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Repair of cisplatin-induced DNA interstrand crosslinks by a replication-independent pathway involving transcription-coupled repair and translesion synthesis

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ABSTRACT

DNA interstrand crosslinks (ICLs) formed by antitumor agents, such as cisplatin or mitomycin C, are highly cytotoxic DNA lesions. Their repair is believed to be triggered primarily by the stalling of replication forks at ICLs in S-phase. There is, however, increasing evidence that ICL repair can also occur independently of replication. Using a reporter assay, we describe a pathway for the repair of cisplatin ICLs that depends on transcription-coupled nucleotide excision repair protein CSB, the general nucleotide excision repair factors XPA, XPF and XPG, but not the global genome nucleotide excision repair factor XPC. In this pathway, Rev1 and Polζ are involved in the error-free bypass of cisplatin ICLs. The requirement for CSB, Rev1 or Polζ is specific for the repair of ICLs, as the repair of cisplatin intrastrand crosslinks does not require these genes under identical conditions. We directly show that this pathway contributes to the removal of ICLs outside of S-phase. Finally, our studies reveal that defects in replication- and transcription-dependent pathways are additive in terms of cellular sensitivity to treatment with cisplatin or mitomycin C. We conclude that transcription- and replication-dependent pathways contribute to cellular survival following treatment with crosslinking agents.

INTRODUCTION

DNA interstrand crosslinks (ICLs) are formed by widely used chemotherapeutic agents [cisplatin, mitomycin C (MMC), nitrogen mustards, nitrosoureas], as well as by endogenous products of lipid peroxidation (1–3). Because ICLs block essential aspects of DNA metabolism, such as replication and transcription, they are highly cytotoxic. The repair of ICLs is of substantial relevance because the efficiency of crosslink repair is a determinant of how tumor cells respond to chemotherapy with ICL-inducing agents (4–6). In vertebrates, ICL repair is primarily triggered by stalled replication forks during S-phase. This pathway requires endonucleases (ERCC1-XPF, MUS81-EME1, FAN1), translesion synthesis polymerases (Polζ, Rev1), Fanconi anemia (FA) proteins and homologous recombination factors (7,8).

However, there is increasing evidence for the existence of a second ICL repair pathway that is independent of replication and recombination, and that occurs primarily in the G0/1 phase of the cell cycle (9,10). Replication-independent ICL repair was originally described in bacteria (11,12). A similar pathway is conserved from yeast to mammals and is believed to involve nucleotide excision repair (NER) factors to recognize and incise ICLs, and the translesion polymerases Rev1 and Polζ to bypass an unhooked ICL intermediate (13–17). Because the cells deficient in NER factors (other than ERCC1-XPF) are only mildly sensitive to ICL-generating agents, it has been suggested that the replication-independent pathway plays a relatively minor role in vertebrates. However, it is also possible that the importance of the G1 pathway in removing ICLs is underappreciated because ICLs are more readily tolerated in G1 than in the S-phase, where stalling of a replication fork is highly toxic. In contrast, ICL toxicity in G1 may only be manifest if it blocks the transcription of an essential gene.

We set out to characterize the replication-independent repair of ICLs and to study its relation to transcription and to the NER-dependent repair of single-stranded

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lesions, such as intrastrand crosslinks generated by the same agents. We decided to study DNA adducts generated by cisplatin because it is the clinically most important crosslinking agent and because the relative distribution of 1,2-d(GpG), 1,2-d(ApG) and 1,3-d(GpNpG) intrastrand crosslinks, and 1,2-d(GpC) ICLs, generated by this compound (~65%, 25%, 5–10% and 2–5% of total adducts, respectively) is known (18). The recognition and repair of cisplatin intrastrand and ICLs by NER has been investigated by biochemical approaches, and it was found that only intrastrand crosslinks are repaired by NER in cell extracts (19,20). The repair of site-specific intrastrand cisplatin adducts in mammalian cells has, furthermore, been studied in a plasmid-based reporter system in mammalian cells (21). To address the mechanism of repair of the three different cisplatin adducts, 1,2-GpC interstrand, and 1,3-GTG and 1,2-GG intrastrand crosslinks, we developed a reporter assay that reflects conditions of repair in the G1 phase of the cell cycle. We found that under our experimental conditions the repair of cisplatin ICLs, but not intrastrand crosslinks, is dependent on a transcription-coupled NER (TC-NER) pathway and the translesion synthesis polymerases Polζ and Rev1. We show that inhibition of replication-dependent ICL repair further sensitizes the cells with a defect in TC-NER, suggesting that cisplatin ICLs are repaired in the G1 and S-phases of the cell cycle.

