NOVEL ROLE OF BASE EXCISION REPAIR (BER) IN MEDIATING CISPLATIN CYTOTOXICITY*

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Using isogenic mouse embryonic fibroblasts and human cancer cell lines, we show that cells defective in BER display a cisplatin specific resistant phenotype. This was accompanied by enhanced repair of cisplatin interstrand crosslinks (ICLs) and ICL-induced DNA double strand breaks (DSBs), but not intrastrand adducts. Cisplatin induces abasic sites with a reduced accumulation in uracil DNA glycosylase (UNG) null cells. We show that cytosines that flank the cisplatin ICLs undergo preferential oxidative deamination in vitro, and AP endonuclease 1 (APE1) can cleave the resulting ICL DNA substrate following removal of the flanking uracil. We also show that DNA polymerase β has low fidelity at the cisplatin ICL site after APE1 incision. Down-regulating ERCC1-XPF in BER deficient cells restored cisplatin sensitivity. Based on our results, we propose a novel model in which BER plays a positive role in maintaining cisplatin cytotoxicity by competing with the productive cisplatin ICL DNA repair pathways.

Cisplatin is used in the treatment of a variety of malignancies including testicular, ovarian, cervical, head and neck, and lung cancers. It forms different types of DNA adducts including monoadducts, intrastrand adducts and interstrand crosslinks (ICLs). These DNA adducts distort the DNA helix thereby blocking DNA replication and transcription, causing G2 arrest and apoptosis. Cells use multiple DNA repair mechanisms to repair/remove the cisplatin damaged DNA. These DNA repair systems are detrimental to the cytotoxic efficacy of the drug (1). Increased DNA repair capacity of cancer cells is an important mechanism of cisplatin resistance (2). Repair of the cisplatin intrastrand adducts is primarily via the nucleotide excision repair (NER) pathway which is a versatile pathway for the removal of a variety of bulky lesions, whereas repair of the cisplatin ICLs is less understood (3,4). Although ICLs comprise ~5-10% of the total cisplatin DNA adducts formed, they are believed to be a significant cytotoxic lesion. The structure of cisplatin ICL shows a unique feature in which the cytosines adjacent to the cross-linked guanines are flipped extrahelical and are exposed to the cellular environment (5). This has a significant impact in the way these adducts are recognized and repaired.

It appears that several pathways are involved in the repair of ICLs including Nucleotide Excision Repair, Homologous Recombination (HR) and Fanconi Anemia pathway (FA) (4,6,7). The ICL DNA repair pathways have been shown to be both replication-dependent (8) and replication-independent (9). Recent evidence suggests a possible role of BER in ICL repair (10), where targeting BER showed enhanced sensitivity to crosslinking agents such as cisplatin, mitomycin C and psoralen (11-13). The involvement of BER has also been demonstrated in the lesion bypass of cisplatin DNA adducts (14,15). This has clinical relevance as many cancer types have been shown to have deregulated BER protein levels (11,16,17). An Oncomine™ (Compendia Bioscience, Ann Arbor, MI) search revealed that cisplatin resistant cells display enhanced as well as reduced expression of BER proteins in tumor samples and cancer cells. This suggests that loss of BER may cause an up-
regulation of effective cisplatin DNA repair or increase adduct tolerance.

Based on the recent studies targeting BER proteins to sensitize cells to antitumor agents and due to the existing conflicting observations, we evaluated the effect of defective BER on cisplatin cytotoxicity using isogenic mouse embryonic fibroblasts (wild-type, Polβ−/− and Ung−/−) and human cancer cells (MDA-MB-231, HeLaS3 and A2780). We also utilized methoxyamine (MX), a small molecule inhibitor, which binds to abasic sites and inhibits APE1 cleavage, ultimately blocking BER. We show that blocking BER with MX as well as deficiency of Polβ and UNG conferred resistance to cisplatin treatment. Complementing Polβ in the knock-down cells restored cisplatin sensitivity implicating the role of Polβ in cisplatin resistance. We report that defective BER does not affect the processing of cisplatin intrastrand adducts, but is involved in modulating cisplatin ICL DNA repair. We also show that cisplatin induces abasic sites, which could be in part due to deamination of exposed cytosines flanking the cisplatin specific ICL DNA structure. Utilizing synthetic oligonucleotides containing a site-specific cisplatin ICL, we demonstrate that preferential deamination occurs on the flipped out cytosine bases adjacent to the cross-linked guanines compared to undamaged and intrastrand adduct containing DNA substrates. Following uracil removal, the abasic site formed adjacent to cross-linked guanines serves as a substrate for APE1 incision and also subsequent gap filling by Polβ. Similar to cisplatin intrastrand adduct substrates, Polβ has poor fidelity at cisplatin ICL sites. Based on these observations, we propose a novel model in which the processing events mediated by BER at sites adjacent to cisplatin ICLs compete with the productive cisplatin ICL DNA repair events. ERCC1-XPF deficient cells gained sensitivity to cisplatin in both BER proficient and deficient cells and the resistant phenotype was not observed in BER deficient cells. These data suggest that BER proteins compete with NER and HR in mediating cisplatin cytotoxicity likely as a result of processing the DNA that flanks the cisplatin specific ICL. This results in non-productive ICL DNA repair and leads to persistent cisplatin ICLs which maintain cisplatin cytotoxicity.

