them. By comparing the choice of glycosylases between removal of
uracil on an undamaged duplex DNA and cisplatin ICL substrate, we attempted to fish out the specific selection of proteins which might be imposed due to the structural distortion. Our *in vitro* results clearly established that UNG is the primary glycosylase responsible for the removal of uracil on a cisplatin ICL uracil DNA substrate and possibly other glycosylases can process the uracil on undamaged duplex DNA substrate. Our choice of HeLa extracts for these experiments is influenced by the fact that they have great BER activity in general and moreover, they are human cell lines. We observed similar results with MEF extracts clearly indicating the generality of our conclusions as well as establishing the fact that mouse extracts and human extracts process cisplatin ICL uracils similarly. The relative expression of each glycosylase in each extract might specifically influence the choice of a glycosylase. Several reports have clearly indicated that at least 80% of uracils arising on DNA are processed by UNG [126]. The crystal structure of UNG bound to uracil on a DNA substrate has been solved and it clearly indicated that UNG traverses across the DNA scanning for these lesions upon finding a non-normal DNA base, which is uracil, it flips out the uracil from the duplex DNA into its catalytic center before carrying out the cleavage of the glycosidic bond [127]. The presence of uracil adjacent to cisplatin ICL in an already flipped out conformation might provide UNG with high efficient recognition as well as a rapid rate of processing. This efficiency of the reaction might impede other back up glycosylases to act on the substrate. In the case of an undamaged duplex DNA substrate, even though UNG is abundant and predominant, other backup glycosylases can still function to remove the uracil. Using both UGI, which is a highly specific small molecule inhibitor of UNG family of glycoyslases, as well as MEF extracts deficient in UNG we were able to reach a
similar conclusion with respect to UNG mediated removal of uracil adjacent to cisplatin ICLs. We are currently conducting studies to determine the actual backup glycosylase for undamaged duplex DNA in our reaction conditions.

**Downstream processing by BER after the removal of uracil:**

Generation of abasic sites upon removal of uracil is the subsequent downstream event of BER reaction. The BER reaction is a highly coordinated process in which the product of one reaction becomes the substrate for the next enzyme in the reaction. The classical BER reaction has been demonstrated to proceed through channeling of the substrates. This model is termed as "passing the baton" model [95]. Our studies clearly indicated that abasic sites are indeed formed adjacent to the crosslink, by the demonstration of the fact that addition of methoxyamine blocks the incision of abasic sites in a glycosylase assay. Methoxyamine binds to abasic sites and makes the sites refractory to cleavage by APE1. In a time period of one hour in which the experiment was conducted, methoxyamine was effectively able to block incision on both undamaged as well as ICL uracil substrate. This result clearly indicates that these substrates are getting processed only by monofunctional glycosylases. None of the glycosylases that have an ability to remove uracil have bifunctional activity. Our *in vitro* data with APE1 inhibitor clearly demonstrated the specific requirement for APE1 in our experimental system. Therefore, these experiments shed light on the physical presence and creation of abasic sites adjacent to cisplatin ICL due to processing by glycosylases followed by APE1 cleavage. These results are in general agreement with our recently published report in which we found increased induction of abasic sites upon cisplatin treatment. Abasic sites are extremely cytotoxic lesions and hence their creation is always proceeded by mechanisms that ensure rapid
processing. Our observation of similarity by methoxyamine inhibition of APE1 cleavage for both undamaged and ICL uracil substrate suggests that there is a potential for the generation of common intermediates. The initial processing events might greatly differ due to the distortion created by the crosslink. Moreover, we were able to demonstrate that APE1 has an ability to cleave at abasic sites generated due to processing by upstream BER glycosylases. This cleavage at the abasic sites by APE1 has other functional consequences apart from the nuclease reaction. APE1 has been demonstrated to increase the overall efficiency by directly participating in the BER reaction as well as displacing the glycosylase present on the abasic site. APE1 has been proposed to have a role in the recruitment of pol β to the cleaved abasic sites. Our demonstration of abasic site formation and APE1 recruitment clearly strengthened our overall hypothesis of the involvement of the BER reaction on uracil adjacent to cisplatin ICLs. Apart from its role in BER, APE1 has been shown have roles in transcriptional activation. Hence, we did not pursue using APE1 deficient cells for cisplatin treatment in our cell based studies. The interpretation of results obtained with APE1 knockout cells will be hard to ascertain as APE1 has functions independent of DNA repair. Our methoxyamine and APE1 inhibitor studies with HeLa extracts provided specific direction for the actual involvement of the BER pathway as a whole in processing the flanking DNA adjacent to cisplatin ICLs.