MATERIALS AND METHODS

Generation of oligonucleotides containing a cisplatin interstrand or intrastrand crosslink at defined positions

A short DNA duplex containing a single cisplatin ICL was prepared using the procedure described by Hofr and Brabec (22). Briefly, a 5′-phosphorylated oligonucleotide containing a unique G (Figure 1B, upper strand) was synthesized, purified by polyacrylamide gel electrophoresis (PAGE) and used to generate the cisplatin monoadduct by reacting with cisplatin activated to a monoaquamonochloro derivative, and purified on a MonoQ column. The purified monoadduct was annealed with the 5′-phosphorylated complementary strand and allowed to form the crosslink. The ICL product was purified on a MonoQ column under denaturing conditions.

For the synthesis of the intrastrand adducts, oligonucleotides containing either a unique GTG (Figure 1C) or a GG (Figure 1D) site for platination were allowed to react with activated cisplatin to produce the 1,3- and the 1,2-intrastrand crosslinks, respectively, following published protocols (23). After purification, the site-specifically modified oligonucleotides were allowed to anneal to their complementary strands. All DNA duplexes contained two different 4-nucleotide overhangs (Figure 1B–D) to allow incorporation into a plasmid.

Preparation of site-specifically modified reporter substrates

The luciferase reporter plasmid was derived from the backbone of pCX-NNX-EGFP plasmid (24) (gift of Bevin Engelward, MIT) by replacing the EGFP gene with the *Renilla* luciferase gene from the plasmid phRG-B (Promega). A tandem *Bbs*I restriction site was cloned upstream of the coding region of the reporter gene to yield the pCX-RLuc plasmid (Figure 1A), into which the various crosslink-containing duplexes were ligated. Digest of pCX-RLuc with *Bbs*I yielded two different nonpalindromic 4-nucleotide 5′ cohesive ends complementary to those of the crosslinked duplexes. The linearized pCX-RLuc was separated from the short fragment on a gel filtration column and used to ligate the lesion-containing inserts or the undamaged oligonucleotides. The closed circular form of the plasmid was purified by CsCl-ethidium bromide gradient ultracentrifugation. A detailed protocol for the preparation of ICL-containing plasmids was published in (25).

Cell lines and tissue culture conditions

Simian virus 40 (SV40)-transformed human fibroblasts GM00637 (wild type), GM08437 (XP-F), GM04312 (XP-A), GM 15876 (GM04312 complemented with the full length cDNA of *XPA*), GM 15983 (XP-C) and GM 16248 (GM 15983 stably transfected with *XPC*-cDNA) from Coriell Cell repositories, and XPCS1RO (XP-G) from Stuart Clarkson (Centre Médical Universitaire, Geneva, Switzerland) (26) were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The mouse embryonic fibroblasts (MEFs), wild type and mutants *Rev3*$^{-/-}$ (27), *Rev1*$^{-/-}$ (28), *Msh2*$^{-/-}$ (RH95021) and *Msh2*$^{+/+}$ (RH95673) (29) established by Niels de Wind (Leiden University Medical Center, the Netherlands) were grown in DMEM supplemented with 10% fetal calf serum (FCS). The Cockayne syndrome B (CS-B) fibroblasts CS1AN-SV and CS1AN-SV dtCSB (stably expressing functional and physiological levels of HA-/His_6 double-tagged CSB) from Wim Vermeulen (Erasmus Medical Center, Rotterdam, the Netherlands) (30), the Chinese hamster cells AA8 (ovary, wild type), V79B (wild-type lung fibroblasts) UV41 (XPF), UV5 (XPD), irs1SF (XRCC3) and irs1 (XRCC2) mutants derived from them (kindly provided by Roland Kanaar, Erasmus Medical Center, Rotterdam, the Netherlands), were maintained in DMEM/Nutrient Mixture Ham's F-10 (1:1) and 10% FBS. The 293T Lα cell line was propagated as described (31). The *Polκ*$^{-/-}$ (M6) and *Polκ*$^{+/+}$ (M1) MEFs (32) (a kind gift from Tomoo Ogi and Haruo Ohmori, Institute for Virus Research, Kyoto University, Japan) and the *Polι*-deficient (240-1) and *Polι*-proficient (238-0) MEFs (33) (from Alan Lehmann, Genome Damage and Stability Center, University of Sussex, UK) were grown in DMEM supplemented with 15% FCS. The isogenic pair of xeroderma pigmentosum variant (XP-V) human fibroblasts (GM02359-hTERT) and complemented with the *Polη* cDNA (GM02359-hTERT+XPV), kindly provided by Marila Cordeiro-Stone (University of North Carolina at Chapel Hill) (34), were maintained in DMEM supplemented with 2× the concentration of MEM nonessential amino acids, 10% FCS and 200 μg/ml G418. The XP30RO (XP-V) fibroblasts and the corrected cells (XP30RO+ *Polη* cDNA) from Alan Lehmann (35) were