**Experimental Procedures**

Additional details about the Experimental Procedures can be found in the Supplemental Information.

**Cell lines-** Wild-type (92TAg), Polβ null (88TAg) and UNG null (202TAg) primary mouse embryonic fibroblasts were kindly provided by Dr. Sam Wilson (NIEHS). MEF cells were cultured in high glucose DMEM (Atlanta Biologicals Inc.) supplemented with 10% FBS plus antibiotics at 37°C in a humidified atmosphere under 10% CO2. MDA-MB-231 cells were grown in RPMI-1640 containing 10% heat inactivated FBS and gentamycin (10µg/ml). MDA-MB-231 Polβ knockdown cells (Polβ lentiviral shRNA) were grown in the presence of 0.5µg/mL puromycin and the wild-type Flag Polβ reconstituted in Polβ knockdown cells were grown in 0.5µg/mL puromycin + 700µg/mL geneticin. The development and characterization of the MDA-MB-231/Polβ-KD cells and the corresponding Flag-Polβ complemented cells were described previously (18). HeLaS3 and A2780 cells were cultured in DMEM and RPMI-1640 respectively.

**Colony survival assay-** Cells were (~400) treated with increasing concentrations of drugs for 2 hr. 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 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.

**MTS assay-** MTS assay was performed according to the manufacturer’s instructions (Promega). The percentage of cell survival was calculated from the average OD of treated cells/ average OD of control cells x 100. The experiment was done in triplicate for each drug concentration.

**Cisplatin Intrastrand adduct measurement-** The repair kinetics of cisplatin intrastrand adducts was assessed by ELISA using a specific monoclonal antibody against cisplatin-intrastrand adducts (ICR4, kindly provided by Michael J. Tilby, University of Newcastle, UK) (19). Genomic DNA was coated on 96 well ELISA plates and
probed with ICR4 antibody. 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 1 M 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 as described previously (20).

Cisplatin Interstrand crosslink measurement- Alkaline comet assay was used to analyze the repair of cisplatin interstrand cross-links (ICLs) as described (20,21). Cell suspensions (~10000 cells) were embedded on a microscopic slide, lysed and incubated in ice-cold alkaline solution for 20 min. Electrophoresis was carried out for 25 min at 28 V, 300 mA. Slides were neutralized and stained with SYBR green (Trevigen). 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 time 0 hr.

\[ \gamma H2AX \] immunofluorescence- Double-strand break (DSB) repair was assessed by monitoring the nuclear \( \gamma \)-H2AX foci by immunofluorescence. Cells were fixed, permeabilized and probed with monoclonal anti \( \gamma \)-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 % \( \gamma \)-H2AX foci positive nuclei.

Abasic site quantification- Abasic sites were measured using DNA damage quantification kit from Dojindo Molecular Technologies, Inc according to the manufacturer’s instructions. The number of abasic sites in the drug treated samples per 100,000 bp was determined.

Cytosine deamination assay- The cytosine deamination assay was performed by treating the oligonucleotide substrates with sodium bisulfite according to the protocol described in the EZ DNA Methylation kit (Zymo Research). Briefly, 100 fmol of each substrate was mixed CT conversion reagent and loaded onto the Zymo-Spin IC columns. After desulphonation, the bound DNA was eluted. The eluted DNA substrates were incubated with UDG (10 min, 37°C), to remove any uracils that had formed and further incubated with APE1 (30 min, 37°C), to cleave the abasic sites that were generated after uracil removal. The reaction mixtures were denatured by heating at 95°C for 5 min and then loaded onto sequencing gels. The products were resolved on a sequencing gel. The gel was dried and exposed to X-ray film.

APE1 cleavage assay- Four different DNA substrates were used for the cleavage assay. The DNA substrates were either left untreated or treated with cisplatin to form cisplatin ICLs as described previously (22). Both undamaged substrate and ICL substrate containing an abasic site adjacent to guanine were also prepared. 50 fmol of each of the DNA substrates with and without an abasic site were incubated with APE1 for 1 hr at 37°C and resolved on a sequencing gel. The gels were dried and exposed to X-ray film.

Polβ extension and fidelity assay- DNA substrates containing a uracil adjacent to guanine were treated with UDG and subsequently with APE1, and used for the extension studies. Undamaged and cisplatin ICL DNA substrates (5 nM) were incubated with individual radiolabeled dNTPs (66 nM) as well as a pool of dNTPs in the presence of Polβ at 37°C for 1 hr. The reaction products were resolved on a sequencing gel, dried and exposed to X-ray film.

Transfection with siRNAs- Transfections were carried out as described previously (20). Briefly, MDA-MB-231 cells were seeded in 6 well plates and two transfections were done at 24 hr interval to target XPF and ERCC1 based on the manufacturer’s instructions.

Statistical analysis- Statistical analysis was performed by student’s t test using R.2.7.0 software.

