A Dissertation

Entitled

The Role of Base Excision Repair and Mismatch Repair Proteins in the Processing of Cisplatin Interstrand Cross-Links.

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Science

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An Abstract of

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Cisplatin is a well-known anticancer agent that forms a part of many combination chemotherapeutic treatments used against a variety of human cancers. Despite successful treatment, the development of resistance is the major limitation of the cisplatin based therapy. Base excision repair modulates cisplatin cytotoxicity. Moreover, mismatch repair deficiency gives rise to cisplatin resistance and leads to poor prognosis of the disease. Various models have been proposed to explain this low level of resistance caused due to loss of MMR proteins. In our previous studies, we have shown that BER processing of the cisplatin ICLs is mutagenic. Our studies showed that these mismatches lead to the activation and the recruitment of mismatch repair proteins. The role of MMR proteins in the processing of cisplatin ICLs has not been studied before. Here, we show that MMR proteins are required to maintain cisplatin ICLs on the DNA leading to increased cellular sensitivity. We distinguished between the requirement of different downstream MMR proteins for maintaining cisplatin sensitivity. We also show that, MutSα heterocomplex is required to maintain cisplatin sensitivity whereas MutSβ
complex has no effect. These results can be correlated with the increased repair of cisplatin ICLs and ICL induced double-strand breaks (DSBs) in the resistant cells. Moreover, we show that MLH1 proficient cells displayed a cisplatin sensitive phenotype when compared with the MLH1 deficient cells and the ATPase activity of MLH1 is essential to mediate this effect. Based on our results, we propose that different recognition heterocomplexes are required to mediate cisplatin cytotoxicity and an entire functional MMR pathway is essential to maintain a cisplatin sensitive phenotype. In addition, targeting XPF-ERCC1 and signaling kinases, including ATM and ATR lead to sensitization of BER and MMR deficient cells to cisplatin highlighting the potential use of their respective small molecule inhibitors in cisplatin combination therapy in the treatment of BER and MMR deficient or mutated tumors.
I lovingly dedicate my dissertation to my parents, Sudhakar Sawant and Shubhada Sawant, and my sister Harshada Sawant for always supporting my decisions and for their unconditional love and faith in my abilities.
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List of Abbreviations

APE1 ......................................................... Apurinic/apyrimidinic endonuclease 1
CSA ........................................................... cockayne syndrome A
CSB ........................................................... cockayne syndrome B
DSB .......................................................... double-strand break
EXO I ........................................................ Exonuclease I
FA .............................................................. Fanconi Anemia
FEN1 ......................................................... flap endonuclease I
HNPCC ...................................................... hereditary non-polyposis colorectal cancer
HR ........................................................... homologous recombination
ICLs .......................................................... Interstrand crosslinks
IDLs .......................................................... insertion deletion loops
KD ............................................................ knockdown
LP-BER ..................................................... long patch BER
MBD4 ......................................................... methyl CPG domain binding protein
MMC ........................................................ mitomycin C
MMR ........................................................ Mismatch repair
MLH1 ........................................................ MutL homolog 1
MSH ........................................................ MutS homologues
MSI .......................................................... microsatellite instability
Neil 1 ........................................................ Nei endonuclease VIII-like 1
NER ........................................................... Nucleotide excision repair
PARP1 ....................................................... Poly ADP ribose polymerase
PCNA ........................................................ proliferating cell nuclear antigen
Polβ .......................................................... Polymerase beta
RFC .......................................................... replication factor C
ROS ........................................................ Reactive oxygen species
RT........................................................................................................... room temperature

SMUG1........................................single-strand selective monofunctional uracil DNA glycosylase
SSBs............................................................... single-strand breaks
ssDNA........................................................................ single-stranded DNA

TC-NER.............................................................. transcription coupled NER
TDG................................................................. thymine/uracil mismatch glycosylase
TFIIH................................................................. Transcription factor II H
TLS........................................................................... Translesion synthesis

UNG............................................................................. uracil DNA glycosylase

WT................................................................................. wild type
List of Symbols

α ............................ Alpha
β ............................ Beta
γ ............................ Gamma
δ ............................ Delta
ε ............................ Epsilon
μ ............................ Micro
η ............................ Eta
ζ ............................ Zeta
Chapter 1

Literature review

Cisplatin, as a chemotherapeutic agent, was discovered serendipitously. Since its discovery, it has been widely used in the treatment of a number of human malignancies. Platinum-based drugs like cisplatin are used against various solid tumors which include genitourinary, colorectal and non-small cell lung cancers [1]. Cisplatin has been found to be particularly effective in the treatment of testicular cancer with a cure rate of nearly 100% when the tumors are detected in an early stage and when used in combination with bleomycin and etoposide [2]. It has become central to the treatment of testicular germ cell tumors. However, cisplatin based chemotherapy has been associated with increased nephrotoxicity, ototoxicity as well as nausea and vomiting [3]. In addition, cancer cells exhibit either inherent or acquired resistance to cisplatin treatment leading to poor prognosis [4, 5]. Attempts have been made to synthesize new platinum agents that would compensate for the drawbacks of cisplatin. Currently, only four platinum agents have been registered as marketed drugs which include cisplatin, carboplatin, oxaliplatin and nedaplatin. Out of the new compounds, only oxaliplatin has been approved by the FDA for the treatment of colorectal cancers. Recently formulated monofunctional platinum
agent, phenanthriplatin has showed a greater efficacy in an NCI 60 cell tumor panel screen [6]. In addition, developments of nano particle based technologies that can efficiently deliver cisplatin in small quantities and that are only activated near the target are underway [7, 8]. In spite of the new advances, it is important to further study platinum agents and understand how resistance to platinum is developed in different types of cancers. Therefore, it becomes imperative to understand the molecular mechanisms that are central to the acquired and intrinsic resistance to platinum agents and use this information for the development of new targets that can potentiate cisplatin chemotherapy. **The main objective of this dissertation is to understand such mechanisms and elucidate the pathways that can be targeted to improve platinum therapy.**

1.1 Biological activity of platinum agents

The transport of the platinum compounds across the cell membranes is the first step for successful chemotherapy. Transport of cisplatin has been known to occur through both active and passive mechanisms through the cell membrane. Once cisplatin enters the aqueous solution inside the cell, it undergoes aquation reactions. In this reaction, the chloride groups are replaced with water molecules. The high chloride concentration (~100 mM) in the extracellular fluid prevents the formation of mono or diaquo cisplatin species. However, inside the cell, the chloride concentration ranges from 2 to 30 mM. In this scenario, the hydrolysis of cisplatin takes place in an efficient manner leading to the formation of aqouted cisplatin in which one or both the chloride groups are replaced with water molecules. This leads to the formation of [Pt(H$_2$O)$_2$(N H$_3$)$_2$]$^{2+}$ cation. This aquated cisplatin is very reactive towards the nucleophilic centers in DNA as H$_2$O is a much better
leaving group compared to chloride ions. It is observed that ~42% of the total platinum is maintained in the form of these aquated species [9]. These cationic molecules rarely diffuse back out of the cell through the lipid bilayer membrane. Such trapped forms of activated cisplatin, therefore, increase the chances of binding to its biological targets such as DNA, which leads to increased cytotoxic potential of the drug.

Early studies have shown that cisplatin enters the cell through passive diffusion. However, recent research has established a role of active transporters in the cell membrane that are responsible for the uptake and transport of cisplatin and thus, affecting the cytotoxicity of the drug. Import and export transport systems that are involved in the maintenance of copper homeostasis have also been shown to be engaged in the transport of platinum drugs [10]. The copper transporting p-type ATPase, ATP7B, is responsible for the efflux of heavy metals outside the cell. It was observed that, overexpression of ATP7B gave rise to resistance to cisplatin in ovarian cancer cell lines, possibly due to enhanced efflux of the drug outside the cell [11]. On the other hand, studies in yeast have shown that lack of ctr1, which is a protein involved in copper influx, also gave rise to cisplatin resistance as it lead to decreased accumulation of the drug inside the cell which reduced the amount of drug available to target the DNA [12]. However, studies also showed that increased influx of cisplatin due to overexpression of ctr1 did not give rise to any change in the level of cisplatin inflicted DNA damage [13]. These observations suggest that the effects of the copper transporters on the cytotoxic potential of cisplatin are still uncertain. In addition, the delivery of platinum through the cell membrane has also been facilitated by organic cationic transporters (OCTs) [14]. These transporters have been implicated in cisplatin nephrotoxicity. Studies have shown that renal secretion
of cisplatin has been abolished in the absence of transporters OCT1 and OCT2, thus providing protection against the cisplatin induced renal damage [15]. Many of the studies on these transporters have suggested OCT2 as the major transporter that protects against renal toxicity [16]. Other transport systems that might play a role in the transport of cisplatin are MRP2/cMOAT (multidrug associated protein 2)(cananicular multispecific organic anion transporter), MRP1 and p-glycoproteins [17, 18]. These observations, therefore, illustrate that platinum enters the cell through passive diffusion as well as through active transport mechanisms.

1.2 DNA is a primary biological target of platinum agents

DNA is the primary target of platinum agents. As far as other biomolecules are concerned, the levels of platinum atoms that bind to proteins and RNA are much less, and they do not exhibit any inhibitory effects. In addition, the increased turnover of proteins and RNA molecules might be responsible for the minimal effects of such binding on the cellular processes [19]. Moreover, the levels of platinum DNA adducts correlate significantly well with the sensitivity of the cells to the drug [20].

1.3 Non-DNA targets of cisplatin

Glutathione is an important antioxidant that protects the cell from the damage caused by reactive oxygen species such as free radicals and peroxides. It is a tripeptide that maintains the redox environment of the cells and provides protection from the xenobiotic substances [21]. Glutathione binds to cisplatin in a reaction catalyzed by glutathione-S-transferase (GST) resulting in the inactivation of the drug. Increased expression of this enzyme can lead to increased inactivation of the drug giving rise to
cisplatin resistance. A significant correlation was found between an increased concentration of glutathione or GST with increased resistance to cisplatin in cervical and lung cancer cell lines [22, 23]. However, other studies pointed out that no correlation could be demonstrated between the expression of GST and the response rate or survival after platinum treatment in patients with cervical and non-small cell lung cancer [24, 25].

In addition, metallothionines are thiol rich proteins that can also bind to cisplatin leading to drug inactivation as these proteins are involved in metal homeostasis and detoxification processes in the cell [21]. A relationship between increased expression of metallothionines and elevated resistance has been investigated with conflicting results. Therefore, the metallothionines are considered to be a minor factor in the development of platinum drug resistance.

1.4 Types of adducts formed by cisplatin

The reaction of cisplatin with DNA leads to the formation of different types of adducts. Cisplatin reacts with the N7 atoms of guanine and adenine situated in the major groove of the double helix. These sites are easily accessible and are reactive nucleophilic centers for platinum DNA binding. The adduct formation takes place through the formation of monofunctional adducts which later are converted to bifunctional adducts which include intrastrand adducts and interstrand crosslinks. The intrastrand adducts are formed on the same strand of DNA. These adducts include 1,2-d(GpG) intrastrand adducts (60-65%) and 1,2-d(ApG) intrastrand adducts (20-25%). 1,3-d(GpXpG) intrastrand adducts form about 5-10% of the total adducts. The interstrand crosslinks (ICLs), which form about 5-10 % of the total adducts, are formed between two adjoining strands of DNA at GpC
sites. The binding of cisplatin occurs with the N7 atoms of guanine residues in the adjoining DNA strands. The resulting structure produced by an ICL is a bulky structure. It distorts the DNA and the cytosine residues are flipped extrahelical. The crystal structure also indicates that the DNA is unwound 70° and bent 47° causing significant structural distortion [26]. This structure formed by cisplatin ICLs is distinct from the ICLs formed by other DNA cross-linking agents including oxaliplatin and mitomycin C.

1.5 Mechanisms of cisplatin resistance and the role of DNA repair pathways in the removal of cisplatin adducts

Impaired blood flow and decreased blood pressure can hinder effective delivery of the drug to the distant tumor cells [27]. As noted earlier, mechanisms of decreased drug influx and increased drug efflux can also result in decreased sensitivity of the drug. In addition, the drug inactivating mechanisms also contribute to the development of cisplatin resistance. Furthermore, decreased expression of pro-apoptotic proteins and increased expression of apoptotic inhibitors can also result in tolerance to the drug. In addition, platinum adducts can also be bypassed and can go unrecognized as the cells can utilize translesion polymerases to tolerate the DNA damage. However, in vivo studies using lung cancer mouse models have shown that enhanced DNA repair is a predominant mechanism for the development of cisplatin resistance [28]. A number of studies have shown the importance of enhanced DNA repair as an important mechanism for the development of resistance [29, 30]. Studies in ovarian cancer cells have also reported increased repair of platinum ICLs and as pointed out by the authors, this is likely a major mechanism for clinical resistance to cisplatin [31].
1.6 Nucleotide excision repair and repair of cisplatin intrastrand adducts

Nucleotide excision repair (NER) is one of the repair pathways that removes bulky damaged bases from the DNA (Figure 1)[32]. NER is a complex biochemical process that requires the participation of several proteins [33]. NER detects the helical distortion in the DNA produced by the bulky adducts caused by exogenous agents like UV light and many DNA damaging agents used in chemotherapy [34]. In the global genomic NER pathway, this damage is recognized by the XPC protein, which is in a complex with another protein called HHR23B. This leads to the binding of several downstream NER proteins including XPA, RPA, TFIIH and XPG. XPA is a zinc finger protein that facilitates specific recognition of the base damage along with RPA, which is a single-stranded DNA (ssDNA) binding protein. Bulky base damages promote some degree of unwinding of the DNA structure, thereby promoting binding of RPA to the single-stranded regions. The recognition of the damage is followed by the stepwise assembly of the rest of the NER proteins. Among these are the Transcription factor II H (TFIIH) which is a subcomplex of RNA polymerase II. TFIIH, therefore, is a part of NER as well as the basal transcription machinery of the cell. It consists of six subunits and contains two DNA helicases XPB and XPD that unwind the DNA duplex near the damaged bases. This unwinding causes the formation of a bubble structure in the DNA localized around the damaged site. These junctions facilitate the incision of the DNA at both these ends by endonucleases XPF-ERCC1 and XPG. XPG endonucleolytic activity cuts the DNA strand at the 3’ side of the base damage. On the other hand, XPF-ERCC1 is a heterodimeric protein that cleaves the 5’ end of the damaged DNA strand. This leads to the removal of a patch of about 27-32 nucleotides containing the damage. The excision of
this oligonucleotide fragment from the genome takes place as the repair synthesis is initiated by DNA polymerases. The new DNA synthesis requires DNA polymerases δ and ε along with the accessory factors PCNA, RPA and RFC. The ends of the newly synthesized strands are then ligated by DNA ligase. In transcription coupled NER (TC-NER), RNA polymerase acts as a sensor and initiates the repair process. In this type of pathway, the adducts are repaired preferentially on the transcribed strand. The damage recognition is independent of XPC and it involves cockayne syndrome A and B (CSA and CSB) proteins. In conclusion, this entire process converts the damaged DNA to its native chemistry and conformation. Cisplatin intrastrand adducts are a good substrate for this repair process due to structural distortions produced by the adducts. Increased NER of intra adducts has been shown to give rise to cisplatin resistance. On the other hand, decreased expression of NER proteins, for example XPA and XPF-ERCC1, results in increased cellular sensitivity to cisplatin [35-37]. As a consequence, cisplatin sensitivity in testicular cancer cells has been attributed to the decreased expression of these proteins and thus, reduced repair of cisplatin adducts from the DNA [30].
Figure 1: Mammalian nucleotide excision repair. a. Recognition, b. Unwinding, c. Incision, d. Synthesis and ligation

Modified from: Proposed model for mammalian nucleotide excision repair (NER)

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1.7 Repair of ICLs: a complicated process that involves the contribution from multiple DNA repair pathways

A number of ICL inducing agents are used in the clinic for the treatment of various types of malignancies in combination with other agents. These include cisplatin, oxaliplatin, mitomycin C, psoralens and alkylating agents like nitrogen mustards. The ICL formation can also occur naturally as a result of lipid peroxidation, byproducts of acrolein, crotonaldehyde R and beta unsaturated aldehydes [38, 39]. The high cellular cytotoxicity caused by these agents is attributed to the covalent linkages formed between two complementary strands of the double helix. The ICLs produce a molecular constraint in the helix and when unrepaired, they form a definite block to the cellular processes like DNA replication and DNA transcription which renders them highly cytotoxic and genotoxic. Due to this property, the repair of ICLs also becomes a complicated process compared to the repair of monoadducts or intrastrand adducts which are formed only in one strand of DNA. In most organisms, ICL repair takes place via two DNA repair pathways, which can be either error free or error prone (Figure 2) [40, 40, 41]. The error prone pathway is recombination independent where the crosslink is excised on one strand by NER proteins and the resulting gap is filled by translesion polymerases. The crosslink is then removed by a second round of NER [42, 43]. This pathway appears to be less responsible for the repair of ICLs in cycling mammalian cells, however, this pathway may be the only resort for the removal of ICLs in the G1 phase or in non-replicating cells. A second repair pathway is initiated with a similar excision process but is dependent on the homologous recombination (HR) pathway for the completion. As the HR pathway
requires a copy of homologous donor template, this pathway takes place in the S phase of the cell cycle and can be initiated when the incoming replication fork detects an ICL.