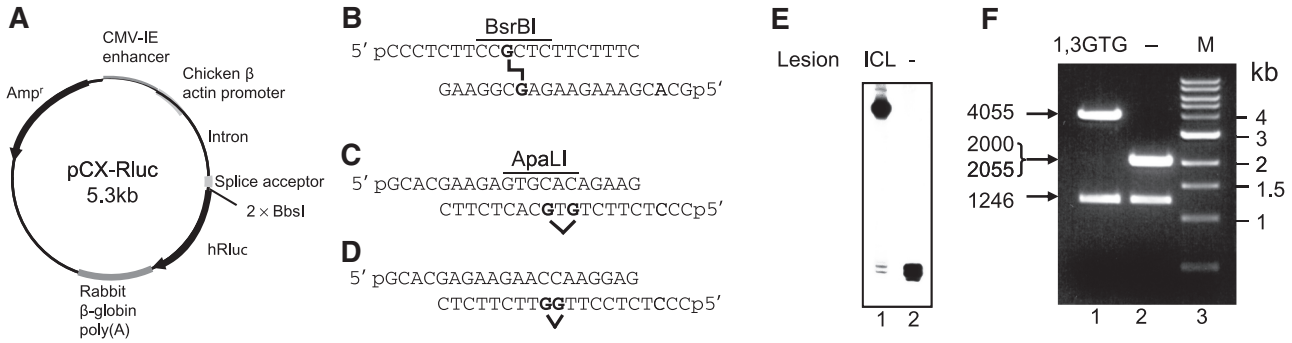

Figure 1. Construction of the plasmid containing site-specific cisplatin crosslinks. (**A**) pCX-RLuc plasmid with two *Bbs*I sites cloned between the splice acceptor and the *Renilla* luciferase (h*Rluc*) reporter gene. (**B**) The sequence of the cisplatin ICL oligonucleotide with the crosslinked guanine bases shown in bold. The ICL is located within a *BsrB*I restriction site. (**C**) The sequence of 1,3-GTG cisplatin intrastrand crosslink located within an *ApaL*I restriction site. The modified bases are shown in bold. (**D**) The sequence of 1,2-GG cisplatin intrastrand crosslink (modified bases in bold). (**E**) Analysis of the cisplatin ICL containing substrate. A 40-bp fragment was released from the plasmid by *Sac*I digestion and labelled with α-[^{32}P]-dCTP. The fragments released from the cisplatin ICL (lane 1) or unmodified control plasmid (lane 2) were analysed by 12% denaturing PAGE. (**F**) Restriction analysis of the 1,3-GTG-cisplatin intrastrand crosslink substrate. *ApaL*I digest releases 3 fragments from unmodified control plasmid (1246, 2000 and 2055 bp, the last two are not separated in agarose gel) (lane 2). In the plasmid with the 1,3-GTG cisplatin intrastrand crosslink, the *ApaL*I site located at the position of the lesion is completely blocked (*ApaL*I digest yields fragments of 1246 and 4055 bp) (lane 1). Lane 3: 1 kb DNA Ladder (NEB).

grown in DMEM supplemented with 10% FBS. Two isogenic pairs of SV40-transformed FA fibroblasts, PD20.F (FA-D2) and corrected (PD20.F+FANCD2), GM6914 (FA-A) and corrected (GM6914+FANCA) (36), kindly provided by Alan D'Andrea, and the EUFA030 (FA-J) and corrected (EUFA0030+FANCJ) (kindly provided by J. de Winter) (37), were grown in DMEM supplemented with 15% FBS. All cell lines were cultured at 37°C in a CO_2 humidified atmosphere.