RESULTS

Defective BER has differential effects on sensitivity of DNA damaging agents. We examined cisplatin induced cytotoxicity in Polβ deficient, UNG deficient and MX treated cells. Defective BER resulted in a cisplatin resistant phenotype compared to parental wild-type (wt) cells (Fig 1). We observed ~2 - 2.5 fold increase in cisplatin resistance in Polβ and Ung null cells when
comparing IC_{50} values (Fig 1A). This is in accordance with previous reports where Polβ−/− cells were resistant to cisplatin treatment (23). In HeLaS3, A2780 and wt MEF cells, MX treatment conferred resistance to cisplatin (Fig 1B, 1C and 1A). MX binds to abasic sites and MX-adducted sites are refractory to APE1 incision (24). Inhibiting APE1 activity and ultimately BER through MX treatment resulted in a cisplatin resistant phenotype in our study. It has been shown that MX protects against alkylating agents in CHO cells (25). In the MDA-MB-231 cells, down regulation of Polβ also showed cisplatin resistance. Polβ knock-down cells complemented with wt Polβ restored cisplatin sensitivity (Fig 1D). This rescue experiment clearly indicates that down-regulation of Polβ mediates cisplatin resistance.

In order to assess whether the effect we observe is a general phenomenon of platinum DNA damaging agents, we assessed oxaliplatin cytotoxicity in these cell lines (Supplemental Fig S1). We observed minimal changes in cell sensitivity to oxaliplatin in the BER deficient/inhibited cells. This shows that the resistant phenotype we observe during defective BER is specific to cisplatin. To assess whether the resistant phenotype in BER deficient cells is general to DNA damage or is cisplatin specific, we utilized transplatin, a trans isomer of cisplatin, mitomycin C, which induces ICLs and an intrastrand specific agent, UV light (Supplemental Fig S2). Wt+MX, Polβ−/− and Ung−/− MEF cells were hypersensitive to mitomycin C and to a lesser extent to transplatin and UVC. These results suggest that the resistant effect that we observe in BER deficient/inhibited cells is not a consequence of general intrastrand or ICL DNA damage, but rather specific to cisplatin.

**BER is not involved in the repair of cisplatin intrastrand adducts.** Cisplatin cytotoxicity is attributed to the DNA adducts that are formed following treatment. The change in sensitivity to cisplatin that we observe should relate to the amount of intrastrand and/or ICL DNA adducts. Therefore, we evaluated the mechanism of BER mediated cisplatin resistance by assessing the repair of cisplatin intrastrand adducts and ICLs during defective BER. The intrastrand adduct repair at various time intervals was calculated as the percent of adducts repaired over time, relative to the percent of adducts present at the initial time point, 2 hr post-incubation (Supplemental Fig S3). In BER proficient and deficient cells, the levels of intrastrand adducts were elevated at 12 hr and repaired efficiently from 24 to 48 hr. During the time course of repair, we observed no significant difference in intrastrand adduct removal when comparing these cell lines. These data indicates that the increased cisplatin resistance that we observe during defective BER is not due to increased intrastrand adduct DNA repair. It should be noted that cells deficient in NER are defective in the repair of the intrastrand adducts (20,21).

**Defective BER enhances cisplatin ICL DNA repair.** We then investigated the effect of BER on cisplatin ICL DNA repair by a modified version of the alkaline comet assay. The comet assay has been used to evaluate DNA interstrand crosslink induction and repair *in vivo* at the single cell level (20). The repair kinetics of cisplatin ICLs was evaluated over a period of 0, 24, 48 and 72 hr post-treatment and was expressed as the percentage of crosslinks that remained at that particular time point (Fig 2). Cisplatin ICLs were removed in wild-type parental cells from 0 to 48 hr (Fig 2A). There was no observable difference in the repair of ICLs at 24 hr. At 48 hr, however, there was a significant decrease in cisplatin ICLs in the Polβ−/−, Ung−/− and MX treated wt MEF cells compared to wt cells. MX treatment in HeLaS3 and A2780 cells also resulted in a significant decrease in ICLs at the 48 and 72 hr time point when compared to wt parental cells (Fig 2B and 2C). Polβ deficiency in MDA-MB-231 cells also displayed less ICLs at 48 and 72 hr, while complementation with Polβ resulted in slower cisplatin ICL repair similar to wt cells (Fig 2D). Considering the lethality of the ICLs, we believe that the faster repair of ICLs is the contributing factor that leads to cisplatin resistance in BER deficient/inhibited cells. NER has been suggested to be involved in unhooking of cisplatin ICLs and also in the later stages of ICL processing (26,27). Accordingly, persistence of cisplatin ICLs and enhanced cytotoxicity are reported in NER deficient cells (20). We also investigated the role of oxaliplatin ICL repair in MEF and MDA-MB-231 cells (Supplemental Fig S4). Oxaliplatin ICLs were repaired from 24 to 72 hr in parental cells. The ICL repair rate in BER deficient cells was
identical to the parental cells at all the time points. Therefore, it can be concluded that BER is not involved in oxaliplatin ICL processing, consistent with defects in BER having no effect on cell sensitivity to oxaliplatin (Supplemental Fig S1).