**Figure 2**

**Figure 2: Models for ICL repair.** a. Recombination dependent ICL repair. b. Recombination independent ICL repair (Modified from: *Mutagenic repair of DNA interstrand cross-links*)[40]

**1.8 NER dependent processing and translesion repair synthesis of the ICLs**

Repair of ICLs appears to take place in all phases of the cell cycle. Increasing evidence has shown that, ICL repair can occur independent of replication. In the absence
of a homologous donor sequence, lesion bypass polymerases seem to play a major role in resolving the crosslink structure. Most ICLs are recognized by GG-NER [44], however, cisplatin ICL removal has been recently shown to be dependent exclusively on TC-NER and translesion polymerases. Reporter assays using plasmids containing site-specific cisplatin ICLs have shown that recombination independent ICL repair is error free and is dependent on the CSB, Pol ζ and Rev1 proteins [45]. In support of these studies, downregulation of Rev3, an essential component of Pol ζ increases cellular sensitivity to cisplatin and to other platinum agents in lung tumors and in HeLa cells [43, 46, 47]. In addition, overexpression of Rev1 in ovarian cancer cell line 2008 has been shown to lead to cisplatin resistance. This overexpression was also associated with increased mutagenesis [48]. Plasmid reactivation assays also indicated increased repair of platinum damaged DNA. However, even though this study did not differentiate between the repair of intra and interstrand crosslinks, the overall involvement of Rev1 in the repair of cisplatin adducts and its effect on cytotoxicity cannot be refuted [43, 47]. Studies by Scharer and colleagues have shown that in addition to Rev1 and Pol ζ, a number of other TLS polymerases can bypass ICLs, and this bypass is dependent on the degree of distortion produced by the ICL inducing agent [49]. NER has been shown to be essential for the recombination independent repair of ICLs produced by mitomycin C (MMC) [50]. MMC crosslinked luciferase plasmids showed inability of XPA, XPF, XPG and ERCC1 mutants to repair the ICL, indicating that the recognition and excinuclease activites of NER are essential for the repair of ICLs in the G1 phase. Additionally, the helicase activity of XPD was also shown to be important for the repair. A majority of the insights into the mechanism of ICL repair have come from the studies involving wild type and
repair deficient cell lines and their response to crosslinking agents. Most NER deficient cell lines with an exception of XPA and XPF-ERCC1 are less sensitive to cross-linking agents [51, 52]. These studies indicated that an intact NER pathway and TLS polymerases are essential for the replication/recombination independent repair of ICLs produced by numerous crosslinking agents.

1.9 Mechanism of the recombination dependent repair of ICLs

ICL repair can be initiated in S phase as a consequence of the complete blockage of the replication fork due to the ICL. This triggers a complex response which results in the removal of the crosslink and reconstruction of the replication fork. Studies in Xenopus egg extracts using site-specific ICL DNA substrates have provided strong evidence for replication dependent ICL repair [53-55]. The ICL prevents unwinding of the two DNA strands, thus, resulting in stalled replication forks. As the replication fork approaches the ICL, the leading strand stalls approximately 20-40 nucleotides from the lesion. As the replication fork encounters the ICL, it leads to the signaling and activation of the Fanconi Anemia signaling (FA) pathway. Further processing by the FA proteins is essential for the repair of ICLs. Recent studies have identified several HR proteins as a part of the FA pathway. These include FANCD1/BRCA2 and FANCO/Rad51C among others. According to one study, stalling of the replication fork leads to fork regression and the formation of a DNA DSB. These DSBs are formed independent of XPF-ERCC1, indicating that the initial DSBs are formed as a result of replication fork collapse [56]. Consequently, this formation of a DSB provides a substrate for XPF-ERCC1 which cleaves at the 3’ end of the ICL. This leads to the unhooking of the ICL from one of the strands followed by gap filling by one of the translesion polymerases. Similar to the G1
phase ICL repair, Rev1 and Pol ζ likely play key roles in this process. The leading strand is thus extended, and it can be ligated to the first downstream Okazaki fragment giving rise to an intact DNA that can serve as a donor template for the recombinational repair and replication restart later [55]. The remaining ICL is removed by NER and in the absence of damage the resulting gap can be filled by the replicative polymerases. The repair of the DSB is mediated by HR pathway which involves 3’ end resection and strand invasion followed by replication restart. RAD51 is shown to play a key role in this process [54]. In addition to NER proteins, deficiencies in the RAD51 family of proteins, BRCA1 and BRCA2 which take part in HR also showed hypersensitivity to the ICL inducing agents indicating that these proteins are involved in the repair of the crosslinks [51].

1.10 FA pathway and ICL repair

Fanconi Anemia (FA) is a rare, hereditary syndrome characterized by congenital abnormalities and increased risk for hematological and squamous cell cancers. There are 14 complementation groups that have been associated with the disease. The products of these FA genes act in a common cellular pathway that is essential for the replication dependent repair of ICLs. Out of these FA genes, 7 gene products form an FA core complex which includes FANCA, FANCB, FANC, FANCE, FANCG and FANCL. Cells deficient in the FA pathway are hypersensitive to crosslinking agents, indicating that these proteins play a crucial role in the repair of ICLs. The FA pathway is considered to be involved in the coordination of DNA repair pathways such as HR, NER and TLS. ICLs that are encountered in the S phase of the cell cycle are recognized by FANCM which further activates the FA core complex and has ubiquitin ligase activity known as
FANCL. The core complex is responsible for the monoubiquitylation of FANCD2 and FANCI. The entire FA core complex is essential for this process. The monoubiquitylation of FANCD2 and FANCI stabilizes them on the chromatin. FAN1 is a 5’ endonuclease associated with FANCD2, and it has been shown that lack of FAN1 leads to cisplatin sensitivity. This study highlighted the role of FAN1 as one of the nucleases that are required for the initial unhooking of the ICL [57]. Increased levels of XPF-ERCC1, MUS81-EME1 and FA proteins have been observed in the resistant tumors associated with chronic cisplatin exposure. These observations point towards the importance of these nucleases in the increased repair of ICLs and consequently in increased resistance to the platinum agents [58-60].

1.11 Base excision repair (BER): short and long patch repair pathways

BER is required for the repair of damaged bases that are generated due to endogenous damage to the DNA. BER mainly deals with the repair of base lesions that are generated in abundance in cells every day. Endogenous DNA damage is caused predominantly by some of the products generated during normal metabolic processes. These damaged lesions are caused by various events like deamination, alkylation and oxidation of individual bases. Water and reactive oxygen species form a major group of endogenous reactants that damage DNA in a variety of ways. In addition, lipid peroxidation products also attack DNA and form a substrate for BER. Water generates hydrolysis products which are responsible for the loss of purines and pyrimidines leading to the generation of abasic sites [61]. Deamination of cytosine and 5-methylcytosine gives rise to uracil and thymine, respectively. Similarly, deamination of adenine gives rise to hypoxanthine and guanine deaminates to xanthine. Reactive oxygen species (ROS)
are formed as a by-product of oxidative metabolism. ROS cause oxidation of the bases and lead to the formation of some of the highly mutagenic products including 8-oxoguanine [61, 62]. All these products, if left unrepaired, can have serious cytotoxic and mutagenic effects [63]. BER is essential for the removal of such endogenous damage as well as base damage caused by exogenous environmental agents. The importance of BER is highlighted by the fact that deficiencies in the major enzymes involved in this pathway lead to embryonic lethality. BER proteins are involved in both direct and indirect protein-protein interactions with other BER and non-BER proteins. BER forms an intricate system that is highly regulated and the enzyme activities are linked to the cell cycle, DNA replication and other cellular processes. However, the basic mechanism of BER remains a highly conserved process (Figure 3). BER is initiated by DNA glycosylases which recognize the damaged base. There are multiple DNA glycosylases that detect different types of base damages. The damage recognition is followed by cleaving of the glycosidic bond connecting the damaged base to the DNA phosphodiester backbone. Two types of DNA glycosylases are found in nature. The monofunctional glycosylases include uracil DNA glycosylase (UNG), thymine/uracil mismatch glycosylase (TDG), single-strand selective monofunctional uracil DNA glycosylase (SMUG1) and methyl CPG domain binding protein (MBD4). These all have uracil as a common substrate. Methyl purine DNA glycosylase also known as AAG deals with the removal of a wide variety of damaged purine bases. The other class of glycosylases contains lyase activity that cleaves the DNA phosphate backbone 3’ of the abasic site created after the glycosylase reaction. These are called bifunctional glycosylases and include hOGG1, which removes oxidized purines like 8-oxoguanine, and hNTH1 which removes oxidized pyrimidines. The DNA
glycosylase hMYH1 is responsible for the recognition and repair of the 8-oxoG/ adenine mismatches. The damage recognition and base removal by DNA glycosylases is followed by the recruitment of Apurinic/apyrimidinic endonuclease (APE1), which cleaves the DNA phosphodiester backbone 5’ of the abasic site and leaves a free 3’ OH group. Further BER processing can take place via either short patch or long patch BER pathways. In the short patch repair pathway, Polymerase beta (Polβ) carries out the DNA synthesis. Polβ has polymerase and lyase activity. It uses its lyase activity to cleave the dRP residue 3’ of the AP site. After DNA synthesis, DNA ligase III/XRCC1 seals the gap, and the short patch BER mechanism is complete [64]. Short patch BER typically involves single nucleotide synthesis by Polβ. However, in some cases, strand displacement synthesis requires incorporation of multiple nucleotides. In these cases, the long patch BER (LP-BER) pathway is employed, which involves DNA synthesis by replicative polymerases like polymerases δ and/or ε with the help of proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). The strand synthesis on the damaged strand involves displacement and creation of a 5’ flap junction. This flap junction is removed by flap endonuclease I (FEN1) and the gap is again sealed by DNA ligase I to complete the long patch repair [65]. The choice between short patch or long patch repair pathway is dependent upon a number of factors like whether the AP site is refractory to the lyase activity, the type of DNA glycosylase that removed the damaged base and also the ATP concentration during the reaction. If the 5’ end is not processed, then a polymerase switch can occur, bringing pol δ/ ε to continue the strand synthesis. The long patch pathway can also be initiated by the presence of replication and repair protein factors that interact with the proteins of LP-BER (e.g RFC, PCNA and RPA). The
employment of either of these sub-pathways is also influenced by the ratio of different BER proteins available for the reaction. Poly ADP ribose polymerase (PARP1) acts like a scaffolding protein and facilitates the recruitment of BER proteins like Polβ and XRCC1. Therefore, excess PARP1 has been shown to block LP-BER [66]. On the other hand, overexpression of APE1 promotes long patch repair [67]. To summarize, the basic mechanism of BER and its sub-pathways has been depicted in Figure 3. BER involves processes that are highly coordinated via protein-protein interactions where correct proteins for each individual steps are recruited in a timely fashion [68].

1.12 Base excision repair and cisplatin cytotoxicity: Role of BER in the processing of bulky lesions?

BER proteins have been implicated in the cytotoxic response to the ICL inducing agents. Recent evidence suggests an emerging link between BER proteins and ICL processing. AAG has been shown to recognize psoralen crosslinks and may be required for the enhanced ICL DNA repair process [69]. Nei endonuclease VIII-like 1 (Neil 1) is a DNA glycosylase/endonuclease VIII specific for oxidized and ring fragmented bases. Lack of Neil 1 gives rise to hypersensitivity to the ICL agent psoralen in HeLa cells [70]. In addition, Neil 1 can excise unhooked ICL fragments, suggesting that this glycosylase can play an important role in the removal of psoralen monoadducts as well as in the later stages of ICL repair [70]. Uracil DNA glycosylase (UNG) deficiency gives rise to cisplatin resistance in breast cancer cells as well as in mouse embryonic fibroblasts (MEFs) [71]. This resistant phenotype correlated well with the increased repair of ICLs suggesting a role for UNG in modulating cisplatin cytotoxicity [71].
Figure 3: Basic mechanism of short-patch base excision repair pathway. Modified from The Pathways of Double-Strand Break Repair. Emil Mladenov and George Iliakis. DOI: 10.5772/24572.

1. DNA glycosylase removes the damaged base
2. AP endonuclease cleaves the abasic site
3. Polβ mediated DNA synthesis
4. Ligation by DNA ligase I
In addition, APE1 was shown to cleave the resulting abasic sites from the ICL DNA substrates. Methoxyamine, which binds to the abasic sites and blocks the action of APE1 gives rise to a cisplatin resistant phenotype [71]. In contrast, overexpression of APE1 has been observed in cisplatin resistant head and neck cancer and non-small lung cancer cells [72, 73]. On the other hand, APE/ref-1 expression was unchanged between platinum-sensitive and platinum-refractory ovarian cancers [74]. Polβ has been implicated in modulating cisplatin cytotoxicity. It has been shown that Polβ facilitates the error prone translesion replication synthesis of the intrastrand adducts and thus, gives rise to DNA damage tolerance, which ultimately results in drug resistance [75-77]. On the other hand, recent reports have indicated that cisplatin treatment in Polβ knockout cells resulted in increased cisplatin resistance [78]. In support of this, the data from our lab demonstrated that Polβ knockout MEFs and Polβ deficient human breast cancer cells are resistant to cisplatin [71]. We have described a novel role of Polβ in mediating cisplatin sensitivity. We showed that Polβ has a low fidelity at the sites flanking cisplatin ICLs, and this BER processing interferes with the repair of cisplatin ICLs. Our data suggests that these responses are specific to cisplatin ICLs as the resistant phenotype in the absence of Polβ was seen only with cisplatin and not with any other crosslinking agent including mitomycin C or oxaliplatin.

Other BER proteins like XRCC1 and PARP are also implicated in the processing of ICLs [79]. Lack of XRCC1 gave rise to a sensitive phenotype in hepatocellular carcinoma cell line in response to cisplatin [80]. PARP is essential for the repair of single-strand breaks (SSBs) and its downregulation has been shown to sensitize a number
of cancer cell lines to γ-irradiation and alkylating agents [81]. Photoaffinity crosslinking experiments have revealed that proteins like XRCC1, DNA ligase III and PARP1 bind to the cisplatin ICLs in vitro, highlighting a possible role of these proteins in the processing of cisplatin ICLs [82]. These studies suggest that ICLs can be potential substrates of the BER pathway. In addition, differential response of BER deficient cell lines to different ICL inducing agents point toward the dependence on the structure of the ICL to influence which DNA repair pathway will be activated. Studies that show processing of ICLs by Neil 1 and by UNG reveal both productive and non-productive repair of ICLs by BER proteins.

1.13 Mismatch repair (MMR): a post replicative DNA repair pathway

Replicative polymerases possess 3’-5’ exonucleolytic activity to remove any mismatched bases. However, when such mismatches escape the polymerase proofreading activity, the MMR pathway is activated. The MMR pathway is responsible for maintaining genomic stability by correcting the mismatches generated during replication and by inhibiting recombination between non-identical sequences. MMR is highly conserved through evolution and deficiency of MMR proteins leads to genome wide instability and are therefore, responsible for giving rise to a mutator phenotype. Due to this reason, defects in the MMR system have been associated with an increased risk of cancer. Inheritance of MMR mutations is associated with hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch syndrome. This genetic condition also leads to increased risk for the development of cancers of endometrium, stomach, ovaries, small intestine and upper urinary tract. Sometimes somatic mutations in MMR genes give rise to sporadic cancers. In addition, epigenetic modification of MLH1 has also been
observed in some cases of sporadic cancers [83, 84]. MMR also plays a key role in the cytotoxic response to alkylating agents as well as to platinating agents, including cisplatin and carboplatin. MMR deficient cells have been shown to be around 100 times resistant to cell killing by alkylating agents [85], whereas the fold difference of this resistance to platinating agents is around 2-3 fold [86, 87]. Recent evidence also shows the involvement of the MMR pathway in the early steps of checkpoint and apoptotic responses to DNA damaging agents [88].

Correction of the errors produced by DNA polymerases requires the directing of the MMR system to the newly synthesized DNA strand. In *E. coli*, the new daughter strand is transiently unmethylated at GATC sites, thus providing a means of strand differentiation. These sites are normally methylated by the enzyme deoxyadenine methylase. The activity of this enzyme lags behind the replication fork causing the newly synthesized strand to be partially unmethylated. The recognition of the mismatch is mediated by the MutS homodimer. MutS binding leads to the recruitment of MutL homodimer. The formation of a MutS and MutL ternary complex leads to the activation of MutH endonuclease. MutH binds to the hemimethylated GATC sites and cleaves the unmethylated strand. The UvrD helicase then causes the unwinding of the mismatch containing DNA strand. This unwinding provides access to a number of exonucleases which digest the error containing strand. These nucleases can be 5’ to 3’ or 3’ to 5’ exonucleases and the selection depends upon whether the nearest GATC site lies 5’ or 3’ from the site of the mismatch. The mismatch removal stops the exonucleolytic cleavage, and the resulting gap is filled by DNA PolIII followed by nick sealing by DNA ligase.
1.14 Mismatch repair in mammalian cells

Mismatch recognition in mammalian cells is mediated by MutS homologues (MSH). There are five MSH proteins identified in human cells: MSH2, MSH3, MSH4, MSH5 and MSH6. These proteins participate in the mismatch recognition in the form of heterodimers. The two most common heterodimers required for mismatch repair and present in all cells are MutSα and MutSβ. The most abundant form of heterodimer present in cells is MutSα, which is composed of MSH2 and MSH6. MutSα is required for the recognition of base-base mismatches and single insertion deletion loops (IDLs) [89]. Another heterodimer called MutSβ, which is composed of MSH2 and MSH3 is required for the recognition of longer IDLs [90].

Single nucleotide IDLs are recognized by both MutSα and MutSβ heterodimers giving rise to partial redundancy in their functions. This functional overlap has been helpful in explaining the tumor phenotypes of MSH2, MSH6 and MSH3 null mice. MSH2 forms a common partner of these two heterodimers and lack of MSH2 displays the most severe tumor and mutator phenotype as it lacks all of the mismatch recognition function. MSH6 deficiency displays a less severe mutator phenotype suggesting that MSH3 can pair up with MSH2 to repair IDLs. In addition, MSH3 −/− mice show late stage tumorigenesis indicating that MutSα can repair most of the mismatches generated during DNA replication. In addition, MutSα has also been shown to recognize larger IDLs with reduced affinity [91].

At the site of the mismatch, MutS heterodimers form a sliding clamp that initially recognizes double-stranded DNA and later engages in ATP dependent sliding along the
DNA strand (Figure 4). MSH proteins have one ATP binding site per molecule and in the presence of ADP, the MSH proteins form a tight complex with the mismatch. However, in the presence of ATP, the MSH proteins form a sliding clamp. This movement is essential at the site of the mismatch, as MSH proteins cannot recognize the newly synthesized strand. The MutH protein found in prokaryotic cells is responsible for making a nick in the error containing strand. There is no homologue for MutH in human cells and the activity that helps to recognize the correct strand has not been discovered in eukaryotic cells. Several models exist to explain how the mismatch containing strand might be identified in human cells. Several in vitro studies have shown that the MMR system is activated after it recognizes a nick in the mismatch containing strand, and this nick serves as an entry point for the exonucleases which cleave the damaged DNA strand [92]. These observations support the idea that the strand recognition can be dependent on a single-strand nick. This nick can be provided by gaps between okazaki fragments in the lagging strand during DNA replication. It has been observed that the yeast mismatch repair system corrects mismatches more efficiently on the lagging strand during DNA replication [93]. Furthermore, studies show that MutL has endonucleolytic activity in MMR in yeast as well as in human cells [94, 95]. But, it is still not clear what drives strand differentiation between newly synthesized and parental DNA strands.