Role of DNA Polymerase β and reconstitution of BER reaction:

Our next sets of experiments were directed in elucidating the mechanism of the single nucleotide nick processing and synthesis reaction. Our in vitro experiments with HeLa extracts, MEF extracts and purified proteins clearly highlighted the specific role for Polβ in incorporating nucleotides adjacent to cisplatin ICL. The observation that Polβ was able
to conduct processive synthesis adjacent to cisplatin ICLs significantly demonstrated its high efficiency in incorporation of nucleotides. All of our experiments were conducted in vitro and this may not be completely reflected in living cells. Recent studies have highlighted that several polymerases can conduct translesion synthesis on cisplatin ICL substrates [128]. Pol λ has been demonstrated to have an overlapping role with pol β in the repair of certain oxidative lesions during the BER reaction [94]. The action of pol λ is highly unlikely in synthesizing nucleotides adjacent to cisplatin ICLs. Moreover, our demonstration of the fact that Polβ knock out MEF extracts display little or no incorporation, but the control wildtype MEFs incorporate nucleotides indicating the importance of Polβ. The product of the cleavage reaction of APE1 generates a single nucleotide nick with a 5′ bordered dRP residue. The removal of this residue needs to be facilitated for the efficient downstream processing. The presence of a lyase domain in Pol β makes it the most suitable and efficient candidate for primer synthesis. The key feature of our hypothesis relies on the misincorporation of nucleotides by Polβ to activate MMR. Hence, Polβ lies at the nexus of the two pathways. Lack of fidelity of Polβ was apparent on a cisplatin ICL substrate compared to undamaged DNA substrate raising the possibility that the structural distortion of ICL pushes Polβ into a conformation which is more mutagenic. Incorporation of adenine was more significant compared to incorporation of thymine. The incorrect nucleotides were proficiently incorporated at the ICL site raising a possibility that Polβ generates a significant mutation spectrum at the ICL site in living cells. Repair of ICLs in general has been proposed to occur in two independent mechanisms. One mechanism relies on the stalling of a replication fork to recruit the processes that conduct repair. Other mechanisms involve transcription coupled
repair and translesion polymerase synthesis. These occur in all other phases of the cell cycle [131]. Since Polβ recruitment at the vicinity of the ICL site occurs due to generation of uracil, we anticipate that this proposed mechanism is not dependent on the phase of the cell cycle. An important parameter that needs to be considered is that replication fork stalling not only leads to processes that attempt to remove the crosslink and restart the stalled replication fork, but various polymerases with an ability to incorporate nucleotides past the intermediate processed crosslink. How the presence of equally proficient polymerases impacts the ability of Polβ to incorporate nucleotides is not known. It is reasonable to speculate that even though these polymerases might compete with Polβ in the incorporation, few polymerases possess lyase domain required for efficient removal of dRP residue. All these considerations make Polβ the most attractive candidate for incorporation for the proposed hypothesis. Moreover, it was observed that in the in vitro reconstitution experiment that, upon the addition of aphidicolin, the efficiency of nucleotide incorporation appeared to be higher. This clearly indicates that when the replicative polymerases were blocked, Polβ or other translesion polymerases were able to incorporate nucleotides efficiently. Several important questions still need to be addressed with respect to Polβ's role in ICL repair. We were able to purify in large quantities wild type as well as polymerase dead (D256A) and lyase dead (K72A) versions of Polβ. Primer extension studies with wild type Polβ suggested that the enzyme falters at the ICL site and incorporates incorrect nucleotides even in the presence of the correct nucleotides. Polβ mediated BER proceeds through either single nucleotide short patch mechanism or a multi nucleotide long patch mechanism. Polβ has been proposed to have a role in both the sub-pathways of BER. Our observation that Polβ synthesizes up to
3 correct nucleotides indicates a long patch mode of synthesis. This mode involves inhibition of the lyase activity and utilization of the strand displacement activity of Polβ. As the lyase step involves the removal of dRP lyase domain by forming a schiff base intermediate and has been shown to be the rate limiting step of BER. Further experimentation is clearly required to clarify the importance of the lyase step in BER processing DNA adjacent to cisplatin ICLs. It is not completely clear from our experiments, which domain of Polβ is important for incorporation of nucleotides adjacent to cisplatin ICL. One more facet of long patch synthesis has been known to be exclusively dependent on the replicative polymerases. The observation of proficient synthesis in the presence of aphidicolin by HeLa extracts adjacent to cisplatin ICL might indicate that the incorporation is predominantly through short patch or via translesion polymerases. Another important question arising from our studies is the impact of post translational modifications (PTMs) of Polβ on its ability to synthesize nucleotides past cisplatin ICL. Some studies have suggested that Polβ undergoes acetylation as well as ubiquitination and these modifications impact protein-protein interactions as well as stability [129]. It will also be interesting to determine the effect of these modifications on cisplatin ICL repair.