Defective BER enhances DSB repair. To further support the comet assay and evaluate cisplatin ICL DNA repair, we monitored nuclear γH2AX foci in MEFs and MDA-MB-231 cells. γH2AX localizes to DNA double-strand breaks (DSBs), which are the consequence of DNA replication fork collapse and ICL processing. γH2AX is an intermediate of the major ICL DNA repair pathway and the repair of DNA DSBs correlates with the repair of ICLs (28). The percent of γH2AX foci positive cells was calculated over a period of time after cisplatin treatment (Fig 3). At 24 hr, γH2AX formation peaked in wt cells and was still detectable at 48 hr (Fig 3A). In wt+MX, Polβ+/− and Ung+/− cells, although the induction was similar at 0 hr, γH2AX foci formation was reduced at 24 and 48 hr compared to wt cells. A similar trend was observed in the γH2AX foci formation in MDA-MB-231 cells (Fig 3B). At 0 hr and 24 hr, the % of γH2AX foci positive cells was similar in wt, Polβ KD and Polβ KD + Wt Polβ cells. γH2AX foci formation was significantly decreased in the knock-down cells at 48 and 72 hr compared to wt cells and the knock-down cells complemented with Polβ. BER deficient/inhibited cells showed faster removal of γH2AX foci compared with BER proficient wt cells. The percent of γH2AX foci positive cells correlates with the faster rate of ICL repair as seen in Fig 2. This is in support of our hypothesis, where enhanced repair of ICLs and ICL-induced DSBs is likely responsible for cisplatin resistance in BER defective cells.

Differential induction of abasic sites. Abasic sites are intermediates of the BER pathway, which are formed either spontaneously by hydrolysis of the N-glycosylic bond or following chemical modification of the bases by DNA damaging agents and subsequent base removal by DNA glycosylases (29). In order to assess the involvement of BER in cisplatin cytotoxicity, we tested the ability of cisplatin, oxaliplatin, mitomycin C and transplatin to induce AP sites. Cells were treated with increasing concentration of drugs to determine the number of AP sites generated per 100,000 bases in genomic DNA. We used H2O2 as a positive control in our experiments as H2O2 has been known to cause rapid induction of AP sites (Supplemental Fig S5A) (30). The treatment of cells with cisplatin and oxaliplatin resulted in a concentration-dependent increase in the number of AP sites above the level of basal induction (Fig 4A and 4B). This concentration dependent induction of AP sites was not observed in the cells treated with mitomycin C and transplatin (Supplemental Fig S5B and S5C). Cisplatin induced AP sites were 1-2/100,000 bp higher than oxaliplatin and statistically significant (Fig 4C).

It has been shown that cisplatin and oxaliplatin induce abasic sites due to oxidative damage (31,32). However, in the time course experiment, oxaliplatin (Fig 4C) induces significantly less abasic sites compared to cisplatin at all time points when the cells are treated with the respective IC50 concentrations. This shows that in addition to oxidative DNA damage, there might be additional events that could contribute to the higher level induction of AP sites in cisplatin treated cells. We speculate that the extrahelical cytosines at the cisplatin-ICLs undergo oxidative or protein induced deamination and get converted to uracil, which are removed by UNG to create AP sites. Consistent with this idea, in the Ung null cells, the number of AP sites generated following cisplatin treatment was significantly reduced when compared with wild-type cells (Fig 4D). This difference in the UNG dependent abasic sites is consistent with the level of cisplatin induced ICLs per 100,000 bp. There was no significant difference between the induction of AP sites in untreated wild-type and Ung null cells (4.84 ± 0.06 and 3.85 ± 0.04, respectively). Oxaliplatin treatment resulted in the induction of the same level of AP sites in both cells. These findings suggest that BER may play a role in the processing of cisplatin specific damaged DNA to generate abasic sites.

Cisplatin ICL flanking DNA undergoes preferential deamination. The induction of abasic sites upon cisplatin treatment prompted us to determine whether deamination could occur at the cisplatin damaged ICL sites on the DNA. The bisulfite conversion method was used to assess whether cytosines near the platinum adducts were susceptible to deamination and conversion to uracil. We prepared synthetic DNA substrates.
containing site-specific cisplatin intrastrand GG adducts and ICLs (22). We incubated the DNA substrates with sodium bisulfite for 15 min, without the addition of UDG and APE1 and the DNA remained intact during the entire deamination procedure suggesting that the DNA does not undergo any unexpected modifications during the incubation (data not shown). These substrates were also incubated with sodium bisulfite for 5 and 15 min followed by incubation with UDG and APE1 (Fig 5A). Lanes 1, 5 and 9 represent the DNA substrates alone. When the DNA substrates were incubated with UDG and APE1 without incubation of sodium bisulfite, no cleavage was observed (lanes 2, 6 and 10) indicating that the cleavage is dependent on the presence of uracil. Very little cleavage was observed at the center of the undamaged and the GG substrates following sodium bisulfite incubation (Lanes 3, 4, 7 and 8). However, in the ICL substrate, we observed 3 major products corresponding to the three cytosines that are adjacent to the cross-linked guanine suggesting preferential deamination at the DNA flanking the ICL site (lanes 11 and 12). In all three substrates, we observed loss of the 3’α-dCTP[32P] with increasing time after sodium bisulfite incubation likely as a result of chemical degradation occurring at the DNA termini. These data indicates that the flipped out cytosines adjacent to cisplatin cross-linked guanines undergo preferential deamination, suggesting that BER processing could occur on the flipped out cytosine following oxidative deamination and the generation of an abasic site.