There are three MutL activities present in mammalian cells. Like MutS, these activities also occur in the form of heterocomplexes with MLH1 acting as a common subunit. MutLα is composed of MLH1 and PMS2 and is a primary activity present in the mammalian cells. MutLα comprises ~ 90% of all MLH1 in human cells [96]. MutLα can continue the mismatch repair initiated by both MutSα and MutSβ [97]. MutLβ is
composed of MLH1 and PMS1 heterodimer. This heterodimer does not participate in MMR \textit{in vitro}. However, PMS1 knockout mice displayed microsatellite instability highlighting some role of MutLβ in MMR \textit{in vivo} [98]. MutLγ consists of MLH1 and MLH3 subunits, and it has been shown to support MutSα initiated base-base and single IDL repair to a low level \textit{in vitro} [99]. MutL endonucleolytic activity has been attributed to a divalent metal binding site within PMS2 which is called a DQHA(X2)E(X4)E motif. This motif is evolutionarily conserved but is absent in bacterial systems which use GATC hemimethylation to direct mismatch repair. Mutations in this motif lead to the inhibition of MutL endonuclease activity as well as it abolishes mismatch repair activity in nuclear extracts [94].

Downstream exonucleolytic degradation in MMR is dependent upon the nick generated either 5’ or 3’ to the mismatch. Exonuclease I (EXO1) has been reported to possess 5’ to 3’ exonuclease activity, and this activity has been implicated in MMR \textit{in vivo} and \textit{in vitro}. EXO1 -/- mice are deficient in MMR of mononucleotide repeats. Some of the recent studies have shown that the 3’ to 5’ exonuclease activity of Mre11 is involved in MMR \textit{in vivo} [100]. In addition, Mre11 deficiency leads to the reduction in the efficiency of 3’ directed mismatch repair by ~ 40%, and this effect was rescued by the addition of purified Mre11 protein [101]. EXO1 mediated excision of the damaged DNA strand at a nick can span over 1000 nucleotides from the mismatch, and it stops about 150 nucleotides past the mismatch [102].
In addition to MutL, MutS and EXO I MMR requires other factors for completion. These factors, which include PCNA, RFC, RPA and Polδ, are also involved in DNA replication machinery and have been implicated in mismatch repair in human cell extracts. PCNA is loaded onto the DNA by RFC and is essential for Polδ mediated DNA repair synthesis [103, 104]. In addition, MSH proteins MSH6 and MSH3 have been reported to be associated with PCNA, suggesting that MMR proteins may form a part of replication machinery [105]. RPA binds to ss- DNA and it stabilizes the parent strand while the error containing daughter strand is being removed. After DNA synthesis, DNA ligase I seals the nick, thereby, completing the repair.

1. Mismatch recognition by MutS heterodimers
2. Recruitment of MutL complex
3. Exonuclease activity
4. DNA repair synthesis and ligation

Figure 4: Mismatch repair of a single base mismatch. Figure modified from [106]
1.15 Role of mismatch repair in tumor susceptibility

Knock-out mouse models have been developed for each of the MMR genes. Most of these mouse models display a mutator phenotype and a predisposition to cancer. A mutator phenotype is defined by the high microsatellite instability (MSI) caused by a lack of MMR function in cells. Microsatellites are repeated sequences in DNA. The nucleotide sequences within the repetitive components contain mononucleotide repeats of adenine \((A)_n\) or cytosine-adenine \((CA)_n\) dinucleotide repeats. During DNA replication, due to polymerase slippage, these repetitive sequences may decrease or increase in length. In the absence of a functional MMR pathway, these mutations are fixed in the genome leading to high MSI phenotype. The most widely used markers for the detection of MSI phenotype are 2 mononucleotide repeat markers: BAT25 and BAT26; and 3 dinucleotide repeat markers: D2S123, D5S346 and D17S250 [107].

MMR deficient mice typically develop lymphomas, sarcomas and gastrointestinal tumors. MSH2 deficient mice have shorter life span, and they show high MSI and a cancer phenotype. Lack of MSH3 does not give rise to cancer susceptibility, consistent with the fact that no MSH3 mutations associated with human HNPCC syndrome have been identified. MSH6 deficient mice displaying tumor susceptibility similar to MSH2 deficient mice, however, they do not share the typical MSI phenotype displayed by MSH2 null mice. MLH1 deficient mice exhibit similar characteristics as MSH2 deficient mice. In addition, MLH1 deficient mice are also infertile, consistent with the role of MutL\(\gamma\) complex in meiosis. Exo1 \(-/-\) null mice display shorter life span, an increased susceptibility to develop lymphomas and similar to MLH1 null mice, EXO1 \(-/-\) mice are sterile [108-110].
In addition, epigenetic modifications also give rise to inactivation of MMR genes. For example, studies have shown that hypermethylation of promoter sequences of MLH1 is responsible for lack of MLH1 function in sporadic colon cancers [111]. In these studies, tumor cell lines with MLH1 promoter hypermethylation were examined. These cell lines displayed loss of MLH1 function. When these cell lines were treated with the demethylating agent 5-aza-deoxycytidine, the MLH1 function was restored indicating that epigenetic modifications of MMR genes can be responsible for a mutator phenotype in addition to genetic defects [112].

1.16 Role of mismatch repair pathway in response to DNA damage

In addition to a predisposition to cancer phenotype, mismatch repair deficiency gives rise to resistance to alkylating agents and platinating agents like cisplatin and carboplatin. MMR deficient human cells are ~ 100 fold resistant to the treatment with alkylating agents as compared to MMR proficient cells [113, 114]. The commonly used alkylating agents such as N-methyl-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU) and temozolomide produce N’ substituted bases and methylate O\(^6\) position of guanine (O\(^6\)MeG). O\(^6\)MeG can also be removed by a suicide enzyme called O\(^6\)MeG methyl transferase (MGMT) [115]. In the absence of MGMT and during DNA synthesis, mutations are generated as there is a misincorporation of thymine instead of cytosine. The MMR system recognizes these mismatches, which leads to the activation of cell cycle arrest pathways and cell death pathways [116]. Cells lacking MMR proteins are resistant to treatment with alkylating agents [117-119]. Colorectal tumor cells deficient in MLH1 showed a resistance to MNNG as compared to the chromosome 3 complemented MLH1 proficient sub-line [120, 121].
Cisplatin forms DNA adducts that have been widely shown to be recognized by the MMR system. These adducts, however, are not processed by MMR proteins. The purified hMSH2 protein has been shown to bind to cisplatin adducts, especially to a single 1,2 GG adduct [122]. This binding was specific to adducts formed by clinically relevant anticancer agents like cisplatin, MNNG but not to clinically ineffective transplatin [123]. MLH1 proficient and deficient cells were tested for their response to a number of chemotherapeutic agents like ionizing radiation (IR), topoisomerase inhibitors, MNNG and crosslinking agents like mitomycin C, psoralen and cisplatin. The results showed that MMR deficiency gives rise to cellular resistance only to alkylating agents and cisplatin [87]. Cell lines lacking a functional MSH2 or MLH1 are 2 to 4 fold resistant to cisplatin [86, 87, 124, 125]. This low level of resistance leads to an enrichment of MMR deficient cells in the heterogenous tumor population. In a xenograft model generated using MSH2+/+ and 2 fold resistant MSH2 -/- cells, it was observed that MSH2 deficient cells were less responsive to cisplatin treatment. These data suggested that low level resistance caused by lack of MMR is clinically relevant. In a case where MMR deficiency occurs early in the tumor development, the cells remain resistant to the treatment but no further enrichment would occur after cisplatin treatment. On the other hand, occurrence of a mutation in MMR in the later stages of tumor development, would result in the enrichment of cells that are deficient in MMR following cisplatin treatment. Re-expression of MLH1 in MLH1 deficient cells also leads to an increase in sensitivity to cisplatin [87]. These results suggest that the MMR status of the cells affects the clinical outcome of cisplatin therapy.
Various models have been proposed to describe the mechanism by which MMR deficiency confers cisplatin resistance. One of the models is the “futile cycle” of DNA repair. Studies by Vaismann et al have shown that MLH1 and MSH6 deficient cells displayed 2.5 to 6 fold increased replicative bypass of cisplatin adducts, and these cells showed 2 to 5 fold increased resistance to cisplatin [124]. According to this model, replicative bypass of the adducts leads to the generation of mismatches. This modification is recognized by MutS and MutL complexes. Repair of these mismatches fails because it is directed at the daughter strand. This leads to the removal of the mismatch, but the adduct in the parent strand remains intact. The persistent action of these “futile” cycles of replication and repair leads to the generation of DNA DSBs which signal apoptosis. Oxaliplatin adducts were not recognized by mismatch repair proteins and there was no significant difference between the replicative bypass of these adducts in MMR proficient and deficient cells. The futile cycle model of mismatch repair has been suggested in case of treatment with alkylating agents like MNNG. Studies have shown that methylation damage leads to single-strand breaks in the newly synthesized DNA and is dependent on the mismatch repair status of the cells [126]. MMR attempts to correct $\text{MeG/T}$ mismatches that arise during DNA replication. Due to the persistence of $\text{MeG}$ in the template strand, repair synthesis cannot take place and this creates single-stranded gaps. During a second round of replication, these gaps cause replication fork collapse and lead to the activation of recombination pathways and cell cycle arrest to allow repair. These data implicate a futile repair model where MMR makes repeated, unsuccessful attempts to correct the mismatches and in this process, strand breaks are produced which cause cell cycle arrest and eventually apoptosis [127, 128].
Another model to describe drug resistance is the direct signaling model. According to this model, the MMR system acts as DNA damage sensor and can activate signal transduction pathways [129, 130]. MMR proteins are considered to be associated with the replication machinery, but MMR proteins can also be recruited to DNA damage independent of replication [131]. MMR proteins take part in a post replicative repair pathway, but their expression remains constant throughout the cell cycle [132]. Studies have shown that MutS proteins bind to damaged DNA as well as to damaged DNA containing a mismatch [133]. MMR proteins are reported to be recruited at the sites of DNA damage in human cells [134]. In this report, MMR proteins were shown to be recruited immediately to the site of single-strand breaks, DSBs and UV induced DNA damage in human cells indicating that MMR plays a role in a cellular response to a variety of DNA damage. In addition, genetic evidence of the direct signaling model has been provided by the generation of “separation of function” mutants of MMR proteins [88, 135-137]. It was shown that DNA repair and DNA damage induced apoptosis functions are uncoupled in the case of a missense mutation G674A in the ATPase domain of MSH2. In this case, cells with this mutation remain responsive to chemotherapy even in the absence of mismatch repair activity while still maintaining the function of signaling apoptosis [88, 138]. Consistent with this idea, MLH1 G67R and MSH6 T1217D ATPase missense mutations also represent separation of function mutants where they exhibit mismatch repair deficiency while still retaining sensitivity to chemotherapeutic agents. These data implied that DNA damage response function and mismatch repair mediated by MMR proteins are independent of each other [135, 136]. Moreover, PMS2 has been reported to interact with p73 which is a p53 related protein.
This interaction is essential for stabilization of p73 and cisplatin mediated stimulation of p73 apoptotic function. These data indicate the role of PMS2 in DNA damage induced apoptosis in addition to mismatch repair [139]. In a similar vein, studies have shown that PMS2 R20Q variant is defective in inducing p73 mediated apoptotic response to cisplatin further strengthening the role of MMR proteins in DNA damage response [137]. Cisplatin has been shown to induce ATR mediated DNA damage response leading to apoptosis. MSH2 was recognized to be one of the ATR binding proteins. Furthermore, ATR activation and its recruitment to the nuclear foci was inhibited in MSH2 deficient cells. This report concluded that ATR activation and the downstream Chk2 signaling in response to cisplatin treatment is dependent on MSH2 [130]. In another study, cisplatin induced apoptotic signaling was shown to be dependent on the MMR status of the cells. The localization of cytochrome C to cytoplasm as well as cleavage of caspase-9 and caspase-3 was reported to be reliant on the presence of MMR proteins. Additionally, this response was found to be independent of p53 [140]. It remains to be understood whether MMR mediated apoptosis is dependent on p53. Studies have shown that this response can be both dependent and independent of p53. Studies using a number of cell lines containing MMR mutations showed that increased sensitivity in MMR proficient cells was independent of p53 status [28, 140, 141].

These studies establish a definite role of MMR proteins in DNA damage response after cisplatin treatment. MMR proteins have been shown to recognize both intrastrand adducts and ICLs formed by cisplatin [82, 123, 142]. However, how MMR proteins process different adducts formed by cisplatin and how they mediate DNA damage signaling in response to different types of adducts is currently unknown. MMR seems to
play a role in both postreplicative mismatch repair as well as in DNA damage response leading to apoptosis. The repair function allows faithful completion of replication, and the apoptosis signaling function is required for the induction of cell death in heavily damaged cells. Therefore, both these functions are required to maintain genomic stability. MMR dependent responses to chemotherapy agents are clinically relevant, and thus, further studies on MMR dependent signaling complex are required for the assessment of the exact role of MMR in DNA damage response.

1.17 Role of ATR and ATM signaling in cisplatin cytotoxicity

DNA damage by platinum or other chemotherapeutic agents initiates a response that activates DNA repair mechanisms that are specific for the type of lesions produced. Along with these repair processes, DNA damage response also involves additional processes. The generation of highly cytotoxic lesions involves activation of cell cycle checkpoints and cell cycle arrest. Checkpoints form a cellular surveillance mechanism and consist of pathways that coordinate DNA repair processes and cell cycle transitions. Phosphoinositide-3- kinase related (PI3K) kinases such as ATM, ATR and DNA-PK form central components of the checkpoint machinery. ATR is activated in response to ssDNA and stalled replication forks. On the other hand, ATM and DNA-PK respond to DNA DSBs. Recruitment of ATR and ATM to the damaged site leads to the activation of a number of downstream substrates including checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) respectively. Cisplatin treatment has been reported to activate checkpoint signaling components including the ATR-Chk1 pathway. Studies have shown that ATR plays a critical role in tumor cell survival however Chk1 inhibition did not sensitize cells to cisplatin [143]. Moreover, one study showed that ATR activation in response to
cisplatin can be initiated by RPA/9-1-1 complex. The hMSH2 mediated ATR activation leads to Chk2 phosphorylation (instead of canonical ATR-Chk1 pathway) culminating in cell cycle arrest and apoptosis [130]. However, the role of Chk1 cannot be completely ruled out as Chk1 has been reported to play an important role in the repair of ICLs by phosphorylating Rad51 to stimulate HR and by phosphorylating FancE to repair the crosslinks through the FA pathway [144, 145]. SiRNA mediated inhibition of ATR sensitizes tumor cells to cisplatin and carboplatin, both in vitro and in vivo [146]. In addition, ATR inhibition using a highly selective ATR inhibitor, VE-821, leads to cell death, and this compound synergizes well with cisplatin and other chemotherapy agents. This study showed a synthetic lethal interaction between ATR and ATM/p53 signaling pathway, and thus, making them selective for tumor cells. ATM plays a key role in mediating sensitivity to DNA damaging agents especially IR [147]. A recently developed compound, KU60019, inhibited ATM kinase and resulted into radiosensitization of human glioma cells. KU60019 treatment also resulted in inhibition of phosphorylation of downstream DNA damage effectors like p53 and H2AX. However, beyond these studies, little progress has been made in the development of new ATM kinase inhibitors. The effect of ATM kinase inhibitors in combination with platinating agents is not well established. As the repair of cisplatin adducts involves the generation of DSBs that are detected by ATM, these inhibitors might prove beneficial to improve cisplatin chemotherapy in combination treatment.
1.18 Novel role of BER and MMR in mediating cisplatin sensitivity

Cisplatin has been used in the clinic for almost 4 decades since its discovery and FDA approval. It has been used successfully in the treatment of testicular cancer in combination with other anticancer agents. However, cisplatin-based chemotherapy is vulnerable to the development of resistance, recurrence and hence poor prognosis. Cisplatin has been studied extensively but the mechanisms of resistance are still not completely understood. DNA repair plays an important role in modulating cisplatin cytotoxicity. Alteration in DNA repair pathways is one of the major mechanisms of resistance. Therefore, it becomes important to understand how cisplatin adducts are recognized and how different DNA repair pathways are activated.

The cisplatin ICL has a unique structure with cytosine residues flipped away from the double helix [26]. These cytosine residues are more prone to undergo oxidative deamination in the cellular environment. Using cisplatin ICL substrates in vitro, studies from our lab have shown that these cytosine residues undergo preferential deamination to uracil. These residues are removed by uracil DNA glycosylase to form an abasic site. We have observed that cisplatin treatment leads to an increased generation of abasic sites as compared to the other crosslinking agents including oxaliplatin, transplatin and mitomycin C. This abasic site is recognized by APE1 which cleaves at the 5’ side of the abasic site to generate a free 3’ OH group. This free end is used by Polβ to carry out DNA synthesis. The lyase activity of Polβ is utilized for the removal of 5’ dRP residue that is remaining in the DNA backbone. The BER proteins bind to the regions flanking cisplatin ICL. We believe that this results in non-productive BER processing as BER proteins do not repair the ICLs but interfere with the productive repair of ICLs by NER.
and HR proteins. The repair of ICLs is hampered and in the presence of BER proteins the cells show increased cellular sensitivity to cisplatin. Furthermore, DNA synthesis by Polβ is error prone. We experimentally showed that Polβ can incorporate incorrect nucleotides even in the presence of correct bases. Distortion created by ICL is unique to cisplatin as the DNA helix is not shown to be distorted to this degree in case of other cross-linking agents like oxaliplatin and mitomycin C. Our studies showed that Polβ processing is more mutagenic near cisplatin ICL sites as compared to undamaged DNA duplex. The misincorporation by Polβ generates mismatched bases, and we hypothesize that this will result in MMR protein binding and further MMR processing. We believe that the binding of MMR proteins would inhibit the productive repair of cisplatin ICLs by NER and HR proteins resulting in decreased repair of ICLs and therefore increased cellular sensitivity. 