Reporter reactivation assay

For transfection experiments, cells were plated on 24-well plates in antibiotic-free media and allowed to grow for 24 h to achieve 40–60% confluency at the time of transfection. To minimize the experimental variability caused by differences in cell viability or transfection efficiency, the Dual-Luciferase Reporter Assay System (Promega) was used. The unmodified or site-specifically crosslinked *Renilla* luciferase experimental reporter plasmid were co-transfected with the pGL3-Control Vector (Promega) that contains the firefly luciferase gene and serves as an internal control to normalize the activity of the experimental reporter. Transfections were performed using 10 ng of the reporter and 0.5 ng of the control plasmids (20:1 ratio) and the Lipofectamine Plus Reagent (Invitrogen) (0.6 µl Lipofectamine and 0.9 µl Plus Reagent) for 6 h in serum-free media. For cells that were difficult to transfect, 100 ng carrier DNA was added to improve the transfection efficiency. After removing the transfection media, cells were incubated in complete media for 20 h, then lysed in 100 µl passive lysis buffer provided with the Dual Luciferase Reporter Assay kit (Promega). The lysates were stored at −80°C until they were assayed. The assays for firefly and *Renilla* luciferase activities were performed sequentially within the same sample, according to the procedure recommended by the supplier, using a Labsystems Luminoskan.

Mutation analysis

To analyse the accuracy of the repair at the site of the crosslink, 40 ng of the site-specifically modified plasmid was transfected into cells in 24-well plates, and 20 h after transfection, the DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). The DNA was electroporated into the NER- and recombination-deficient *Escherichia coli* strain AB2480 (*uvrA6recA13*) (38), and the isolated ampicillin-resistant clones were analysed by *BsrB*I digest. Clones that were resistant to *BsrB*I digest at the site of the crosslink were further analysed by DNA sequencing.

RNA interference

The day before transfection, CS1AN-SV and CS1AN-SV dtCSB cells were seeded at a density of 10^5 per well in six-well plates in DMEM/Nutrient Mixture Ham's F-10 (1:1) and 10% FBS without antibiotics. Cells at 30–40% confluency were transfected with 50 pmol siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the procedure of the supplier. The siRNA target sequences were as follows: siLuciferase (CGU ACG CGG AAU ACU UCG A), siFANCD2 (CAG AGU UUG CUU CAC UCU CUA). Cell pellets were collected 48 h after transfection to check the efficiency of siRNA-mediated downregulation. The following primary antibodies were used for Western blots: FANCD2 (Fl17, Santa Cruz), CSB (H-300, Santa Cruz), TFIIH p89 (S-19, Santa Cruz).

Cell survival assays

The human fibroblasts were seeded in triplicates in six-well plates at a density of 500 cells per well. Following incubation for 8 h, cells were treated with the indicated concentrations of cisplatin or MMC, and the media was changed after 24 h. For experiments with siRNA-mediated down regulation, cells were plated 24 h after transfection and treated with the drug 16 h later. After 9–16 days, the

colonies were fixed and stained with 0.5% crystal violet in 20% ethanol, and individual colonies with more than 30 cells were counted. The number of colonies obtained with untreated cells was set as 100% survival. Each survival experiment was performed at least three times.