APE1 cleaves abasic sites in the ICL DNA substrate. Since APE1 cleaves at an abasic site formed after the removal of uracil by UDG, we tested whether APE1 could cleave at an abasic site adjacent to a cisplatin ICL. We incubated the undamaged and ICL DNA substrates which do not contain an abasic site with APE1 and observed no cleavage for both substrates (data not shown). This suggests that the presence of an abasic site is a primary requirement for the cleavage by APE1 on cisplatin ICL DNA substrates. In order to further test whether the presence of an abasic site results in cleavage, we prepared undamaged and cisplatin ICL DNA substrates containing a synthetically produced abasic site and incubated them with APE1 (Fig 5B). In a time course experiment, we followed the cleavage of the substrates over a period of time (0, 1, 5, 10, 15, 30 and 60 min) upon incubation with APE1. The DNA substrates are 3’ end labeled by sequenase extension and upon APE1 incision, a labeled 21 base product is expected. We observed an incised product of 21 bases in both the undamaged and ICL DNA substrate upon incubation with APE1 (lanes 2-7 and 9-14, respectively). The efficiency of incision is higher on undamaged DNA with an abasic site compared to the ICL DNA substrate (5X more enzyme) and this could be due to alteration of enzymatic activity resulting from the structural distortion imposed on the double helical structure of DNA by the cisplatin ICL. These data demonstrates that APE1 has the ability to cleave at an abasic site 3’ of the cisplatin ICL.

Polβ extension and lack of fidelity. After APE1 incises at an abasic site, a 5’-deoxyribose-phosphate (5’dRP) moiety would remain unless removed by the lyase activity of Polβ. We tested whether Polβ can synthesize past a cisplatin ICL in the presence or absence of APE1 (Fig 5C). The ICL structure influences the migration of DNA products on a denaturing gel and this could complicate the analysis. Therefore, we treated all ICL substrates with NaCN to remove the cisplatin ICL prior to sequencing gel analysis (33,34). Lanes 1 and 5 represent the control reactions without the addition of the enzymes. The DNA substrates were incubated with APE1 in the absence (lanes 2 and 6) or presence of Polβ (lanes 3 and 7). In the absence of APE1 incision, Polβ had no synthesis activity for both undamaged and ICL substrate (lane 3 and 7). We observed incorporation of the correct dCTP nucleotide by Polβ following APE1 incision (lanes 4 and 8). Interestingly, we observed incorporation of three dCTP nucleotides into the cisplatin ICL DNA and only one dCTP into the undamaged substrate at low concentrations of dCTP (50fmol). However, higher concentrations of α-dCTP[32P] gave rise to equal incorporation in both DNA substrates (data not shown). Additional nucleotide incorporation in the ICL could be the result of enhanced strand displacement synthesis by Polβ, due to the distortion created by the cisplatin ICL DNA structure. These data demonstrates that Polβ has the ability to incorporate nucleotides at an ICL site after APE1 incision.
We also tested the fidelity of Polβ at the ICL site, since Polβ has been shown to have very low fidelity at sites of DNA damage (Fig 5D). We incubated undamaged and ICL DNA substrates with Polβ (after APE1 incision) along with α-dCTP[32P] (lanes 2 and 9), α-dATP[32P] (lanes 3 and 10), α-dTTP[32P] (lanes 4 and 11), and found incorporation of nucleotides in all three cases. This suggests that Polβ can incorporate incorrect bases at the ICL site and this low fidelity nature of Polβ could likely lead to the activation of the mismatch repair (MMR) pathway at the cisplatin ICL processing site. However, these experimental data do not indicate the actual incorporation profile of Polβ when it has equal access to all the dNTPs in the cell. In order to test this, we incubated undamaged and DNA ICL substrates with a pool of dNTPs and observed a similar lack of fidelity (lanes 5-7 and 12-14). We also quantified the incorporation of incorrect base and normalized it to incorporation of the correct base (cytosine). We observed that Polβ misincorporates dATP at ~1.6 fold higher frequency compared to dCTP in the ICL substrate (compare lane 13 to 12). However, dTTP was incorporated at similar frequency (~0.9 fold) as the correct base (compare lane 14 to 12). The quantification results are summarized in a table below Figure 5D. These data collectively indicate that Polβ has a low fidelity at the cisplatin ICL site even in the presence of the correct nucleotides.