The aim of this study is to understand whether BER and MMR can work together in the same molecular mechanism. Our hypothesis is that BER and MMR processing near the ICL sites would interfere with the “actual repair” of cisplatin ICLs and mediate cisplatin sensitivity.
Chapter 2

Overlapping role of BER and MMR proteins in mediating cisplatin cytotoxicity*

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2.1 Introduction

Cisplatin remains one of the most widely used chemotherapeutic agents for the treatment of a variety of cancers. Cisplatin binds to DNA and forms intrastrand and interstrand crosslinks (ICLs). These adducts hamper normal cellular functions like DNA replication and RNA transcription which leads to cell cycle arrest and/or apoptosis. Cisplatin has been used successfully in the treatment of testicular cancer as a part of combination therapy along with other anticancer agents. However, there are various limitations to the clinical use of cisplatin. Cisplatin based chemotherapy is susceptible to the development of resistance, recurrence and hence poor prognosis. The mechanisms of development of resistance are still not completely understood. One of the major mechanisms of resistance is the alteration in the DNA repair capacity of the cells. Therefore, it becomes important to understand how various DNA repair proteins recognize cisplatin adducts and how it leads to the activation of a variety of DNA repair pathways. Nucleotide excision repair (NER) is the major pathway responsible for the repair of cisplatin intrastrand adducts. But the exact mechanisms of cisplatin ICL repair is poorly understood. NER, HR and FA pathway play an essential role in the repair of cisplatin ICLs. Other DNA repair pathways like base excision repair (BER) and mismatch repair (MMR) have also been implicated in mediating cisplatin cytotoxicity. Recent studies from our lab and others have shown that reduced expression of polymerase β (Polβ) gives rise to cisplatin resistance [71, 78]. In addition, MMR deficiency also results in resistance to cisplatin and carboplatin [86]. However, the exact mechanism of how these proteins influence cisplatin cytotoxicity is not well understood.
The MMR pathway maintains genomic stability via post replicative mismatch repair. The mismatches can be recognized by one of the two heterocomplexes. MutSα is composed of MSH2 and MSH6 and recognizes base-base mismatches and single IDLs. On the other hand, MutSβ consists of MSH2 and MSH3 and is required for the repair of IDLs larger than a single base pair. Mismatch recognition by MutS proteins is followed by recruitment of MutL complexes which consist of essential downstream MMR proteins. In addition to its role in repair of mismatches, the MMR pathway also responds to DNA damage produced by a number of DNA damaging agents. MMR machinery is responsible for the recognition of mismatches generated during the treatment with alkylating agents. MMR deficient cells are resistant to treatment with methylating agents [128]. Lack of MMR proteins gives rise to a low level of resistance to cisplatin and carboplatin [86, 124]. Various models have been proposed to explain the mechanism of MMR mediated cisplatin resistance, including futile cycle repair of cisplatin intrastrand adducts, MMR dependent apoptotic signaling and a model involving DNA damage shielding [148, 149]. The postulated hypotheses for these models involve binding of MMR proteins to cisplatin intrastrand adducts. MMR recognition protein MutSα recognizes cisplatin GG intrastrand adduct [122, 123, 142]. The binding of MMR proteins to cisplatin ICLs is not very well studied. Photo-crosslinking experiments and electromobility shift assays have also shown that hMutSβ is one of the proteins that binds to cisplatin ICLs. The pathways involved in the recruitment of MMR proteins to cisplatin ICLs in vivo remain to be explored.

Our previous studies have shown that the BER pathway modulates cisplatin cytotoxicity [71]. Studies have shown that Polβ is capable of bypassing cisplatin
intrastrand adducts which may lead to DNA damage tolerance and increased mutagenicity [150, 151]. Using cisplatin ICL DNA substrates, we have shown that BER proteins process the regions near the ICL site and Polβ has a low fidelity near the DNA flanking the ICL sites. This leads to misincorporation of nucleotides at the sites flanking the cisplatin ICL and these mismatched bases can act as an initiating point for the MMR pathway. Therefore, we targeted BER and MMR pathways separately and together to assess whether these pathways work together in an epistatic manner to mediate cisplatin sensitivity. Using mouse embryonic fibroblasts (MEFs), we show that BER and MMR pathways are epistatic in mediating cisplatin cytotoxicity. Our studies show that, when BER and MMR are downregulated separately there is cisplatin resistance, and when downregulated together, no additional increase in the resistance was seen. This strongly suggests that BER and MMR have overlapping roles in mediating cisplatin sensitivity. We also show that MMR binding occurs downstream of BER processing, and activation of MMR is dependent upon the mutagenic role of Polβ near cisplatin ICL sites. Our data also demonstrate that loss of BER and MMR leads to faster repair of ICLs which appears to be the cause of cisplatin resistance in these cells. Based on our results, we hypothesize that BER and MMR pathways maintain cisplatin sensitivity by competing with the “actual repair” of cisplatin ICLs. BER and MMR deficiency, therefore, leads to faster repair of ICLs and hence cisplatin resistance.
2.2 Materials and Methods

Chemicals and Antibodies

Cisplatin was purchased from Sigma-Aldrich. VE-821 was purchased from Axon Medchem. KU60019 and NU7441 were purchased from Tocris Bioscience. All other chemicals and reagents were from standard suppliers. The antibody for MSH2 was from Calbiochem and α-tubulin was from Sigma-Aldrich. For stock preparation, cisplatin was diluted in 1X PBS and vortexed vigorously to dissolve the drug completely. The stock concentration was 1mM and fresh stocks of cisplatin were prepared before each experiment.

Cell lines

The human breast adenocarcinoma MDA-MB-231 cells were grown in RPMI 1640 containing 10% FBS and antibiotics. MDA-MB-231 Polβ knockdown cells (Polβ lentiviral shRNA) were grown in the presence of 0.5μg/mL puromycin. The MDA-MB-231 Polβ knockdown cells re-expressing WT Polβ and a variant deficient in polymerase activity (D256A) were grown under similar conditions with the addition of 700μg/ml geneticin. The development and characterization of the MDA-MB-231/Polβ-KD cells were described previously [152]. Wild-type (92TAga) and Polβ null (88TAga) primary MEFs were cultured in high glucose DMEM supplemented with 10% FBS plus antibiotics at 37°C in a humidified atmosphere under 10% CO₂. The MSH2 deficient human endometrial carcinoma cell line HEC59 and its chromosome 2 complemented sub-line HEC59+2 were obtained from Dr. Kandace Williams, University of Toledo.
chromosome complemented sub-lines were maintained in DMEM F-12 media supplemented with 400 μg/ml of geneticin.

**siRNA transfection**

ON-TARGET plus SMART pool siRNAs specific for human MSH2, XPF and ERCC1 were purchased from Dharmacon RNAi technologies, Thermo Scientific. The non-targeting control siRNA was used as a control for non-specific effects. siRNA transfection was carried out per the manufacturer’s protocol. Briefly, the cells were plated in 6 well plates in the antibiotic free media. At the time of transfection, the cell density was maintained at 60-70% and two transfections were done with an interval of 24 hrs. Dharmafect transfection reagent 1 and 4 were used for MEFs and MDA-MB-231 cells, respectively. The cells were harvested at 48 and 72 hr timepoints after transfection for the detection of protein and transcript knockdown.

**Western blot analysis**

Cells were harvested at the indicated timepoints after the infection, washed with PBS and lysed in lysis buffer (10 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 1mM EDTA) containing protease inhibitors (stock concentrations 1000X: 0.5 M phenyl methyl sulphonyl fluoride PMSF, 1 mg/ml Leupeptin and 1 mg/ml pepstatin A). The protein concentration was measured using Bradford assay with BSA as a standard. Approximately 50 μg of total protein extract was used for loading. The proteins were separated on 8% SDS-polyacrylamide gels and transferred onto Immobilon-P transfer membranes (Millipore). After blocking in 2% non-fat dry milk, the membranes were probed with primary antibodies recognizing human MSH2, with α-tubulin as loading
control. The primary antibody incubations were carried out overnight at 4°C. The membranes were incubated with appropriate secondary antibodies and the signal was detected by using Enhanced chemiluminescence detection system.

**Real time PCR for the measurement of transcript levels**

At indicated post-transfection time points, cells were harvested and pelleted. RNA was isolated using TRIzol reagent (Invitrogen) by standard procedures. The total RNA was reverse transcribed using MMLV reverse transcriptase enzyme (Invitrogen) as per the manufacturer’s protocol. The primers specific for human and mouse MSH genes were designed and were analyzed using oligoanalyzer 3.0 (IDT technologies). The transcript levels were quantified using iQ SYBR green supermix (Bio-Rad) in iCycler iQ System, with GAPDH as an endogenous control for human cells and B actin for mouse cells. The percent transcript knockdown was determined from $2^{\Delta\Delta CT}$ values.

**Colony survival assay**

The cells were plated at a density of ~ 500 cells per dish. Cells were treated with increasing concentrations of cisplatin for 2 hr in serum free media. After treatment, fresh media was added and the cells were allowed to grow for 7-14 days. Colonies were fixed with 95% methanol and stained with 0.2 % crystal violet. Colonies with ≥50 cells were counted using a light microscope, and colony survival was expressed as the ratio of the average number of colonies in drug treated cells versus control cells x 100. The experiment was done in triplicate for each drug concentration. Statistical analyses were performed using student’s t test comparing wild type and knock down cells.
**Drug treatment**

The cells were treated with VE-821, KU60019 or NU7441 alone to assess their effect on cell viability (Data not shown). From these curves, a concentration (IC\(_{20}\)) was chosen, so as to have minimum influence on cell viability with the inhibitors alone. The cells were pretreated with this concentration for 2 hrs followed by treatment with increasing concentration of cisplatin for 2 additional hrs. The cells were kept in the inhibitor containing media for total 24 hrs. The colony survival assays were performed as described.

**ELISA**

Cisplatin intrastrand adduct measurement was carried out using an antibody that detects the major cisplatin GG adduct. This antibody was kindly provided by Michael J. Tilby, University of Newcastle, UK. Cells were treated with cisplatin for 2 hr in serum free media. After completion of 2 hr treatment, the drug containing media was replaced with complete media. The cells were then harvested at timepoints 0, 12, 24, 48 hr post treatment. The repair kinetics of cisplatin intrastrand adducts was assessed by ELISA. The cell pellets were stored at -80 °C until needed. The genomic DNA was harvested using DNeasy blood and tissue DNA isolation kit from Qiagen. The DNA concentrations were measured using a nanodrop instrument. The genomic DNA was coated onto 96 well ELISA plates in a coating buffer containing 1M NaCl, 50 mM sodium Phosphate buffer, pH 7.0 and 0.02% Sodium Azide. The coating reaction was done overnight at 4 °C. The next day the plate was washed using a wash buffer containing 0.1% Tween-20 in PBS. After 3 washes of 10 mins each the blocking buffer (1% BSA) was added to the plates
and incubated for 1 hr at room temperature (RT). The plate was washed and was incubated with primary antibody ICR4 which is specific for cisplatin GG adducts. After incubation with HRP conjugated goat anti-rat antibody (Calbiochem), TMB (1 step ultra TMB-ELISA, Thermo Scientific) was added. The reaction was stopped with 2M sulfuric acid and absorbance was measured at 450 nm (Spectramax M5 plate reader, Molecular Devices). The % intrastrand adducts were calculated using OD 450 nm where the 0 hr time point was used as 100% intrastrand adducts in each cell line.

**Alkaline comet assay**

Alkaline comet assay was used to analyze the repair of cisplatin interstrand cross-links (ICLs) as described [153, 154]. This assay is also called single-cell gel electrophoresis as the analysis can be done at the single cell level. In this method, the cells were treated with cisplatin for 2 hrs in serum free media and the assay was performed at 0, 24, 48 and 72 hr time points post-treatment. At the start of each time point, the cells were treated with 50 μM hydrogen peroxide (Fisher) for 15 mins in serum free media. Cells were trypsinized, harvested and were resuspended in cold PBS. Cell suspensions (~10000 cells) were embedded on a microscopic slide in 0.5% ow melting point (LMP) agarose. A total three layers of normal melting agarose (without cells) and low melting agarose (with cells) were used to embed the cells. Each layer of agarose was allowed to solidify at RT for 10 mins each. The cell lysis was carried out in a lysis buffer containing 2.5 M NaCl, 100mM EDTA, 10mM Tris Base, NaOH to pH 10 and 1% triton X100 for 1 hr at 4 C. The lysis step removes all the cellular proteins. After the lysis, the slides were incubated in ice-cold alkaline solution for 20 min to allow the DNA to unwind. The denatured DNA is then subjected to electrophoresis in a buffer containing 0.3 M NaOH and 1 mM EDTA.
Electrophoresis was carried out for 30 min at 28 V, 300 mA. Slides were neutralized and stained with SYBR green (Invitrogen).

During electrophoresis, broken DNA fragments migrate further than the supercoiled undamaged DNA. The resulting structure resembles a comet with a bright fluorescent head nucleus and the length and intensity of the tail part is determined by the DNA strand breakage produced in the cell. Cisplatin treatment generates covalent ICLs between two adjoining strands of DNA. Hydrogen peroxide treatment leads to the formation of DNA DSBs. The presence of a covalent linkage in the DNA retards its migration during electrophoresis. Hence, the DNA comets from these cells have a reduced tail moment. On the other hand, lack of ICLs in the DNA due to increased repair leads to their uninhibited migration during the electrophoresis giving rise to a larger tail moment. The comets were scored using a Nikon epifluorescence microscope. At least fifty cells were analyzed per slide using Komet Assay Software 5.5F (Kinetic Imaging, Liverpool, UK). The data was expressed as the percentage of ICLs that remained at a particular time point normalized to 100% at 0 hr.

Immunofluorescence

Cisplatin ICL processing leads to the formation of DSBs during the later stages of repair. As the DSBs are formed, ATM protein undergoes autophosphorylation and activation which in turn phosphorylates H2AX in the chromatin near the damage. The phosphorylated H2AX is called γ-H2AX. These events can be detected as foci at the damage site in the nucleus. This phosphorylation event can be monitored using an antibody against γ-H2AX. The dephosphorylation of γ-H2AX is correlated with repair of
the ICL induced DSBs. DSB repair was assessed by monitoring the nuclear γ-H2AX foci by immunofluorescence. The cells were plated in 60mm plates containing coverslips in complete media. The next day cisplatin treatment was carried out for 2 hr in serum free media. The cells were assayed at 0, 24, 48 and 72 hr post-treatment. For each timepoint, cells were fixed using 4% paraformaldehyde in PBS, permeabilized using 0.3% Triton X100 and blocked using 10% goat serum in PBS. The primary monoclonal anti γ-H2AX antibody was used at a dilution of 1:500 (Millipore). Washes were done using 0.1% Triton X 100 in PBS. The coverslips were then incubated with secondary Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody (Invitrogen). The nuclei were stained with DAPI. The coverslips were mounted on the glass slides using DAKO anti-fade reagent. The edges of the coverslips were sealed with nail polish. The images were then visualized using a Nikon Eclipse T2000-U microscope. Foci were counted in at least 200 cells at each time point per condition in each cell line and results are expressed as % γ-H2AX foci positive nuclei.

**Preparation of ICL DNA substrates and biotin DNA pull downs**

Biotinylated ICL DNA substrates were used for the pull down assays. These substrates were designed and synthesized to include undamaged DNA, ICL substrate, ICL substrate containing uracil. The individual DNA oligos were purchased from IDT and were gel purified to remove any degradation products. The substrates for ICLs were designed to contain a single G in the top strand for the formation of a single ICL. The platination reaction was carried out using platination buffer containing 10 mM sodium phosphate pH 7.5 and 30 mM NaCl at a drug to nucleotide ratio of 15:1 for ICL substrates. The reaction was carried out at 37 ºC for around ~15 hrs to ensure adduct formation. After the reaction
was complete, the unreacted cisplatin was removed by filtration through tip20 columns from Qiagen. The substrates were then ethanol precipitated in the presence of 0.3 M sodium acetate pH 5.2 and annealed to the complementary strand to generate a double-stranded DNA substrate. The annealing reaction was carried out in annealing buffer containing 10 mM Tris base pH 7.5 and 100 mM NaCl. The annealed substrate was then subjected to overnight dialysis at 37 °C in 100 mM sodium perchlorate and 10 mM Tris-HCL (pH 7.5) to enhance the crosslinking reaction. The dialyzed substrate was then ethanol precipitated and was sequencing gel purified. In a sequencing gel containing urea, the two DNA strands are separated. However, the presence of an ICL in the DNA retards the migration of the DNA leading to separation of the ICL product from uncrosslinked DNA. The crosslinked substrate runs as a smear near the top of the gel, and this smeared area can be excised from the gel. The DNA is eluted from the gel and ethanol precipitated. Undamaged substrates were annealed without platination reaction.

BER processing of ICL substrates was carried out by treatment with purified UDG, APE1 and DNA Polβ in the presence of dNTPs. The reaction was carried out at 37 °C for 1 hr. The substrates were then recovered using ethanol precipitation. This BER processing of the ICL U substrates leads to either correct or incorrect base insertion next to the cisplatin ICL.

The biotinylated substrates were then bound to streptavidin beads (Dynal biotech) in the presence of DNA binding buffer containing 20 mM HEPES pH 7.8, 2 mM DTT, 0.001 % NP40, 100 mM NaCl and 200 mM MgCl₂ at 4 °C for 30 mins. The unbound DNA was washed off using 3 washes with binding buffer. The tubes were kept in a magnetic separation stand (Promega) during the washing steps. The beads were then incubated
with hMSH2-hMSH6 cell extract prepared from baculovirus infected SF9 insect cells. In the same step, poly dI-dC competitive inhibitor (Sigma Aldrich) was added at 30 fold excess concentration to reduce non-specific binding. The tubes were incubated for 1 hr at 4 ºC. To increase the stringency of binding, the next step of washes were done using binding buffer containing 200 mM NaCl. The bound proteins were eluted in 1M NaCl. The supernatants were collected from magnetically separated beads. The protein precipitation was carried out using 10% trichloroacetic acid (TCA). The pellets were washed using cold acetone, resuspended in 1X SDS buffer and were loaded onto 8% SDS gels, transferred onto PVDF membrane (Milipore) and probed for MSH2 using western blot analysis.