RESULTS

Construction of plasmids containing site-specific cisplatin crosslinks

The strategy to prepare site-specifically modified plasmids is based on a tandem *Bbs*I restriction site, which was cloned between the splice acceptor and the coding region of the *Renilla* luciferase reporter gene, yielding the pCX-RLuc plasmid (Figure 1A). The two different nonpalindromic 5′ overhangs formed after *Bbs*I digestion allow for the ligation of either unmodified or lesion-containing oligonucleotides with complimentary cohesive ends (Figure 1B–D). This design ensures the incorporation of a single insert into the plasmid in a controlled orientation. In the case of 1,2-GG and 1,3-GTG cisplatin intrastrand crosslinks, the orientation of the *Bbs*I sites was adapted to specifically incorporate the lesion into the transcribed strand. After optimization of the ligation procedure, to decrease the formation of multimeric species, the purified closed circular form was obtained with a reproducible yield of 20–50%. The analysis of the substrates by denaturing PAGE (a 40 bp fragment containing the ICL) (Figure 1E) or restriction analysis (inactivation of the restriction site *ApaL*I by the 1,3-GTG cisplatin intrastrand adduct) (Figure 1F) confirmed the presence of the lesion in the modified plasmids. The very low amount of uncrosslinked material detected with the cisplatin ICL substrate (<2%) is probably generated during the sample analysis, as it is known that the stability of cisplatin ICL is affected in short DNA fragments (39). However, the cisplatin ICL incorporated into a plasmid has a good stability and the activity of different restriction enzymes (*BsrB*I, *Sap*I, *Ear*I) whose recognition and/or cutting site overlap with the ICL were completely blocked (data not shown). The amount of uncrosslinked material contaminating the crosslinked substrate plasmid is estimated to be 1.1%, as determined by quantitative PCR, with a pair of primers flanking the ICL site (data not shown).

Reactivation of transcription in cisplatin ICL containing plasmids depends on NER

The reporter plasmid contains the cisplatin ICL in an actively transcribed region, blocking expression of the reporter gene; therefore, the ICL needs to be removed to restore transcription. To quantitatively estimate the cisplatin ICL repair (measured as the level of reporter gene expression), the cisplatin ICL plasmid (*Renilla* luciferase) was cotransfected into mammalian cells with an internal control reporter (firefly luciferase). After normalization of the *Renilla* luciferase activity to that of the internal control, the relative repair was estimated as the percentage of the activity obtained with an identical reporter plasmid that did not contain a lesion (set to 100%). Previous studies using a similar reporter plasmid with psoralen, MMC or alkyl ICLs showed a dependence of the repair of the ICL on the NER genes (13,16,17). We therefore tested the level of reactivation of reporter gene expression of the ICL-containing plasmid in wild type or NER-deficient human fibroblast cell lines. The level of repair was ~60% in the wild-type cells (compared with the non-damaged control), and was decreased to ~20% in fibroblasts from xeroderma pigmentosum patients with mutations in the *XPA*, *XPF* and *XPG* genes, demonstrating that the removal of the crosslink was dependent on functional NER (Figure 2A). Reporter gene expression was restored to the level of the wild-type cells in an XP-A cell line complemented with the wild-type *XPA*, confirming that the reduced repair of the cisplatin ICL was indeed due to a defect in NER and not an indirect effect. Similar levels of ICL repair were found in Chinese hamster ovary (CHO) cell lines with mutations in the XPF (UV41) or XPD (UV5) genes (Supplementary Figure S1A).

We then addressed how the NER-dependent repair of ICLs compared with that of intrastrand crosslinks in our reporter assay. Previous biochemical studies revealed that the 1,3-GTG intrastrand adduct is more efficiently repaired by NER than the 1,2-GG intrastrand adduct, as the structural alteration induced in the DNA by the former triggers NER more efficiently (19,20,40). We investigated the reactivation of the *Renilla* luciferase reporter gene in plasmids containing the 1,3-GTG or 1,2-GG cisplatin intrastrand adducts on the transcribed strand, similar to previous studies (21). The repair of the 1,3-GTG adduct showed dependence on the NER factors XPA and XPG, similar to cisplatin ICLs, and repair was restored to near wild-type level in the XP-A complemented cells (Figure 2B). In the XP-F cells, the 1,3-GTG adduct was repaired close to wild-type levels, possibly due to the significant residual NER activity present in these cells (~10% of normal post UV unscheduled DNA synthesis (41)), which appears to be sufficient for the removal of the 1,3-GTG intrastrand, but not the ICL. However, the XPF-mutant CHO cell line UV41 showed a similar level of deficiency as the human XPA and XPG and the CHO UV5 cell lines (Supplementary Figure S1B), demonstrating the involvement of XPF in the repair of 1,3-GTG intrastrand adducts. In contrast, expression of the reporter gene blocked by an 1,2-GG cisplatin intrastrand adduct is fully reactivated (80–100%) in either wild-type or NER-mutant cells (Figure 2C). It is possible that this reflects bypass of 1,2-GG cisplatin ICLs by RNA polymerase II as observed previously in cell extracts under certain conditions (42). We note that the absence of XPF had a slightly greater impact on repair rates of 1,2-GG and 1,3-GTG adducts in a previous study (21). However, the impact of XPF deficiency on 1,3-GTG repair was less than of the absence of XPA or XPG described here. The reason for these differences is not clear. We note that significant residual reporter gene reactivation is found in the absence of NER, which may be due to repair and tolerance pathways, such as mismatch repair (MMR) and translesion synthesis (5,6).