**DISCUSSION**

Base excision repair is one of the major pathways involved in the removal of damaged bases and DNA single-strand breaks. In addition to the non-bulky lesions, evidences suggest that crosslinks are also substrates for the BER machinery. This could be an important determinant linking BER to ICL processing. Significant efforts have also been made in identifying the role of BER in cisplatin cytotoxicity. Polβ and APE1 are over expressed in various cancer cells which relates to cisplatin resistance (11,16). Over-expression of Polβ leads to bifunctional DNA damage tolerance and facilitates the error-prone translesion synthesis over the adducts that otherwise would block DNA replication and kill the cells (14,36). However, in the same studies that showed over-expression of Polβ in tumor cells, some of the tumor samples showed reduced levels of Polβ (17). In our study, we observed that down-regulation of DNA Polβ in human cancer cells as well as MEFs resulted in enhanced resistance to cisplatin. Raaphorst et al (2002) observed that Polβ null cells were more resistant to cisplatin compared to the wt cells. Another study showed that a Polβ inhibitor conferred resistance to cisplatin in wt MEFs (37). Also, cisplatin resistant cells expressed decreased levels of UNG in certain cancers (Oncomine™, Compendia Bioscience, Ann Arbor, MI) and consistent with this, UNG deficiency resulted in a cisplatin resistant phenotype in the current study. We also utilized methoxyamine (MX) to inhibit the endonuclease activity of APE1 by binding to the abasic sites in the BER pathway. A study by Yan et al (2007) has shown that combined treatment of temozolomide with MX induced topoisomerase (topo) II expression and subsequently enhanced temozolomide sensitivity (38). MX treatment in our study consistently
conferred cisplatin resistance in normal as well as cancer cells. In the study by Yan et al, topo II cleaved the MX adducts when located at topo II cleavage sites in vitro. Further studies are warranted to identify the effect of MX on topo II induction following cisplatin treatment to fully address if the cisplatin resistance that we observe is solely due to APE1 inhibition. BER deficiency/inhibition showed minimal response to oxaliplatin, transplatin, mitomycin C and UVC. This indicates that cytotoxicity of crosslinking agents in the context of BER is not general, but cisplatin specific.

As stated earlier, enhanced repair of DNA lesions has been implicated as one of the important mechanisms of resistance to cisplatin. Therefore, we assessed the effect of defective BER on the repair capacity of cisplatin intrastrand adducts and ICLs. There was no significant difference between the repair kinetics of intrastrand adducts in BER proficient and deficient cells. Polβ is capable of bypassing and synthesizing past cisplatin intrastrand adducts which revealed a possible role of Polβ in adduct tolerance (15). The alkaline comet assay revealed that cisplatin ICLs are repaired at a faster rate in Polβ deficient and MX treated cells compared to wild type parental cells. This faster ICL repair is consistent with the γH2AX data. γH2AX foci is the molecular marker for DNA DSB repair in general and the repair of DNA interstrand crosslinks induced by several anticancer agents (28,39). This effect was observed at 48 hr and 72 hr but not 24 hr, which could likely be due to the requirement of cytosine deamination at the cisplatin ICL prior to an involvement of BER (Model).

Our in vitro data demonstrate that the cytosines flanking a cisplatin ICL undergo preferential deamination (Fig 5A). The structure of DNA containing a cisplatin ICL has been solved and demonstrates a significant distortion to the duplex DNA structure. The cytosines adjacent to the cisplatin ICL are extrahelical and flipped away from the duplex and the DNA is also unwound 70° and bent 47° which causes significant structural alterations to the DNA (40,41). It has been shown that the extrahelical cytosines flipped out of the DNA double helix are more susceptible to deamination (42). Also, duplex DNA protects cytosines from undergoing deamination as cytosines in mismatched and single-stranded DNA are 100-200 fold more likely to undergo oxidative deamination (43,44). The ICLs induced by platinum drugs form unique DNA structures. The transplatin ICL structure results in no extrahelical cytosines, while in the oxaliplatin ICL structure the flanking cytosines are not as exposed as in cisplatin ICLs and protein recognition is altered (45-47). The ICL structure is also distinct from those formed by mitomycin C (MMC) and psoralen, and down-regulation of BER proteins resulted in hypersensitivity to these crosslinking agents (48,49). These structural differences suggest that cisplatin could elicit different responses than other crosslinking agents.

We believe that the flipped out cytosine residues in the cisplatin ICL DNA structure results in preferential oxidative deamination of the extrahelical cytosines which convert to uracils. There can also be protein-induced deamination as these events occur normally in a cell, which could ultimately be influenced by the structure of the cisplatin ICL DNA (50). In our in vitro studies, we used sodium bisulfite to monitor the deamination reactions as it readily converts accessible cytosines to uracil. In Figure 5A, we are able to demonstrate that under these reaction conditions, the cytosines flanking the cisplatin ICL undergo preferential deamination compared with an undamaged DNA substrate as well as a DNA substrate with a cisplatin intrastrand adduct. Based on these data, we expect the exposed cytosines flanking a cisplatin ICL to undergo preferential deamination in vivo. Cytosine deamination events are usually followed by removal of uracil by uracil DNA glycosylases (UNG or SMUG1) which generate abasic sites. Reduced induction of AP sites (~50%) in Ung null cells upon cisplatin treatment supports the in vitro data and the hypothesis that cytosine deamination can occur at cisplatin-ICLs.