**DNA substrates used for in vitro assays**

Cisplatin ICL is formed by annealing VS IC 2.1 & VS IC 2.2
VS IC 2.1 is platinated before the annealing.

VS IC 2.1 - 5’- CTCTTCCCCATCTCCTTCTCCGCTCCTCCCTCCCTCCCTCCCT-3’
VS IC 2.2 -
AGGGAGGGGAAGGGAGGAGGGCGGAAGGAGATGGGAAGAG/BIOTIN

Undamaged duplex DNA is formed by annealing VS IC 2.1 & VS IC 2.2
Cisplatin ICL U substrate:
VS 2.1U – 5’CTCTTCCCCATCTCCTTTTG/ideoxyU/CCCTCCTTTCTCCCTCCCTCCCT
VS 2.2 -
AGGGAGGGGAAGGGAGGAGGGCGGAAGGAGATGGGAAGAG/BIOTIN
2.3 Results

**siRNA transfection for MSH2 and MLH1 knockdown:**

WT and Polβ -/- MEFs were transfected with siRNA directed against MSH2. The cells were transfected twice with an interval of 24 hours and were harvested at 48, 72, 96 and 120 hrs after first transfection. The protein was extracted and western blot analysis was done to check for protein expression. Protein levels were found to be reduced to 90-95% in both wild type (WT) and Polβ MEFs at all time-points (Figure 5) (Supplementary Figure S1 in [155]). In addition, we quantified transcript levels of MSH2 and found that 90-95% of the transcript knockdown was achieved at 48 and 72 hr time point post transfection.

**Cisplatin cytotoxicity in BER and MMR deficient cells**

To assess the effect of BER and MMR knockdown on cell viability in response to cisplatin treatment, we performed colony survival assays (Figure 6) (Figure 1 A in [155]). Polβ null cells showed resistance to cisplatin as compared to WT cells. When MSH2 was knocked down in WT MEFs, cisplatin treatment gave rise to low level of resistance as compared to the WT cells. These results are consistent with the literature [23, 24]. However, when MSH2 was knocked down in BER deficient Polβ null cells, no significant increase in the resistance was observed. We performed similar studies in WT and Polβ KD MDA-MB-231 cells, and we did not see any additive increase in the level of resistance in response to cisplatin (Figure 1B in [155]). From these results, we conclude that BER and MMR might have an overlapping role in mediating cisplatin sensitivity.
Rate of cisplatin intrastrand adduct repair does not affect cisplatin cytotoxicity in BER and MMR deficient cells

Cisplatin resistance is developed when there is an increased repair of DNA adducts. Increased repair of cisplatin adducts has been correlated with decreased cisplatin efficacy. As described earlier, NER is the major pathway responsible for the repair of cisplatin intrastrand adducts. In our recent publication, we have shown that BER proteins are not involved in the intrastrand adduct repair [71]. MMR proteins have been shown to bind to cisplatin DNA adducts [122]. Our goal was to study whether BER and MMR status of the cells affects intrastrand adduct repair. We studied the repair kinetics of platinum intrastrand adducts after downregulation of BER and MMR pathways separately and together. The cells were transfected for the knockdown of MSH2 and were treated with cisplatin for 2 hrs. The genomic DNA was harvested at the specified time points using Qiagen DNeasy DNA isolation kit. The DNA was then coated onto the 96 well plates using a DNA binding buffer. To assess the repair of cisplatin intrastrand adducts, we performed an ELISA using a monoclonal antibody specific for 1,2-GG intrastrand adduct which is the major adduct formed by cisplatin (ICR4 antibody, Dr. Michael Tilby, University of Newcastle, UK). After using the appropriate HRP conjugated secondary antibody, the HRP signal was converted to a colored product. The percentage of adducts at 0 hr time point were normalized to 100%. The number of adducts increased significantly at 12 hr time point. However, there was an efficient repair of these adducts from 12 hr to 48 hr time point. Similarly, we also knocked down MSH2 in WT and Polβ deficient MDA-MB-231 cells and studied intrastrand adduct repair. The results show that loss of BER and/or MMR displayed no significant difference in the rate of adduct
removal when compared to the WT cells. These data indicate that BER and MMR are not involved in the repair of cisplatin intrastrand adducts. Moreover, Intrastrand adduct repair does not contribute to the increased resistance in BER and/or MMR deficient cells (Figure 7A) (Supplementary Figure S3 in [155]).

**Cisplatin interstrand crosslink (ICL) repair**

As intrastrand adduct repair kinetics are unchanged in BER and MMR deficient cells, we studied cisplatin ICL repair in these cells. We have utilized alkaline comet assay to examine the repair kinetics of ICLs. These results show that cisplatin ICLs are repaired efficiently from 0 to 72 hr time points. At 24 hr time point, there was no significant difference between proficient and deficient cells. However, from 48 to 72 hr BER and/or MMR deficient cells showed increased repair of ICLs. ICL repair in mammalian cells requires the participation of both NER and HR pathways. But downregulation of BER and/or MMR resulted in increased repair of the ICLs (Figure 7B) (Figure 2A in [155]). Studies have shown that increased ICL repair is associated with increased resistance to cisplatin [31]. These results demonstrate that the increased resistance that we see in the BER and MMR deficient cells is likely due to faster repair of ICLs as compared to the WT cells. Several studies have suggested MMR protein binding to ICLs which points to a possible role of MMR proteins in cisplatin ICL processing. Deficiency of both BER and MMR shows similar repair kinetics as compared to the cells that are deficient in BER and MMR alone and no increased repair capacity was observed in the absence of both BER and MMR. These results imply that BER and MMR play an overlapping role, and they work together in the same mechanism to maintain cisplatin sensitivity.
Cisplatin ICL induced double strand break (DSB) repair

Cisplatin ICL processing is known to give rise to DSBs [156]. The repair of DNA DSBs thus can be correlated with cisplatin ICL repair [157]. Histone H2AX is phosphorylated in response to DNA DSBs and is a part of foci formed at sites of these breaks due to colocalization of DNA damage sensor proteins [157]. The dephosphorylation or disappearance of these foci indicates the repair of cisplatin ICL induced DSBs. After transfection, the cells were grown on coverslips and were treated with cisplatin. The immunofluorescence assay was performed at specified time points to monitor the repair of DSBs. The cells were probed with anti γH2AX monoclonal antibody for 1 hour. Fluorescent secondary antibody alexafluor-488 was used and the coverslips were then treated with DAPI. At least 200 cells were counted and were plotted as percent foci positive cells at each post treatment incubation time point. Our results show that the number of foci positive cells peaks at 24 hr and they are efficiently repaired from 48 to 72 hr time point. When BER and MMR were downregulated separately and together, faster repair of cisplatin induced DSBs was observed as compared to the BER and MMR proficient WT cells. No increased DSB repair capacity was seen when BER and MMR were downregulated together. These results suggest that the increased resistance observed in the absence of BER and MMR is due to the increased repair of cisplatin ICLs and ICL induced DSBs (Figure 7C) (Supplementary Figure S4 in [155]).

MMR requires polymerase activity of Polβ to mediate cisplatin cytotoxicity

DNA Polβ is a 39 KDa polypeptide that contains a 31 KDa polymerase domain. Asp 256 is one of the three active site aspartates in the polymerase domain, which is critical for
nucleotidyltransferase mechanism. D256A mutation has been shown to completely abolish polymerase activity of Polβ [158]. This mutant lacks the polymerase activity while still retaining the dRP lyase activity. To understand the importance of polymerase domain of Polβ, we re-expressed WT Polβ and a mutant deficient in polymerase activity (D256A) in Polβ knockdown cells. We wanted to understand the involvement of MMR in response to cisplatin in context of these cells. For this reason, we knocked down MSH2 using siRNA and we performed colony survival assays using increasing concentration of cisplatin. The Polβ KD and MSH2 KD cells showed increased resistance to cisplatin. In addition, downregulation of both Polβ and MSH2 did not give rise to any additional increase in cisplatin resistance indicating that these proteins work in the same pathway to mediate cisplatin cytotoxicity. The D256A cells showed cisplatin sensitive phenotype indicating that the polymerase dead mutant can mediate cisplatin sensitivity but loss of Polβ can give rise to cisplatin resistance. Interestingly, when we knocked down MSH2 in D256A mutant cells we did not see any resistance phenotype, indicating that the involvement of MMR is dependent on polymerase activity and is likely dependent on misincorporation at cisplatin ICL sites (Figure 8) (Figure 3C in [155]).

**In vitro Binding of MMR proteins to cisplatin ICL DNA with a mismatch**

Our data suggested that BER and MMR play an overlapping role in mediating cisplatin sensitivity. In this mechanism, our data support the hypothesis that BER and MMR proteins are required to maintain cisplatin sensitivity as they block the repair of cisplatin ICLs. Our previous studies have shown that BER processing occurs at the site near cisplatin ICLs. In addition, Polβ incorporates incorrect nucleotides in the flanking region near cisplatin ICLs even in the presence of correct nucleotides [71].
hypothesized that mutagenic role played by Polβ is responsible for the activation of MMR. Error prone DNA synthesis mediated by Polβ in the region flanking the ICL is essential for generation of a mismatch and for subsequent MMR binding. We hypothesized that this binding by BER and MMR blocks the productive repair of ICLs. To test this hypothesis, we checked for binding of MMR proteins after BER processing of ICL substrates. For this experiment, we generated undamaged uracil and cisplatin ICL DNA substrates containing a uracil adjacent to the ICL. The substrates were processed in a BER reaction containing UDG, APE1 and Polβ proteins in the presence of dNTPs. The BER processing involves the removal of uracil by UDG followed by cleavage by APE1 and DNA synthesis by Polβ. We used these substrates in biotin-streptavidin pull-down experiments using purified hMutSα protein (Figure 9). We observed increased recruitment of MSH2 on the undamaged uracil substrate compared undamaged processed substrate (Lane 3, G:U unprocessed). This is consistent with previous studies that showed increased hMSH2-hMSH6 binding to a G:U mismatch [159]. BER processing at this site can result in the incorporation of a correct nucleotide by Polβ. This results in a reduced binding of hMSH2-hMSH6 at this site (Lane 4, G:U processed). In the case of ICL DNA substrates, however, BER processing leads to increased retention of hMSH2-hMSH6 on the ICL substrates (Lane 6, ICL G:U processed) as compared to unprocessed substrates (lane 5, ICL G:U unprocessed). These results suggest that, at the sites near cisplatin ICL, MMR binding occurs as a result of misincorporation of nucleotides by Polβ.

Our data suggest that MMR binding near cisplatin ICLs occurs downstream of BER processing. The role of MMR in mediating cisplatin sensitivity is dependent on the mutagenic processing by Polβ. BER processing near the cisplatin ICLs and subsequent
MMR binding blocks the repair of ICLs and ICL induced DNA double strand breaks, and thus, BER and MMR play epistatic roles in mediating cisplatin sensitivity (Figure 10) (Figure 5C in [155]).

Targeting XPF-ERCC1, ATM and ATR sensitizes BER and MMR deficient cells to cisplatin

Our data shows that BER and MMR deficiency gives rise to cisplatin resistance. Signaling kinases like ATR and ATM play an essential role in signaling DNA damage followed by cisplatin treatment. ATR inhibition results in cisplatin sensitivity and this inhibition was shown to be synthetic lethal with ATM/p53 signaling defect [160]. We utilized an ATR inhibitor VE-821, to study its cytotoxic effect in BER proficient and deficient human breast cancer cells and MEFs. We found that ATR inhibition sensitized Polβ deficient cells to cisplatin, and they were as sensitive to cisplatin as the WT cells. ATR inhibition also sensitized MEFs to cisplatin under both BER proficient and deficient background (Figure 11A).

In addition, in response to DSBs, ATM detects DNA damage and causes phosphorylation of downstream target effectors such as Chk2, p53, H2AX and 53BP1. These effectors cause cell cycle arrest and facilitate the repair of DNA damage. ICL resolution leads to the formation of DSBs and persistence of DSBs makes cells more sensitive to platinum treatment. ATM plays a key role in the repair of these breaks, so we asked whether inhibiting ATM kinase function will result in sensitization to cisplatin chemotherapy. For this purpose, we used ATM kinase inhibitor KU60019 at IC20 concentration. We pretreated cells with this inhibitor before cisplatin treatment and kept
the cells in inhibitor containing media for a total of 24 hrs. Our colony survival assays show that, ATM inhibition sensitizes human breast cancer cells to cisplatin under both BER proficient and deficient background (Figure 11B). These results show that ATM and ATR inhibitors can be utilized for the sensitization of BER and possibly MMR deficient cells to cisplatin. However, when we pretreated the cells with DNA-PK inhibitor NU7441, we did not see any sensitive phenotype in response to cisplatin (Figure 11C).

XPF-ERCC1 is a structure specific endonuclease which plays a key role in the repair of cisplatin adducts. It is required as a part of the NER pathway for the removal of cisplatin intrastrand adducts. Studies show that XPF-ERCC1 also plays an essential role in providing substrates for homologous recombination and cisplatin ICL repair. We have previously shown that XPF-ERCC1 knockdown results in an increased sensitive phenotype in BER deficient human cancer cells [71]. For this reason we wanted to study whether downregulation of XPF-ERCC1 would also sensitize MMR deficient cells to cisplatin. Inhibition of XPF-ERCC1 in HEC59 cancer cells lines sensitized the cells to cisplatin, indicating that XPF-ERCC1 inhibitors could potentially be used in cisplatin combination therapy for BER and MMR deficient or mutated cancers (Figure 12).
2.4 Discussion

Cisplatin and related platinum compounds are widely used anticancer agents for the treatment of solid tumors. The therapeutic effect is mediated by intrastrand adducts and ICL DNA leading to stalled replication forks which result in cell cycle arrest and apoptosis. Despite initial favorable response, platinum therapy often results in development of resistance. This results in poor prognosis and recurrence of the disease. Therefore, it becomes crucial to understand these mechanisms of cisplatin resistance. Several studies have indicated that the rate of DNA repair is one of the major determinants of cisplatin resistance and increased repair of cisplatin adducts leads to enhanced resistance to the drug. Deficiency or loss of MMR proteins gives rise to resistance to cisplatin. Cisplatin treatment results in the selection of resistant cells that are MMR deficient. We have also shown that BER deficiency also contributes to a cisplatin resistant phenotype. The mutagenic role played by Polβ in bypassing cisplatin adducts suggests a role for the subsequent activation of the MMR pathway. For this reason, we studied the effect of downregulation of BER and MMR pathways on cisplatin cytotoxicity and their potential involvement in a single mechanistic pathway to mediate cisplatin cytotoxicity.

Colony survival assays showed that loss of BER and MMR resulted in ~2 fold resistance to cisplatin which is consistent with the literature. Interestingly, when both BER and MMR pathways were downregulated together, no additional resistance to cisplatin was seen. Therefore we hypothesized that BER and MMR pathways play an overlapping role and function together in the same mechanism. This is the first study that
shows a possible epistatic role of two DNA repair pathways to mediate cisplatin cytotoxicity in mammalian cells.

Numerous studies have shown that MMR proteins bind to cisplatin intrastrand adducts. However, when we checked for the repair of cisplatin GG intrastrand adducts in BER and MMR deficient cells, we did not see any significant difference in the rate of repair in these cells. These data revealed that BER and MMR pathways are not involved in the repair of cisplatin intrastrand adducts. Moreover, these studies also demonstrated that the increased resistance seen in the BER and MMR deficient cells is not due to any change in the rate of repair of intrastrand adducts.

In addition, BER and MMR deficiency results in the increased repair of cisplatin induced ICLs as compared to the WT cells. We show that increased repair of cisplatin ICLs contributes to increased resistance to cisplatin. MSH2 has been previously shown to be involved in the recognition and processing of psoralen ICLs and loss of MSH2 results in hypersensitivity to psoralen. However, in our study, MSH2 deficiency resulted in increased repair of ICLs indicating that ICLs produced by different crosslinking agents are processed differently. In the case of cisplatin, MMR deficiency gives rise to resistance due to increased repair of ICLs and ICL induced DSBs.

We have previously shown that the BER pathway plays a novel role in processing a uracil adjacent to the cisplatin ICL. This uracil is likely produced as result of the deamination of extrahelical cytosines induced by the distorted structure of cisplatin ICLs. Uracil acts as a substrate for the BER pathway and if BER processing leads to nucleotide misincorporation and generation of a mismatch, this can act as a nucleation point for the
MMR pathway. Using ICL DNA substrates containing uracil, we studied the binding of MutSα after BER processing. We observed increased retention of MSH2 on the BER processed ICL substrates indicating that binding of MMR proteins occurs downstream of BER processing. Furthermore, the mutagenic role of Polβ is essential for activation and recruitment of MMR proteins. This is consistent with our data using polymerase dead D256A mutant cell lines in which MMR knockdown results in no cisplatin resistance [155]. We believe that BER and MMR binding near the cisplatin ICL sites interferes with the actual processing of the cisplatin ICLs and therefore, lack of these pathways gives rise to increased repair of ICLs and increased cellular resistance to the drug. This is a first report that shows that MMR processing in the regions near cisplatin ICLs, affects the repair of ICLs and influences cisplatin cytotoxicity. This study highlights a novel mechanism where MMR binding occurs downstream of BER processing and these two pathways play an epistatic role to mediate cisplatin cytotoxicity (Model Figure 10) (Figure 6 [155]).