**Activation of MMR at the site of mismatch generated due to misincopration by Pol β:**

Our findings display MMR under a new light with respect to crosslink and cisplatin repair. Several studies have been conducted to address the importance of MMR and cisplatin resistance. These studies have ascertained the role of MMR in cisplatin resistance to be due to either its function in recognizing and binding to intra adducts.
formed by cisplatin or else due to its involvement in direct damage signaling [109]. No function of MMR has been ascribed in cisplatin ICL repair so far. Our results support an alternative and equally interesting hypothesis in which MMR gets activated at the cisplatin ICL site only when a mismatch is generated by the aberrant processing of BER. A recent report by Zhu and Lippard attempted to identify all the proteins that uniquely recognize cisplatin ICL substrates and found that the MMR damage recognition protein MSH2-MSH3 binds directly to it [117]. The functional impact and the mechanism of the crosslink were not addressed in this report. In contrast, when we conducted a biotin-pull down experiment by increasing the stringency of the reaction conditions by increasing the concentration of the salt, we were not able to detect any retention of MSH2 on cisplatin ICL DNA substrates. In the similar reaction conditions, we were able to observe retention of MSH2 on a cisplatin ICL substrate containing a mismatch adjacent to the crosslink. We have utilized cisplatin ICL with a G/T mismatch in our studies as the binding of MMR damage recognition proteins is highest for G/T mismatch followed by G/A mismatch. The mobility shift experiments corroborated our results with the biotin immunoprecipitation experiment. We were unable to determine whether the MSH2 retained on the mismatch was in complex with MSH6 or MSH3. All of our studies with HeLa extracts and purified proteins clearly pointed to the role of BER processing and lack of fidelity of Polβ. If these processing reactions culminate in the generation of a mismatch, this site will be the substrate for the MMR damage recognition proteins. We have not systematically addressed the extent of mutation produced by Polβ. If Pol β incorporates multiple incorrect nucleotides adjacent to ICL on both strands of DNA, then it will be interesting to determine as to how MMR coordinates repair on both strands.
This scenario raises the possibility that MMR might get recruited to both strands and make futile attempts to resolve the mismatches. The exact mechanistic details of the resolution of ICL repair are lacking. After damage recognition by MutS α or MutS β, the completion of reaction requires the presence of a nick. If a nick is generated at the vicinity of the crosslink by endonucleases like XPF-ERCC1 or MUS81, it will serve as an entry point for EXO1 mediated degradation. The generation of a nick usually occurs on one strand which will lead to initial unhooking of the crosslink. This implies that the strand containing a nick has more probability of completing the MMR reaction as compared to the other strand. It is accepted that a nick can be generated on the other strand eventually. This delay in nick generation during the resolution of the crosslink might endow MMR machinery with different rates of action on both the strands. A recent study indicated that MSH2-MSH6 complex always exists in the replisome coupled to replication, as the downstream processing protein MLH1-PMS2 is not coupled to replication [130]. This suggests that there might be a differential recruitment of MMR during replication dependent ICL repair and independent ICL repair. Further studies need to be performed to define the molecular mechanism of action of MMR at cisplatin ICLs. All the scenarios discussed above need to be considered in arriving at a conclusion for the mechanism of MMR in crosslink repair. Our data support a novel and unanticipated role of MMR in which MMR acts downstream of BER and is dependent on Polβ misincorporation and influences the repair of cisplatin ICLs.
Overall summary:

Cisplatin based chemotherapy is still one of the most widely used treatment protocols for the treatment of variety of cancer types. ICLs formed by the drug are formed at relatively minor percentage. Recent studies have demonstrated that the repair capacity of ICLs correlated with the sensitivity of various tumor cell lines indicating the importance of these less frequent lesions. DNA repair of ICLs have been proposed to occur through multiple mechanisms. But, the exact mechanistic details of all the proteins involved and the array of steps by which the lesion is resolved remain unclear. The work presented in this thesis highlights a role for BER and MMR repair pathways in the processing of cisplatin specific ICLs. Possibilities do not exist for the participation of these pathways in ICL repair unless a substrate for these pathways is formed as an intermediate during processing of ICLs. We proposed a mechanism in which BER actively gets recruited and acts in ICL repair due to a unique structural distortion created by cisplatin ICL. Using indirect approaches, we were able to demonstrate that cytosines adjacent to cisplatin ICLs get converted to uracil. This conversion calls for the action of glycosylases with an ability to remove the uracils. We demonstrated that UNG is the primary glycosylase responsible for the removal of uracil on an ICL but not on an undamaged substrate highlighting the proposition that the structure and nature of the distortion greatly influences the selection of the protein. We further were able to demonstrate that the subsequent downstream steps involve the action of APE1 and DNA synthesis by Polβ. An interesting finding emanating from the studies of Polβ synthesis past cisplatin ICL is the fact that it lacks fidelity and incorporates incorrect bases even in the presence of the correct bases. This was followed by providing a link in which MMR damage recognition proteins act on the
cisplatin ICL upon misincorporation by Polβ. We showed that MSH2-MSH6 complex does not directly bind to cisplatin ICL, but binds only when a mismatch is present in the vicinity of the crosslink. Overall, these studies highlight a novel mechanism in which BER acts adjacent to cisplatin ICLs and MMR gets recruited and acts downstream of the BER in processing the sequence flanking the DNA. Our studies provide a direction to explain the mechanism of resistance to cisplatin upon downregulation of BER and MMR raising an intriguing possibility that these processing events adjacent to crosslink might lead to the competition with the repair processes on the crosslink. This scenario, if it occurs in cells, would lead to persistent cisplatin ICLs and sensitivity. Understanding the exact mechanisms of the crosslink repair will help in the design and development of platinum analogues with an ability to evade these processes.
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