In the BER pathway, the abasic sites are cleaved by APE1. Cisplatin ICL DNA substrates containing an abasic site can be cleaved by APE1 but not as effectively as the undamaged DNA substrate in the current study. We speculate that the distortion created at the ICL site might be the reason for the decreased efficiency of APE1 cleavage. When incubated with the APE1 cleaved cisplatin ICL substrate, Polβ was able to incorporate both correct and incorrect nucleotides at the incision site. Previous studies have shown that Polβ was able to carry out synthesis past the
major cisplatin intrastrand adduct and the efficiency of incorporation past the adduct was also dependent on sequence context around the adduct (14,51). This indicates that translesion synthesis by Polβ might lead to cisplatin DNA damage tolerance and increased mutagenicity. Our data also suggest that Polβ could display a mutagenic spectrum on the cisplatin ICL DNA substrate. Incorporation of incorrect nucleotides by Polβ at the ICL site will lead to the generation of mismatched bases. Mismatch repair damage recognition proteins MSH2/6 and MSH2/3 are involved in recognizing mismatched bases and insertion/deletion loops, respectively. Several reports show that MMR proteins recognize both cisplatin intrastrand adducts as well as ICLs. However, they are not believed to play an active role in the repair of the DNA lesions. A key question that remains to be answered is how the MMR pathway contributes to cisplatin resistance. We speculate that BER helps maintain cisplatin ICL levels to achieve cisplatin sensitivity by processing the flanking DNA at the ICL site and competing with productive ICL DNA repair mechanisms (Model). It has been shown that increased repair of ICLs is associated with cisplatin resistance (27) and persistence of ICLs enhances cisplatin sensitivity (20). We assessed the influence of the NER and HR pathway on cisplatin cytotoxicity in a BER-deficient or inhibited background. ERCC1-XPF is a critical component of NER and also required in HR to complete ICL DNA repair (26,35). Down-regulation of ERCC1-XPF resulted in hypersensitivity to cisplatin in wild type parental cells consistent with previous studies (20,27).

ERCC1-XPF deficiency in BER defective cells restored cisplatin sensitivity from its resistant phenotype and there was no significant difference in cisplatin sensitivity between BER proficient and deficient cells. This supports our hypothesis that BER competes with productive ICL DNA repair pathways including NER and HR to maintain persistent cisplatin ICLs.

REFERENCES

**FOOTNOTES**

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The abbreviations used are: Ape1, Apurinic/apyrimidinic (AP) endonuclease; BER, Base excision repair; ICL, interstrand crosslinks; DAPI, 4',6-Diamidino-2-Phenylindole; DSBs, double strand breaks; ELISA, enzyme linked immunosorbent assay; MX, methoxyamine; MEF, mouse embryonic fibroblasts; MMC, mitomycin C; Pol β, polymerase beta; UNG/UDG, Uracil DNA glycosylase.

**FIGURE LEGENDS**

**Fig. 1.** Cisplatin cytotoxicity. Cells were treated with increasing doses of cisplatin, incubated for 2 hr and cytotoxicity was determined by MTS and clonogenic assay. For wt+MX, 20mM methoxyamine was added prior to cisplatin treatment. Results are represented as mean ± SD from 3 independent experiments.

**Fig. 2.** Repair of cisplatin ICLs. Cells were treated with cisplatin for 2 hr and comet assay was performed as described at different time intervals (0, 24, 48 and 72 hr). For wt+MX, 20mM methoxyamine was added prior to cisplatin treatment. 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 wt and wt+MX; wt and *Polβ<sup>−/−</sup>*, wt and *Ung<sup>−/−</sup>*; wt and Polβ KD; wt and Polβ KD + Wt Polβ. NS – non significant; * - P< 0.05.
Fig. 3. Repair of γH2AX foci. Cells were treated with cisplatin for 2 hr and immunofluorescence was performed as described at different time intervals (0, 24 and 48 hr). For wt+MX, 20mM methoxyamine was added prior to cisplatin treatment. 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 wt and wt+MX; wt and Polβ−/−; wt and Polβ KD; wt and Polβ KD + Wt Polβ. NS – non significant; * - P< 0.05.

Fig. 4. Differential induction of abasic sites. Cells were treated with increasing doses of cisplatin (A) and oxaliplatin (B) for 2 hr. Genomic DNA was isolated and abasic sites were measured using aldehyde reactive probe conjugated to biotin and represented as number of AP sites/100,000 bp. (C) AP sites were quantified at indicated time points. (D). AP sites were quantified in wt and Ung null cells. Results are represented as mean ± SD from 3 independent experiments. Statistical analysis was done by student’s t test and statistical comparisons are made between cisplatin vs oxaliplatin. * - p<0.05, and NS - non significant.

Fig. 5. (A) Cytosine deamination at a cisplatin ICL. Lanes 1-4 represent undamaged DNA substrate, lanes 5-8 represent cisplatin GG substrate and lanes 9-12 represent cisplatin ICL substrate. The lane marked L, represents a 10 bp DNA ladder (B) APE1 incises abasic sites on both undamaged and cisplatin ICL substrates. DNA substrates containing abasic sites were prepared similarly as previous substrates. Lanes 1-7 represent undamaged DNA substrates containing an abasic site. Lanes 8-14 represent DNA substrates containing an abasic site next to cross-linked guanine. (C) DNA Polymerase β has the ability to extend past the cisplatin ICL site. Lanes 1-4 represent undamaged and lanes 5-8 represent Cisplatin ICL DNA substrates. (D) Polymerase β incorporates incorrect nucleotides even in the presence of correct nucleotides. Lanes 1-7 represent undamaged and lanes 8-14 represent cisplatin ICL DNA substrates. A table representing the radioactive intensity ratios of the misincorporated bases normalized to the correct base (cytosine) is shown below the gel.