Our results show that BER and MMR deficiency in tumor cells involves increased repair of cisplatin ICLs. Therefore, we asked whether inhibiting the proteins involved in these DNA repair pathways would sensitize BER and MMR deficient cells to cisplatin. We targeted ATR and ATM signaling kinases in BER proficient and deficient cells human cancer cells. We found that ATR and ATM inhibition lead to sensitization of these cells to cisplatin making them an attractive target for cisplatin combination therapy in cisplatin resistant cells that are deficient in BER or MMR. DNA-PK also plays a key role in non-homologous end joining pathways of DNA DSB repair. The role of DNA-PK in processing of cisplatin mediated DNA damage is not clearly understood. Cisplatin
adducts themselves have been postulated to inhibit DNA-PK activity [161]. In one study, shRNA mediated downregulation of DNA-PK activity lead to the sensitization of cancer cells to cisplatin [162]. On the other hand, it has also been reported that cells deficient in DNA-PK activity are markedly resistant as compared to their WT counterpart [163]. Moreover, reports have shown that DNA-PK inhibition sensitized breast cancer cells to ionizing radiation and doxorubicin [164]. However, when we inhibited DNA-PK using NU7441, we observed increased resistance to cisplatin as compared to the untreated cells. However, the mechanism of cisplatin resistance in DNA-PK deficient cells remains to be clearly established.
Figure 5, Supplementary Figure S1 in [155]: siRNA mediated downregulation of MSH2 in wildtype (A) and Polβ null (B) MEFs. Cells were transiently transfected with siRNA directed against MSH2 as described in Materials and Methods. Proteins were extracted at indicated time points and probed with MSH2 antibody with α-tubulin as a loading control. Control cells were treated with nontargeting siRNA (Ctl si).

(C) Percent transcript knockdown in wildtype and Polβ-/- MEFs. Cells were transiently transfected with siRNA directed against MSH2. At 48 and 72 hr post transfection, total RNA was extracted from cells and the transcript levels were quantified as described in Materials and Methods with β-actin as an endogenous control. The percent transcript knockdown was determined from $2^{\Delta\Delta CT}$ values with non-targeting siRNA as controls.
**Figure 6**

![Graph showing colony survival assay](image)

**Figure 6, Figure 1A in [155]: Cisplatin cytotoxicity** - Colony survival assays in wild type and Polβ null MEFs. Control and MSH2 siRNA transfected cells were treated with increasing doses of cisplatin and cytotoxicity was determined by clonogenic assays. Results are represented as mean ± SD from 3 independent experiments.
Figure 7

A  Intrastrand adduct repair

Post-incubation time
% of intrastrand adducts
0 hr 12 hr 24 hr 48 hr

Wt + siCtrl
Wt + siMSH2
Pol β -/- + siCtrl
Pol β -/- + siMSH2

B  ICL Repair

Post-incubation time
% of ICLs
0 hr 24 hr 48 hr 72 hr

Wt + siCtrl
Wt + siMSH2
Pol β -/- + siCtrl
Pol β -/- + siMSH2

C  DSB repair

Post-incubation time
% γ H2AX foci positive cells
0 hr 24 hr 48 hr 72 hr

Wt + siCtrl
Wt + siMSH2
Pol β -/- + siCtrl
Pol β -/- + siMSH2
**Figure 7A, Supplementary Figure S3: Repair of cisplatin intrastrand adducts** in MEFs. Cells were treated with cisplatin and ELISAs were performed as described in Materials and Methods at different time intervals (0, 12, 24, 48 hr). The percentage of intrastrand adducts present at 0 hr were normalized to 100%. Results are represented as mean ± SD of three independent experiments. Statistical analysis was performed by student’s t test and comparisons are made between wildtype and proficient cells vs deficient cells. NS – non-significant; * - P< 0.05.

**Figure 7B, Figure 2A in [155]: Repair of cisplatin ICLs** in MEFs. Cells were treated with cisplatin and comet assays were performed as described at different time intervals (0, 24, 48 and 72 hr). The percentage of interstrand crosslinks present at each time point was calculated using olive tail moments. Results are represented as mean ± SD of three independent experiments. Statistical analysis was performed by student’s t test and comparisons are made between wildtype and proficient cells vs deficient cells. NS – non-significant; * - P< 0.05.

**Figure 7C, Supplementary Figure S4 in [155]: Repair of DSBs** in MEFs. Cells were treated with cisplatin and immunofluorescence assays were performed at different time intervals (0, 24, 48 and 72 hr). The percentage of foci positive cells were counted for each timepoint. Results are represented as mean ± SD of three independent experiments. Statistical analysis was performed by student’s t test and comparisons are made between wildtype and proficient cells vs deficient cells. NS – non-significant; * - P< 0.05.
**Figure 8**

Figure contributed by Dr. Anbarasi Kothandapani

**Figure 8, Figure 3C in [155] Cisplatin cytotoxicity**, Cells were transfected with siRNA for MSH2, treated with cisplatin and colony survival assays were performed. Results are represented as mean SD from three independent experiments.
**Figure 9, Figure 6C in [155]: Interaction of MSH2 with cisplatin ICL DNA processed with BER proteins.** Undamaged and single cisplatin ICL biotinylated DNA duplexes incubated with BER proteins UDG, Ape1, and Polβ were bound to streptavidin magnetic beads and were incubated with equal amounts of overexpressed hMSH2-hMSH6 insect cell extract. The proteins bound to the DNA beads were eluted and immunoblotted with the antibody against MSH2.
Figure 10: BER and MMR play an epistatic role in mediating cisplatin cytotoxicity. BER processes the DNA flanking the ICL and Polβ produces a mutagenic spectrum at the incision site. Incorporation of incorrect nucleotides would generate mismatched bases and act as a nucleation point for MMR protein binding and further processing. Both BER and MMR processing would result in non-productive repair of cisplatin ICLs. As a result of blocking productive ICL repair pathways, there are persistent cisplatin ICLs which enhance cisplatin cytotoxicity.
**Figure 11:** Targeting signaling kinases in BER proficient and deficient cells. The cells were pretreated with IC20 concentration of VE821 (A), KU60019 (B) and NU7441 (C), followed by cisplatin treatment for 2 hrs. The inhibitors were kept in the media for a total of 24 hrs. The colony survival assays were performed with increasing concentrations of cisplatin. Results are represented as mean ± SD from 3 independent experiments.
Figure 12

Figure contributed by Dr. Anbarasi Kothandapani

**Figure 12: Downregulating XPF-ERCC1 in MMR proficient and deficient cells**
siRNA mediated downregulation of XPF-ERCC1 was carried out in HEC59 and HEC59 +2 (A) cells. The colony survival assays were performed using increasing doses of cisplatin. Results are represented as a mean SD of 3 independent experiments.
Chapter 3

ROLE OF DOWNSTREAM MISMATCH REPAIR PROTEINS IN THE PROCESSING OF CISPLATIN INTERSTRAND CROSS-LINKS

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Running title: Differential role of MMR proteins in modulating cisplatin cytotoxicity

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3.1 Introduction

The DNA mismatch repair system, which is involved in the post replicative repair of mismatches plays a crucial role in the maintenance of genomic stability [165]. In addition to the recognition of mismatches, MMR proteins have also been involved in the recognition and processing of DNA damage inflicted by a number of chemotherapeutic agents, including cisplatin, carboplatin, alkylating agents and 5-Fluorouracil [86, 125, 166-168]. The MMR pathway is composed of recognition proteins with MSH2 as a common partner in two heterocomplexes, namely MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3) [169]. MutSα is required for the repair of base-base mismatches and one base pair insertion deletion loops (IDLs). On the other hand, MutSβ carries out the repair of IDLs with single or multiple base pairs. The mismatch recognition step is followed by the recruitment of downstream MMR proteins MutLα (MLH1-PMS2), Exonuclease I (EXO1), DNA Polδ and DNA ligase.

MMR has also been shown to participate in the DNA damage response after treatment with certain chemotherapeutic agents. Loss of MMR proteins has been associated with resistance to a number of anti-cancer agents (e.g. alkylating agents and cisplatin) [86, 168]. Various models have been proposed for the possible role of MMR in maintaining drug sensitivity. The adducts formed by alkylating agents can result in the generation of mismatches. It has been suggested that MMR proteins take part in futile cycles of repair of these mismatches in the daughter strand. It is believed that, the resulting strand breaks signal apoptosis and loss of this function gives rise to drug resistance. In addition, MMR proteins have been shown to bind to certain types of DNA damage and activate the DNA damage response, eventually resulting in cell death.
However, these studies did not differentiate between different types of adducts that are formed by cisplatin, namely intrastrand adducts, which are formed within the same DNA strand, versus ICLs, which are formed between adjoining strands of DNA. MutSα proteins have been shown to recognize cisplatin intrastrand adducts [122]. In addition, MutSβ was found to be one of the proteins that interact with cisplatin ICLs [82]. However, the exact role of these proteins in the processing of cisplatin adducts has not been clearly evaluated.

Recent studies have shown that MSH3 is required for the repair of DNA DSBs induced by cisplatin and oxaliplatin treatment [170, 171]. Thus, the MMR pathway has been shown to be required for the sensitization of colorectal cancer cells to cisplatin and oxaliplatin, and this effect is believed to be independent of canonical MMR processing. However, other studies have shown that MSH3 proficient cells, which were more resistant to chemotherapy, expressed higher levels of NER proteins which could explain the reason for increased resistance [172]. Thus, the exact role of MSH3 in modulating platinum cytotoxicity remains to be clearly determined.

In our previous studies, we have shown that loss of base excision repair (BER) and MMR proteins gives rise to resistance to cisplatin and these two pathways take part in the same mechanism to mediate cisplatin sensitivity (Chapter 2) [71, 155]. In the absence of these proteins, increased repair of cisplatin ICLs was observed which leads to decreased cellular cytotoxicity and cisplatin resistance. We also showed that this mechanism is dependent upon the low fidelity of Polβ, which leads to misincorporation of bases and generation of base mismatches. These mismatches, in turn, activate the mismatch repair pathway. In this report, we distinguish between the requirements of
different downstream MMR proteins to mediate this effect. We show that, in contrast to previous studies, there is a clear distinction between MMR recognition heterocomplexes where MutSα is required to maintain cisplatin sensitivity, and MutSβ seems to play no role at least in breast cancer cell lines and mouse embryonic fibroblasts. Moreover, we show that the ATPase activity of MLH1 is required for maintaining a cisplatin sensitive phenotype, highlighting the importance of the mismatch repair activity in the processing of cisplatin ICLs.

3.2 Materials and Methods

Chemicals and Antibodies

Cisplatin, oxaliplatin and myricetin were purchased from Sigma-Aldrich. All other chemicals and reagents were from standard suppliers. Antibodies directed against MSH3, MSH6 and MLH1 were from BD Pharmigen and α-tubulin was from Sigma-Aldrich. For the stock preparation, cisplatin and oxaliplatin were diluted in 1X PBS and vortexed vigorously until the drug dissolved completely. The stock concentration was 1 mM. Cisplatin was prepared fresh before each experiment. Stock for oxaliplatin was stored at -80 ºC up to 6 months and thawed at room temperature (RT) when needed.

Cell lines

The human breast adenocarcinoma MDA-MB-231 cells were grown in RPMI 1640 containing 10% FBS and antibiotics. MDA-MB-231 Polβ knockdown cells (Polβ lentiviral shRNA) were grown in the presence of 0.5μg/mL puromycin. The development and characterization of the MDAMB-231/Polβ-KD cells were described previously [152].
Wild-type (92TAg) and Polβ null (88TAg) primary mouse embryonic fibroblasts were cultured in high glucose DMEM supplemented with 10% FBS plus antibiotics at 37ºC in a humidified atmosphere under 10% CO₂. The HCT116 cells were grown in DMEM F-12 media with 10% FBS, antibiotics and 600 μg/ml of geneticin. The human colon cancer HCT116 cell lines were characterized by Dr. Anatoly Zhitkovich, Brown University. These HCT116 cells are deficient in MLH1. These cells were reconstituted with WT MLH1, S44L ATPase mutant MLH1 and S44F ATPase mutant MLH1. The MLH1 deficient human colon cancer cells HCT116, chromosome 3 complemented sub-line HCT116+3 were obtained from Dr. Kandace Williams, The University of Toledo. The chromosome complemented sublines were maintained in 400μg/ml of geneticin.

**shRNA transfection**

Mission shRNA plasmid bacterial stocks directed against human MSH6 and MSH3 were obtained from Sigma Aldrich. The plasmid DNA was purified using a plasmid purification maxi prep kit from Qiagen. Lentiviral particles were packaged using 293FT cells with the help of 3rd generation packaging plasmids PMD2G, PMDLG/RRE and PREV/RRE. Lipofectamine 2000 reagent (Invitrogen) was used for the transfection of the plasmid DNA. The media was changed after 24 hrs of transfection. The viral particles were harvested 48 hr and 72 hr after the transfection by centrifugation followed by filtration through 0.2 micron filters. The viral stocks were stored as aliquots at -80ºC for future use. At the time of the experiment, the viral stocks were used along with polybrene (Sigma Aldrich) for the knock down of proteins of interest. Cells were harvested at the 72 hr timepoint post transduction to check for protein and transcript knockdown.
siRNA transfection

ON-TARGET plus SMART pool siRNAs specific for human MSH3 and MLH1 were purchased from Dharmacon RNAi technologies, Thermo Scientific. The non-targeting control siRNA was used as a control for non-specific effects. siRNA transfection was carried out as per the manufacturer’s protocol. Briefly, the cells were plated in 6 well plates in the antibiotic free media. At the time of transfection, the cell density was maintained at 60-70% and two transfections were done with an interval of 24 hrs. Dharmafect transfection reagent 1 and 4 were used for MEFs and MDA-MB-231 cells, respectively. The cells were harvested at 48 and 72 hr timepoints after transfection for the detection of protein and transcript knockdown.

Western blot analysis

Cells were harvested at 96, 120, 144 hrs after the infection, washed with PBS and lysed in lysis buffer (10 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing protease inhibitors (0.5 M phenyl methyl sulphonyl fluoride PMSF, 1 mg/ml Leupeptin and 1 mg/ml pepstatin A). The proteins were separated on 8% SDS-polyacrylamide gels and transferred onto Immobilon-P transfer membranes (Millipore). After blocking (2% non-fat dry milk), the membranes were probed with primary antibodies recognizing human MLH1, with α-tubulin as loading control. The membranes were incubated with appropriate secondary antibodies and the signal was detected by using Enhanced chemiluminescence detection system.
Real time PCR for the measurement of transcript levels

At indicated post-transfection time points, cells were harvested and pelleted. RNA was isolated using TRIzol reagent (Invitrogen) by standard procedures. The total RNA was reverse transcribed using MMLV reverse transcriptase enzyme (Invitrogen) as per the manufacturer’s protocol. The transcript levels were quantified using iQ SYBR green supermix (Bio-Rad) in iCycler iQ System, with GAPDH as an endogenous control. The percent transcript knockdown was determined from $2^{-\Delta\Delta CT}$ values.

Colony survival assay

Cells (~500) were treated with increasing concentrations of cisplatin for 2 hr. After treatment, fresh medium was added, and the cells were allowed to grow for 7-14 days. Colonies were fixed with 95% methanol and stained with 0.2 % crystal violet. Colonies with $\geq 50$ cells were counted, and colony survival was expressed as the ratio of the average number of colonies in drug treated cells versus control cells x 100. The experiment was done in triplicates for each drug concentration. Statistical analyses were performed using student’s t test comparing wild type and knock down cells.

Alkaline comet assay

Alkaline comet assay was used to analyze the repair of cisplatin interstrand cross-links (ICLs) as described [153, 154]. Cell suspensions (~10,000 cells) were embedded on a microscopic slide, lysed and incubated in ice-cold alkaline solution for 20 min to allow the DNA to unwind. Electrophoresis was carried out for 30 min at 28 V, 300 mA. Slides were neutralized and stained with SYBR green (Invitrogen). The comets were scored
using a Nikon epifluorescence microscope. At least fifty cells were analyzed per slide using Komet Assay Software 5.5F (Kinetic Imaging, Liverpool, UK). The data was expressed as the percentage of crosslinks that remained at that particular time point normalized to 100% at 0 hr.

**Immunofluorescence**

Double-strand break (DSB) repair was assessed by monitoring the nuclear γ-H2AX foci by immunofluorescence. Cells were fixed using 4% paraformaldehyde, permeabilized and probed with monoclonal anti γ-H2AX antibody (1:500, Millipore). The images were visualized using a Nikon Eclipse T2000-U microscope. Foci were counted in at least 200 cells at each time point per condition in each cell line and results are expressed as % γ-H2AX foci positive nuclei.

**MTS assays**

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was used to evaluate cisplatin or oxaliplatin cytotoxicity. Cells were treated with increasing concentration of cisplatin for 2 hr. After 72 hr, 20 μL of combined MTS/PMS solution [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate] was added to each well and incubated for 2-4 hr at 37°C. The absorbance was measured at 490 nm in Spectramax M5 plate reader (Molecular Devices). The percentage of cell survival was calculated from the average OD of treated cells/average OD of control cells x 100. Statistical analyses were performed using student’s t test comparing wild type and knock-down/ mutant cells.
Drug treatment

The cells were treated with myricetin alone to assess its effect on cell viability (Data not shown). From these curves, a concentration of 10 µM (IC$_{20}$) was chosen, so as to have minimum influence on cell viability with Myricetin alone. The cells were pretreated with this concentration for 2 hrs followed by treatment with increasing concentration of cisplatin for 2 additional hrs. MTS assay was then performed as described.

3.3 Results

Requirement of different MMR recognition heterocomplexes to maintain cisplatin sensitivity

To differentiate between the role of MutSα and MutSβ in mediating cisplatin sensitivity, colony survival assays were performed using wild type and Pol β deficient MDA-MB-231 cells. We knocked down MSH3 or MSH6 using shRNA lentivirus directed against these proteins. The knockdown efficiency was analyzed at both the protein and transcript level using western blot analysis and real time PCR, respectively (Figure 13 A-F). The level of knockdown was found to be 80 – 90 % compared to the controls. Using clonogenic assays, we found that MSH6 deficient cells were resistant to cisplatin as compared to the wild type cells (Figure 14 A). Loss of Polβ also gave rise to cisplatin resistance which is consistent with our previous results (Chapter 2) [71, 155]. However, down regulation of MSH6 in Pol β deficient cells did not give rise to any additive increase in the degree of resistance reinforcing the epistatic role of MMR and BER proteins in mediating cisplatin cytotoxicity. In addition, we also performed clonogenic assays using MSH3 knockdown cells, and we found that down regulation of
MSH3 did not have any effect on cisplatin cytotoxicity. Similar to the wild type cells, MSH3 deficient cells maintained a cisplatin sensitive phenotype (Figure 14 B). Comparable results were observed in wild type and Polβ null mouse embryonic fibroblasts (Supplementary Figure S1). These data indicate that MSH6 is required for mediating cisplatin sensitivity, whereas, MSH3 does not influence cisplatin cytotoxicity in the cell lines used in this study.

Previously, we have shown that BER and MMR processing of the ICLs is dependent upon the unique structure produced by the cisplatin ICL. This distorted extrahelical structure is not produced to the same extent by any other platinating agents. For this reason, we studied cell viability in response to oxaliplatin in MSH3 and MSH6 knockdown cells using MTS assays (Supplementary Figure 2A, 2B). Cell survival studies showed no statistical difference in the oxaliplatin cytotoxicity highlighting the fact that the role of MMR proteins in modulating chemosensitivity is specific to cisplatin. In support of these studies, Zdraveski et al have shown that the oxaliplatin adducts are not recognized by MMR proteins [173].

**Differential role of MMR recognition proteins in cisplatin ICL repair**

Enhanced DNA repair has been shown to be a major mechanism in the development of cisplatin resistance [174]. In previous studies, MMR proteins have been shown to bind to the cisplatin GG adducts [122, 142]. In our previous studies, we have shown that downregulation of MSH2, which is a common partner of MutSα and MutSβ heterocomplexes, does not influence the rate of repair of cisplatin intrastrand adducts (Chapter 2) [155]. Based on our previous observations, we did not expect MSH3 and
MSH6 to be involved in the repair of cisplatin intrastrand DNA adducts. As the repair of intrastrand adducts was expected to be unaffected, we hypothesized that the rate of repair of cisplatin ICLs would influence cisplatin cytotoxicity. For this reason, we studied cisplatin ICL DNA repair using a modified alkaline comet assay in MSH3 or MSH6 knock down cells. Using this assay, we observed increased repair of cisplatin ICLs over the time course of 0-72 hr in all cell types with no difference in the repair rate up to the 24 hr time point. At the 48 hr and 72 hr time point, we observed increased repair of cisplatin ICLs in Polβ deficient cells consistent with our previous studies [71, 155]. However, MSH6 knockdown in both WT and Polβ deficient cells showed increased repair of cisplatin ICLs as compared to WT cells suggesting the involvement of MSH6 in cisplatin ICL processing (Figure 15 A). Moreover, we did not see any additive increase in the repair capacity in the absence of both MSH6 and Polβ compared to the repair capacity in the cells lacking either MSH6 or Polβ alone. In addition, we also performed similar assays using MSH3 knockdown cells. In contrast to MSH6 knockdown cells, we did not see any change in the rate of repair of cisplatin ICLs in these cells (Figure 15 B). It should be noted that the modified alkaline comet assay used in this report is sensitive to the initial unhooking of the ICL from the double-stranded DNA and therefore, this assay can represent the involvement of MMR proteins in the initial stages of the ICL repair. Thus, these data suggested that MSH6 interferes with or inhibits the repair of cisplatin ICLs whereas MSH3 does not participate in the cisplatin ICL processing which leads to a similar rate of repair of ICLs as observed in WT cells. Moreover, increased repair rates have been shown to be one of the mechanisms of cisplatin resistance and poor prognosis,
and these data correlate well with the increased cisplatin resistance observed after down regulation of MSH6 (Figure 14A).

Cisplatin ICL processing leads to the generation of DNA DSBs. The ATM kinase recognizes the DSBs and phosphorylates a histone variant H2AX at serine 139. This causes recruitment of downstream DSB repair proteins. Phosphorylated γH2AX proteins form distinct nuclear foci and can be studied using an immunofluorescence assay. In this assay, we observed induction of γH2AX foci after cisplatin treatment followed by a decrease in γH2AX positive cells from 24 hr to 72 hr time points indicating DNA repair. The Polβ deficient cells showed a decreased percentage of γH2AX foci positive cells at 48 hr and 72 hr as compared to WT cells consistent with our previous report [71]. MSH6 knockdown cells also showed decreased percentage of γH2AX foci positive cells and therefore, increased repair of cisplatin ICL induced DSBs as compared to the WT cells (Figure 16 A). These data validate the involvement of MSH6 in the processing of cisplatin ICLs. However, in contrast to some of the other studies done in colorectal cancer cells lines [170, 171], MSH3 knockdown cells did not show any change in the rate of repair DNA DSBs as compared to WT breast adenocarcinoma cell lines used in this study (Figure 16 B). These results suggest that loss of MSH3 does not affect the repair of cisplatin ICLs and ICL induced DSBs.

**MutL homolog 1 (MLH1) plays a key role in maintaining cisplatin sensitivity**

MLH1 is the human homologue of the E. coli MMR gene MutL. The MMR pathway involves recognition of a base mismatch or insertion/deletion loop by a MutS homolog followed by recruitment of a MutLa heterodimeric complex consisting of
MLH1 and PMS2 [169]. To understand the importance of MLH1 in mediating cisplatin cytotoxicity, we knocked down MLH1 using siRNA in MDA-MB-231 breast cancer cells and MEF cell lines. The knock down efficiency at both the transcript and protein level were found to be 80-90% (Figure 17 A, B, C). Colony survival assay was performed to address the effect of MLH1 downregulation on cell viability in response to cisplatin (Figure 18). MLH1 knock down in WT cells showed ~2 fold resistance to cisplatin as compared to the control cells. However, MLH1 knock down in Polβ deficient cells did not give rise to any additional increase in cisplatin resistance indicating an overlapping role of these two distinct DNA repair proteins in the same mechanism to mediate cisplatin sensitivity. To understand the mechanism of resistance, we checked for the effect of MLH1 knockdown on the rate of cisplatin adduct repair. As mentioned earlier, increased rate of repair of intrastrand adducts and/or ICLs is concomitant with increased drug resistance. We performed enzyme linked immunosorbent assays using a monoclonal antibody specific for the cisplatin GG adduct, which is a major intrastrand adduct formed by cisplatin. Knockdown of BER and MMR showed no difference in the repair of intrastrand adducts indicating that these pathways do not influence the repair rate of cisplatin GG adducts (Figure 19A). As cisplatin intrastrand adduct repair was unaffected, we checked for the repair of cisplatin interstrand crosslinks. Modified alkaline comet assays were used to evaluate the rate of repair of ICLs over a period of 0-72 hr time points after cisplatin treatment. Downregulation of Polβ showed a decreased percentage of ICLs at 48 hr and 72 hr time points. MLH1 has shown to be required for signaling DNA damage and activating the DNA damage response pathway in reaction to psoralen crosslinks [175]. In our studies in response to cisplatin, depletion of MLH1 in the WT as
well as Polβ deficient cells resulted in increased repair of ICLs (Figure 19 B). In addition, similar results were observed in an immunofluorescence assay where MLH1 KD cells showed increased repair of ICL induced DSBs as compared to the WT cells (Figure 19 C). These results indicate that increased repair of ICLs accounts for the cisplatin resistant phenotype seen in these cells. Furthermore, we observed similar levels of cisplatin ICL repair in the absence of both BER and MMR pathways as compared to the knockdown of MMR and BER alone. These data indicate an epistatic relationship of these two pathways in mediating cisplatin sensitivity.

**ATPase activity of MLH1 is essential for cellular sensitivity to cisplatin.**

We have shown that BER and MMR play an epistatic role in mediating cisplatin sensitivity (Chapter 2) [155]. This overlapping role is dependent upon the error prone nature of Pol β which generates a mismatch while processing cisplatin ICL flanking DNA, thereby leading to the activation of the MMR pathway. Our results indicate that the complete knockdown of MLH1 gives rise to a cisplatin resistant phenotype, suggesting that a functional downstream MMR pathway is essential to maintain cellular sensitivity to cisplatin. Next, to confirm the involvement of MMR and the actual processing of the mismatch, we utilized human colon cancer cell lines that are deficient in the MLH1 ATPase activity (Figure 20 A). The HCT116 cells are deficient in MLH1. These cells were reconstituted with plasmid vectors expressing WT MLH1, S44L and S44F ATPase mutant MLH1. Owing to a point mutation that affects the serine residue at position 44 in the ATPase domain, these cells are deficient in mismatch repair activity. We utilized these cell lines to differentiate between the effects of possible binding of MLH1 to the mismatch as opposed to the actual MMR processing of the mismatch by MLH1.
We studied the expression of MLH1 in these mutants and found both protein and transcript levels to be comparable to the expression in cells reconstituted with WT MLH1 (Figure 20 B, 20 C). After that, we checked the effect of these mutations on cisplatin cytotoxicity using an MTS assay. The MLH1 deficient empty vector cells showed a cisplatin resistant phenotype as compared to the MLH1 proficient WT cells, consistent with our previous results. Moreover, the MLH1 ATPase mutant cells also showed a cisplatin resistant phenotype indicating the importance of the ATPase activity of MLH1 in mediating cisplatin sensitivity (Figure 20 D). In contrast, previous studies using mouse embryonic fibroblasts have shown that loss of MLH1 ATPase activity still maintains sensitivity to cisplatin, suggesting the direct DNA damage signaling by MMR proteins as the mediator of cisplatin sensitivity [88, 135]. Our studies in human cells, however, showed that this activity is essential for cellular sensitivity, indicating that different ATPase mutations can have a differential effect on cisplatin cytotoxicity. Furthermore, we did not see any significant difference in the survival profiles of these cells in response to oxaliplatin (Supplementary Figure 2C). These results suggest that these effects are specific to cisplatin and can be attributed to the unique structure produced by cisplatin ICLs.

To understand the mechanism of resistance and to appreciate the events taking place near the ICLs, we studied how these ATPase mutations affect the repair of cisplatin ICLs. We used modified alkaline comet assays to study the rate of ICL repair up to the 72 hr time point after cisplatin treatment. MLH1 deficient empty vector cells showed increased repair of cisplatin ICLs at 48 hr and 72 hr time point. Moreover, the MLH1 ATPase mutant cells also showed increased repair of cisplatin ICLs at 48 hr and 72 hr
time points as compared to the MLH1 proficient WT cells (Figure 21 A). In addition, in an immunofluorescence assay MLH1 deficient empty vector cells and MLH1 ATPase mutants also showed increased repair of ICL induced DSBs as compared to the WT MLH1 proficient cells (Figure 21 B). These results indicated that the ATPase activity is essential to maintain cisplatin ICLs on the DNA, and therefore, this increased repair can be correlated with the resistant phenotype observed in the cell survival studies.

This role of MLH1 is dependent on the generation of a mismatch due to BER processing. To understand whether in these cells MMR and BER pathways play an overlapping role, we inhibited BER in HCT116 cells using myricetin. Myricetin is a small molecule inhibitor of the repair function of Apurinic apyridiminic endonuclease 1 (APE1). Pretreatment with myricetin gave rise to a cisplatin resistant phenotype in MLH1 proficient WT cells, which is consistent with our previous findings. However, when we used MLH1 deficient empty vector cells and MLH1 ATPase mutant cells, which are already resistant to cisplatin, myricetin pretreatment did not give rise to any additive increase in the degree of resistance (Supplementary Figure 3A-3C). These results suggest that MMR processing occurs downstream of BER and these two pathways play an epistatic role in mediating cisplatin cytotoxicity.

**Targeting XPF-ERCC1 in MLH1 deficient cancer cells**

Our studies showed that MLH1 deficiency gave rise to cisplatin resistance. Our results suggest that in the absence of MLH1, cisplatin ICLs are repaired at a faster rate compared to WT cells. XPF-ERCC1 is essential for various stages of ICL repair. Our previous studies have shown that downregulation of XPF ERCC1 can be used to sensitize
BER deficient cancer cells [71]. When we knocked down XPF-ERCC1 in MLH1 deficient HCT116 cells, we observed increased sensitivity to cisplatin (Figure 22 A). Similarly, a double knockdown of MLH1 and ERCC1 gave rise to a sensitive phenotype in response to cisplatin (Figure 22 B). These results suggested that inhibition of XPF-ERCC1 can potentiate cisplatin cytotoxicity in MLH1 deficient cancer cells.

In conclusion, our results suggest that downstream MMR proteins play a differential role in mediating cisplatin cytotoxicity. In addition, the ATPase activity of MLH1 is essential for the processing of the BER induced mismatch near the cisplatin ICL region and this MMR processing hinders the productive repair of cisplatin ICLs and thus, gives rise to a cisplatin sensitive phenotype (Model Figure 10, Chapter 2). In the absence of MMR processing, ICLs are repaired at an increased rate giving rise to cisplatin resistance, and these cells can be sensitized by inhibition of XPF-ERCC1.

3.4 Discussion

The role of MutS and MutL homologues in the repair of ICLs has been previously studied using psoralen as a DNA damaging agent [175, 176]. However, the role of individual MMR proteins in the processing of cisplatin ICLs remains unexplored. Cisplatin forms ICLs that have a unique structure as compared to most other platinum agents as well as other cross linking agents. As a consequence, cisplatin treatment leads to the recruitment of different DNA repair proteins and initiation of different DNA repair pathways. Previously, we have shown for the first time the crosstalk between BER and MMR pathways in response to cisplatin ICLs. We have shown that the cisplatin ICL structure, which involves the flipping out of the flanking cytosine residues, makes them
prone to undergo oxidative deamination. These deamination events lead to the conversion of cytosine residues to uracil. Uracil acts as a substrate for the initiation of the BER pathway. BER processing near the cisplatin ICLs involves error prone DNA synthesis by Polβ. The low fidelity nature of Pol β generates a mismatch, which in turn, leads to the activation of the MMR pathway. We have shown an increased recruitment of the MMR protein MSH2 on the BER processed substrates as compared to BER unprocessed substrates [155]. The aim of this work was to understand the requirement of the downstream MMR proteins in mediating cisplatin sensitivity. It was anticipated that this information would help us better understand the mechanism of the crosstalk between BER and MMR pathways near a cisplatin ICL site. Furthermore, it will help us to elucidate the role of MMR proteins in the complex process of cisplatin ICL repair. Repair of ICLs is a complicated process and the exact mechanism of the ICL repair and how different repair proteins play a role is still unknown. From a clinical point of view, increased repair of ICLs has been shown to be associated with resistance to the drug and poor prognosis [31, 157]. Therefore, understanding the mechanisms of cisplatin ICL repair and evaluating the role of different proteins in these pathways becomes clinically relevant.

We are not the first to study the effect of an MSH3 defect on cisplatin cytotoxicity. Recently, it was shown that MSH3 is involved in the repair of cisplatin ICL induced DNA DSBs [170, 171]. In contrast, Vaismann et al. have shown that MSH3 deficiency does not have any significant effect on cisplatin or oxaliplatin cytotoxicity [124]. Consistent with these studies, when we knocked down MSH3 in the breast adenocarcinoma MDA-MB-231 cell line, we did not see any effect on cisplatin or
oxaliplatin cytotoxicity indicating that MSH3 does not influence platinum cytotoxicity at least in this cell line. These observations suggest that the MSH3 deficiency should still retain sensitivity to cisplatin based chemotherapy. Depletion of MSH3 in Polβ deficient cells did not give rise to a cisplatin sensitive phenotype indicating that MSH3 is not essential and does not play any significant role in pathways that maintain cisplatin sensitivity. In addition, MSH6 knockdown cells showed a cisplatin resistant phenotype consistent with the literature [124, 136]. Furthermore, MSH6 downregulation in Pol β knock down cells did not give rise to any additive increase in the cisplatin resistance, indicating that these proteins play an overlapping role and are required to maintain cisplatin sensitivity.

In accordance with the cell survival studies, we wanted to elucidate the mechanism of cisplatin resistance in MSH6 deficient cells. Increased repair of adducts from the DNA has been shown to be one of the major mechanisms of cisplatin resistance. MSH2 forms a common partner in MutSα and MutSβ heterocomplexes. Previously, we have shown that lack of MSH2 does not affect the rate of cisplatin intrastrand adduct repair [155]. For this reason, we did not expect MSH3 and MSH6 to affect intrastrand adduct repair either. When we studied ICL repair in MSH3 knockdown cells, we did not see any change in the rate of cisplatin ICL repair as well as ICL induced DNA DSBs. Zhu and Lippard have shown that MutSβ is one of the proteins that bind specifically to cisplatin ICLs [82], indicating that MSH2-MSH3 complex might play some role in the repair of cisplatin ICLs. Based on these observations, we thought that MSH3 might be one of the proteins that is recruited to the cisplatin ICL site. However, it is not absolutely essential, as lack of MSH3 did not affect cellular response to cisplatin within our
experimental conditions. MSH6 deficiency has been known to cause cisplatin resistance. Various models have been proposed to explain the mechanism. There have been studies that link futile cycles of mismatch repair of cisplatin intrastrand adducts with increased sensitivity. Some other studies have shown that MSH6 is essential to signal apoptosis. These studies have been attributed to all kinds of adducts formed by cisplatin overall. However, we found that MSH6 knockdown resulted in the increased repair of cisplatin ICLs and ICL induced DNA DSBs. These results suggested that the drug sensitizing effects of MMR proteins are dependent on the processing of cisplatin ICLs with MutSα and not MutSβ being required to maintain a cisplatin sensitive phenotype.

Inactivation of MLH1 has been shown to give rise to cisplatin resistance. In our studies, knockdown of MLH1 in Pol β deficient cells did not give rise to any additional increase in the degree of resistance, suggesting that these two proteins are involved in the same pathway that mediates cisplatin cytotoxicity. The difference in the sensitivities could be correlated with the difference in the rate of cisplatin ICL repair and ICL induced DSBs. These results are consistent with an involvement of the entire downstream MMR pathway in maintaining cisplatin sensitivity. We hypothesized that, the MMR pathway tries to repair the mismatch that is generated due to error prone DNA synthesis by Polβ. This mismatch repairing ability of MMR proteins is dependent upon the ATPase domain of these proteins. This activity is essential for the initiation of the repair processes that recruit downstream repair proteins and facilitate the removal of the mismatch. To understand the importance of these processes in response to cisplatin, we utilized the MLH1 ATPase mutant cell lines. MLH1 missense mutations form about 65% of all the MMR mutations associated with HNPCC syndrome [177]. Many of these mutations
occurs in the ATPase domain of this protein giving rise to mismatch repair deficiency and a high level of microsatellite instability [178]. S44F mutations in the ATPase domain have frequently been associated with the HNPCC syndrome [177]. In our cell lines, these mutations did not have any significant effect on the level of mutant MLH1 protein. The absence of ATPase activity led to a cisplatin resistant phenotype, indicating that this mismatch repairing ability represents the essential activity that maintains a cisplatin sensitive phenotype.