Fig. 6. Down-regulation of ERCC1-XPF affects cisplatin and oxaliplatin sensitivity. A,B. Wildtype parental with siControl (closed circles), Polβ deficient with siControl (closed triangles), wt + siERCC1-siXPF (open circles), Polβ deficient + siERCC1-siXP (open triangles) cells were treated with increasing doses of cisplatin (A) and oxaliplatin (B) for 2 hr and cytotoxicity was determined by a clonogenic assay. Results are represented as mean ± SD from 3 independent experiments.
Figure 2

**A**
ICL Repair MEFs

<table>
<thead>
<tr>
<th>Time</th>
<th>wt</th>
<th>wt + MX</th>
<th>Polβ -/-</th>
<th>Ung -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr.</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>24 hr.</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>48 hr.</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>72 hr.</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

**B**
ICL Repair HeLaS3

<table>
<thead>
<tr>
<th>Time</th>
<th>wt</th>
<th>wt + MX</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr.</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>24 hr.</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>48 hr.</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>72 hr.</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

**C**
ICL Repair A2780

<table>
<thead>
<tr>
<th>Time</th>
<th>wt</th>
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</tr>
</thead>
<tbody>
<tr>
<td>0 hr.</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>24 hr.</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>48 hr.</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>72 hr.</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

**D**
ICL Repair MDA-MB-231

<table>
<thead>
<tr>
<th>Time</th>
<th>wt</th>
<th>Polβ KD + WT Polβ</th>
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</thead>
<tbody>
<tr>
<td>0 hr.</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>24 hr.</td>
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<td>80</td>
</tr>
<tr>
<td>48 hr.</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>72 hr.</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>
Figure 3

A

DSB repair MEFs

% γ-H2AX positive cells

Post-incubation time

untreated 0 hr. 24 hr. 48 hr.

Wt Wt + MX Polβ -/

B

DSB repair MDA-MB-231

% γ-H2AX positive cells

Post-incubation time

0 hr. 24 hr. 48 hr. 72 hr.

wt Polβ KD + WT Polβ Polβ KD

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Figure 4

Induction of Abasic Sites

A & B: Induction of abasic sites by Cisplatin and Oxaliplatin. The graphs show the number of abasic sites (AP sites) per 100,000 bases in response to different concentrations of Cisplatin and Oxaliplatin. The x-axis represents the concentration of the drug in micromolar (μM), while the y-axis represents the number of AP sites.

C: Time course of abasic sites induction. The graph shows the number of AP sites over time (0h, 24h, 48h) for Cisplatin and Oxaliplatin.

D: Abasic sites in wild type (WT) and Ung null cells. The graph compares the number of AP sites in WT and Ung null cells treated with Cisplatin and Oxaliplatin. The data is presented as mean ± standard error of the mean (SEM).
Figure 5

A Cytosine Deamination at a Cisplatin ICL

DNA
UDG
APE1
NaHSO3
Time (min)

<table>
<thead>
<tr>
<th>DNA</th>
<th>Undamaged</th>
<th>GG</th>
<th>ICL</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDG</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>APE1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NaHSO3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Time (min)</td>
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<td>0</td>
<td>5</td>
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</table>

ICL

Undamaged
ICL (reversed)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undamaged</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ICL (reversed)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B APE1 ICL Incision

APE1 incision

Undamaged ICL

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undamaged</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ICL (reversed)</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

D Polβ Lack of Fidelity

APE1 incision

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undamaged</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ICL (reversed)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

Intensity values of misincorporation normalized to incorporation of correct base (cytosine)

<table>
<thead>
<tr>
<th></th>
<th>dATP</th>
<th>dTTP</th>
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<tbody>
<tr>
<td>Undamaged</td>
<td>~0.9</td>
<td>~0.5</td>
</tr>
<tr>
<td>ICL</td>
<td>~1.6</td>
<td>~0.9</td>
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Figure 6

A Colony survival MDA-MB-231

B Colony survival MDA-MB-231
BER is required for cisplatin cytotoxicity

Cisplatin ICL (extrahelical cytosines)

Deamination of extrahelical cytosine
Uracil DNA Glycosylase base removal

Abasic (AB) site generation
APE1 Recruitment

APE1 incision

Polβ recruitment and extension

Polβ correct incorporation
Polβ incorrect incorporation

X = any nucleotide

BER Processing

Non-Productive BER Processing
Competition with Productive ICL Repair
Persistent ICLs
Cisplatin Sensitivity
Novel role of base excision repair (BER) in mediating cisplatin cytotoxicity
Anbarasi Kothandapani, Venkata Srinivas Mohan Nimai Dangeti, Ashley R. Brown, Lauren A. Banze, Xiao-Hong Wang, Robert W. Sobol and Steve M. Patrick

J. Biol. Chem. published online February 28, 2011

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