Previous studies have shown that an MLH1 ATPase G67R mutation still retains sensitivity despite having a defect in the mismatch repair capacity [135]. These studies point toward the role of MMR proteins in the activation of processes that directly signal apoptosis. Different models have been proposed to explain the role of MMR proteins in cisplatin mediated cytotoxicity. The futile cycle model involves the futile repair of base mismatches generated in response to alkylating agents, which gives rise to strand breaks ultimately leading to cell death. In our studies, the MLH1 deficient and MLH1 ATPase mutant cells showed a resistant phenotype in response to alkylating agent MNNG (Supplementary Figure 4). The expression of MGMT in the ATPase mutant cells was comparable to that in the WT cells (Supplementary Figure 5A). On the other hand, a second model involves the role of MMR proteins as DNA damage sensors which directly signal apoptosis in response to alkylating and platinating agents [88, 135, 136, 140]. PMS2 binds to MLH1 and forms MutLα. This complex is involved in all MMR processes. In response to cisplatin treatment, PMS2 has been shown to interact with p73 to signal apoptosis [137, 139]. ATP binding to MLH1 has been shown to enhance the interaction between MLH1 and PMS2. In our cell lines, an ATPase mutation in MLH1
did not have any effect on the stability of PMS2 which would mean the signaling function of PMS2, if any, remained unaffected (Supplementary Figure 5B). Our studies suggest that, the MMR protein binding near the ICL site hinders the repair of ICLs which in turn results in cisplatin sensitivity. The ATPase mutant MLH1 protein lacks the repair capacity but retains its function as a DNA damage sensor, if such an activity is required for cisplatin sensitivity. Cell survival studies in the ATPase mutant cell line show that this activity is essential for mediating cisplatin cytotoxicity. This would predict that either MLH1 does not have any DNA damage signaling role in response to cisplatin ICLs or ATPase mutants do not stick around at the damage site long enough as they are unable to repair the damage. These possibilities will be examined in future studies. Alternatively, our study shows that MLH1 ATPase S44L and S44F mutations behave differently as compared to the MLH1 ATPase G67R mutation studied before, suggesting that different MLH1 ATPase mutations could respond differently to cisplatin therapy.

Initially, we set out to differentiate between the futile repair cycle model and the direct DNA damage signaling model involving MMR proteins in response to cisplatin ICLs. However, one caveat here is that the specific domains in the MMR proteins that are required for sensing and signaling DNA damage are still unknown. These domains can be attributed to any residues in the ATPase domain itself, because of this, the role of MMR proteins as DNA damage sensors cannot be refuted. Therefore, we still cannot make any definite conclusions about the role of direct signaling by MMR proteins in the development of cisplatin resistance. Considering that the MMR deficient ATPase mutants showed increased cisplatin resistance, we believe the mismatch repair ability and MMR
processing near the ICL sites plays a crucial role in maintaining cisplatin sensitivity consistent with our ICL repair data.

When we looked at how these mutations affect the events taking place near the ICL site by using alkaline comet assay, we found that these mutant cells as well as MLH1 null cells showed increased repair of ICLs and ICL induced DNA DSBs. These results suggest that the repair activities by MMR proteins or the MMR processing near the ICL are essential to maintain ICLs on the DNA and therefore, to maintain a cisplatin sensitive phenotype. This increased repair of cisplatin ICLs can be due to reduced binding of the MLH1 mutant proteins at these sites due to an inability to process and repair the mismatch. This, in turn, results in increased repair of ICLs through an enhanced accessibility of NER and HR proteins to the ICL site. On the other hand, the increased repair of ICLs can also be due to increased expression of NER and HR proteins which are involved in the repair of ICLs. In contrast, there have been some other studies which showed that the increased resistance in the MSH3 proficient cells was dependent on the increased expression of XPF and ERCC1 proteins which are involved in ICL repair [172]. Various studies have shown that increased expression of these proteins is responsible for the development of cisplatin and oxaliplatin resistance [30, 36]. We studied the expression of XPF and ERCC1 proteins for a period of 0 to 72 hr after cisplatin treatment, and we did not see any change in the expression levels of these proteins in cisplatin treated (Supplementary Figure 6) or in untreated cells (Data not shown) which rules out enhanced HR or NER protein expression. These studies indicate that the MMR proteins process the Polβ induced mismatch near the cisplatin ICL site, block productive ICL repair and give rise to cisplatin sensitivity (Model figure 10).
Whereas, lack of MMR provides enhanced accessibility to ICL DNA repair proteins which results in increased repair of cisplatin ICLs.
Figures

MSH3 Knockdown in wt MDA-MB-231

A

MSH3 Knockdown in Poβ KD MDA-MB-231

B

MSH6 Knockdown in wt MDA-MB-231

C

MSH6 Knockdown in Poβ KD MDA-MB-231

D

MSH3 Transcript knockdown in MDA MB 231 cells

E

MSH6 Transcript knockdown in MDA MB 231 cells

F

Figure 13: shRNA mediated downregulation of MSH3 in wildtype (A) and Poβ KD (B) MDA-MB-231 cells and MSH6 KD in wildtype (C) and Poβ KD (D) MDA-MB-231 cells. Cells were transfected with shRNA directed against MSH3 or MSH6 as described in Materials and Methods. Proteins were extracted at indicated time points and probed with MSH3 and MSH6 antibody with α-tubulin as loading control.

Percent transcript knockdown in wildtype and Poβ KD MDA-MB-231 cells: MSH3 KD (E) MSH6 KD (F). Cells were transiently transfected with shRNA directed against MSH3 and MSH6. At 96 and 120 hr post transfection, total RNA was extracted from cells and the transcript levels were quantified as described in Materials and Methods with GAPDH as an endogenous control. The percent transcript knockdown was determined from 2^-ΔΔCT values with non-targeting shRNA as controls.
Figure 14: Cisplatin cytotoxicity. Colony survival assay in MDA-MB-231 cells (A). Control (closed circles), MSH3 (open triangles), Polβ (closed squares) and Polβ MSH3 KD (open circles). shRNA transfected cells were treated with increasing doses of cisplatin and cytotoxicity was determined by colony survival assay. (B) Colony survival assay in MDA-MB-231 cells: Control (closed circles), MSH6 (open triangles), Polβ (closed squares) and Polβ MSH6 KD (open circles). Results are represented as mean ± SD from 3 independent experiments.
Figure 15. Repair of cisplatin ICLs in MDA-MB-231 (A) MSH3 KD in MDA-MB-231 (B) MSH6 KD in MDA-MB-231 Cells were treated with cisplatin for 2 hrs and comet assays were performed as described at different time intervals (0, 24, 48 and 72 hr). The data was collected using komet 5.5 software. The percentage of interstrand crosslinks present at each time point was calculated using olive tail moments. Results are represented as mean ± SD of three independent experiments. Statistical analysis was performed by student’s t test and comparisons are made between wildtype and proficient cells vs deficient cells. NS – non-significant; * - P< 0.05.
Figure 16: Repair of γH2AX foci in MDA-MB-231 (A) MSH3 KD (B) MSH6 KD

Cells were treated with cisplatin for 2 hrs and immunofluorescence was performed as described at different time intervals (0, 24, 48 and 72 hr). A minimum of 200 cells were analyzed for each time point. The percentage of γH2AX foci positive cells at each time point was calculated. Results are represented as mean ± SD of three independent experiments. Statistical analysis was performed by student’s t test and comparisons are made between wildtype and proficient cells vs deficient cells. NS – non-significant; * - P< 0.05
Figure 17

A  MLH1 Knockdown in wt MEF

<table>
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B  MLH1 Knockdown in Polβ−/− MEF

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C  Transcript knockdown

% MLH1 transcript knockdown

<table>
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<tr>
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<tr>
<td>72 hr</td>
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**Figure 17: knockdown efficiency** in wildtype (A) and Polβ−/− (B) MEFs, Cells were transiently transfected with siRNA directed against MLH1. At 96 and 120 hr post transfection, Proteins were extracted at indicated time points and probed with MLH1 antibody with α-tubulin as loading control.

**C** Percent transcript knockdown in wildtype and Polβ−/− MEF cells: Cells were transiently transfected with siRNA directed against MLH1. At 96 and 120 hr post transfection, total RNA was extracted from cells and the transcript levels were quantified as described in materials and methods with B actin as an endogenous control. The percent transcript knockdown was determined from \(2^{-\Delta\Delta CT}\) values with non-targeting siRNA as controls.
Figure 18: Colony survival assay in WT and Pol β null MEFs with MLH1 KD. Cells were treated with increasing doses of cisplatin for 2 hrs. Results are represented as mean SD of 3 independent experiments.
Figure 19

A  Intrastrand adduct repair

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<td>24 hr</td>
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<td>48 hr</td>
<td>40</td>
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Wt + siCtrl
Wt + siMLH1
Polβ -/- + siCtrl
Polβ -/- + siMLH1

B  ICL repair

<table>
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<td>48 hr</td>
<td>60</td>
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<td>72 hr</td>
<td>40</td>
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Wt + siCtrl
Wt + siMLH1
Polβ -/- + siCtrl
Polβ -/- + siMLH1
Figure 19: Cisplatin adduct repair in WT and Polβ null MEFs with MLH1 KD (A). ELISA: MLH1 KD in MDA-MB-231 cells. Cells were treated with cisplatin for 2 hrs and genomic DNA was extracted at the indicated time points. ELISA was performed using an antibody specific for cisplatin GG intrastrand adduct and the percentage of GG adducts remaining was calculated as described. Results are represented as mean ± SD of three independent experiments. Statistical analysis was performed by student’s t test and comparisons are made between wild type and proficient cells vs deficient cells. NS – non-significant (B) Alkaline comet assay was performed in WT and Pol β null MEFs as described previously. (C) Immunofluorescence assay results for MLH1 KD in WT and Pol β null MEF cells.
Figure 20

A

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<th>PMS2 binding domain</th>
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<tr>
<td></td>
<td>S44L</td>
<td>S44F</td>
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</tr>
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</table>

B

MLH1 protein Expression

WT 3.6 Empty vector S44L S44F

MLH1

α-tubulin

C

MLH1 Transcript levels

% MLH1 Transcript knockdown

Empty vector WT 3.6 S44L S44F

Figure 20: Graphical representation of the MLH1 ATPase mutants (A), (B) Western blot analysis and (C) real-time PCR were performed to check the expression levels of MLH1 transcript and protein.
**Figure 20 D: MTS assay** was performed by treating the HCT116 cells using increasing concentration of cisplatin for 2 hrs. WT cells (closed diamonds), Empty vector (open triangles), S44L cells (closed squares) and S44F cells (open circles). Results are represented as mean ± SE from 3 independent experiments.
**Figure 21**

**ICL repair**

![ICL repair graph](image)

**DSB repair**

![DSB repair graph](image)

**Figure 21: Repair of interstrand crosslinks in HCT116 cells (A)** Alkaline comet assay was performed as described previously. Repair of DNA DSBs in HCT116 cells was measured using an immunofluorescence assay with an antibody specific for γH2AX as described previously (B)
Figure 22: Downregulating XPF-ERCC1 in MLH1 proficient and deficient cells

siRNA mediated downregulation of XPF-ERCC1 was carried out in HCT116 and HCT116 +3 (A) cells. (Figure contributed by Dr. Anbarasi Kothandapani). (B) siRNA mediated downregulation of MLH1 and ERCC1 separately and together. The colony survival assays were performed using increasing doses of cisplatin. Results are represented as a mean SD of 3 independent experiments.
Supplementary Figure S1, The colony survival assay was performed in WT and Polβ-/- MEF using an MSH3 specific siRNA (A). The cells were treated with increasing concentration of cisplatin to assess cytotoxicity. Results are represented as mean SD of 3 independent experiments. Percent transcript levels show the knockdown efficiency of MSH3 (B)
Supplementary Figure S2

A

MTS assay

B

MTS assay
Supplementary Figure S2. Oxaliplatin cytotoxicity was measured by using MTS assay for MSH3 KD in WT and Polβ deficient MDA-MB-231 cells (A), MSH6 KD in WT and Polβ deficient MDA-MB-231 cells (B) and HCT116 cells (C). The cells were treated with increasing concentrations of oxaliplatin for 2 hrs.
Supplementary Figure S3

A  MTS assay

B  MTS assay
Supplementary Figure S3: MTS assay was used to assess cisplatin cytotoxicity after myricetin pretreatment. The cells were pretreated with myricetin for 2 hrs followed by treatment with increasing concentration of cisplatin in WT 3.6 (A), Empty vector (B) and S44L cells (C).
Supplementary Figure S4: MTS assay was used to assess MNNG cytotoxicity. The cells were treated with MNNG for 45 mins. MTS assays were performed as described in Materials and Methods. The results are represented as mean SD of 3 independent experiments.
**Supplementary Figure S5**

**A**

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<th>S44F</th>
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MGMT  
α-tubulin

**B**

<table>
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</table>

PMS2  
α-tubulin

**Supplementary figure S5: Western blot analysis** for MGMT (A) and PMS2 (B) expression in HCT116 cells. Approximately 50 µg of protein extract was loaded and western blot analysis was performed as described in materials and methods.
Supplementary Figure S6

XPF-ERCC1 expression in HCT116 cells after cisplatin treatment

Supplementary Figure S6: Expression of XPF and ERCC1 was checked in HCT116 cells after cisplatin treatment. The cells were treated with cisplatin for 2 hrs and were harvested over a period of 0 – 72 hr time points. The western blot analysis was performed as described previously.
Chapter 4

Overall summary

Cisplatin is still one of the most widely used chemotherapeutic agents for the treatment of a variety of human malignancies. It forms a basis of a number of treatment protocols and combination therapies. Despite its successful use, cisplatin based chemotherapy often develops resistance. This drug resistance can be acquired or it can be intrinsic in nature. Numerous studies have shown the correlation between increased repair of cisplatin adducts and increased resistance to the drug. Earlier, cisplatin intrastrand adducts were considered to be responsible for the clinical efficacy of the drug. It was believed that these adducts are clinically most relevant as they are formed more frequently than cisplatin ICLs, their cellular levels correlated well with clinical efficacy and clinically ineffective transplatin did not form these adducts. Recent studies have considered cisplatin ICLs to be more cytotoxic as compared to the other types of adducts formed by cisplatin. These ICLs are formed at a lower rate and the synthesis of defined ICL substrates has been difficult, thus limiting the studies related to the biological and clinical importance of cisplatin ICLs. Nonetheless, cisplatin ICL repair rate has been correlated with sensitivity of tumor cells. Furthermore, recent studies have reported that cisplatin ICL repair is dependent on TC-NER, but not GG-NER, outside of replication. These
studies predicted that when cells enter S phase, this would lead to higher relative levels of ICLs compared to intrastrand adducts, thus sensitizing tumor cells. These studies highlight the clinical importance of ICLs. However, the exact mechanism and the repair pathways involved in the repair of ICLs are still not very well understood. Enhanced repair of cisplatin ICLs has been correlated with increased cisplatin resistance. Understanding the mechanisms that affect the repair of ICLs, therefore, is clinically relevant. DNA repair pathways including BER and MMR have shown to modulate cisplatin cytotoxicity. Our studies have shown how these pathways influence cisplatin ICL repair and affect cisplatin cytotoxicity.

Lack of BER and MMR pathways give rise to cisplatin resistance. Studies have shown that cisplatin treatment leads to the enrichment of MMR deficient drug resistant cells. These effects are detrimental to cisplatin based chemotherapy. Our studies showed that, increased resistance in case of MMR and BER deficient cells can be attributed to increased repair of cisplatin ICLs. The rate of intrastrand adduct repair was not affected in BER and MMR deficient background. In addition, we did not see any resistant phenotype in response to oxaliplatin. These results suggested that the influence of BER and MMR status on cisplatin cytotoxicity was cisplatin ICL specific. In addition, our studies highlighted a novel mechanism where two DNA repair pathways are involved in modulating cisplatin ICL repair. Our data suggested that this possibility was created by mutagenic role of Polβ. Polβ mediated misincorporation of nucleotides leads to the generation of mismatches, and these mismatched bases activate and recruit MMR proteins to the regions near the ICLs. We showed that BER processing in these regions is essential for the binding and recruitment of MMR proteins. In support of these
observations, using Polβ D256A polymerase mutant cells, we showed that MMR activation and involvement in this pathway is dependent on the polymerase activity of Polβ. In these studies, Polβ D256A cells which are deficient in polymerase activity were shown to be sensitive to cisplatin. Alterations in Polβ activity and Polβ mutations are a common occurrence in cases of colon cancer. Currently, majority of the colon cancer cases are treated with oxaliplatin. It will be interesting to study how different Polβ mutations respond to cisplatin and whether these subsets of patients can be treated more effectively with cisplatin.

Subsequently, we also studied the requirement of different MMR proteins in this mechanism. We showed that MutSα is required to maintain a cisplatin sensitive phenotype, whereas MutSβ had no role in mediating cisplatin cytotoxicity. In addition, MLH1 is required to maintain a sensitive phenotype, suggesting that the entire MMR pathway is essential for this process. Clinically relevant MLH1 ATPase mutants, which are deficient in MMR also showed increased resistance owing to increased repair of ICLs when compared with WT cells. From our studies, we predict that BER and MMR proteins process the regions that flank the cisplatin ICLs, and this processing interferes with the productive ICL repair by NER and HR proteins. As a consequence, ICLs are maintained on the DNA for a longer time giving rise to increased cytotoxicity in response to cisplatin. When we targeted XPF-ERCC1 in MMR deficient cells, we observed that inhibition of adduct repair sensitizes the cells to cisplatin. Moreover, targeting signaling kinases ATR and ATM also sensitized BER deficient cells to cisplatin, indicating the potential use of these inhibitors in cisplatin based combination therapy. We believe that, understanding the exact mechanisms of the pathways that affect ICL repair will lead to
better treatment protocols and generation of new protein targets that will increase the sensitivity and efficacy of cisplatin based chemotherapy in BER and MMR deficient cancer cells.
Reference List