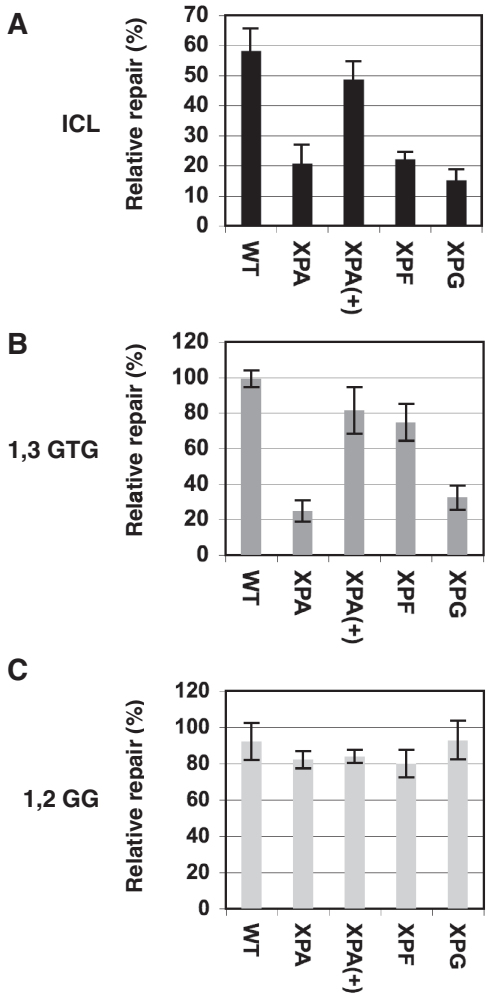

Figure 2. Reactivation of reporter gene expression blocked by a site-specific cisplatin ICL depends on NER. Plasmids containing site-specific cisplatin lesions: ICL (**A**) and 1,3-GTG- (**B**) or 1,2-GG- (**C**) intrastrand crosslink were transfected into normal human fibroblasts (WT, wild type) and fibroblasts from xeroderma pigmentosum patients groups A, F and G. XPA(+) are XP-A fibroblasts complemented with wild-type *XPA*. The relative repair is estimated as percentage of the reporter gene activity compared with the undamaged plasmid, after normalization to an internal cotransfected control. Standard deviations are shown as error bars.

We decided to focus on the 1,3-GTG intrastrand crosslink to distinguish the requirements for the repair of intra- and ICLs in our studies. In the conditions used in our assays, the repair of the 1,3-intrastrand and interstrand cisplatin adducts was thus achieved by an NER-dependent mechanism, although there was a more stringent requirement for a functional XPF for the repair of the interstrand adduct.

The repair of cisplatin ICLs is coupled to transcription

There are two branches of NER: global genome NER (GG-NER), which can detect bulky DNA lesions anywhere in the genome, and TC-NER, which is triggered by lesions in actively transcribed genes that block progression of the transcription machinery (43). While most NER factors are involved in both pathways, XPC-RAD23B, responsible for damage recognition in GG-NER, and CSA and CSB, involved in early stages of TC-NER, have pathway-specific functions (44). We investigated the requirement for these GG-NER- and TC-NER-specific factors in our ICL repair assay. In XPC-deficient cells, cisplatin ICLs were removed at levels similar as the XPC-corrected cells, suggesting that the XPC protein does not play an important role in this repair pathway (Figure 3A). In contrast, CS-B cells displayed significantly reduced efficiency of cisplatin ICL removal, but this defect was corrected in an isogenic cell line complemented with the full-length CSB. These results suggest that the repair of cisplatin ICL proceeds through the TC-NER pathway.

Considering the design of the reporter assay, with the lesion placed as a block to transcription directed from a strong promoter, the dependence on TC-NER is perhaps not unexpected. However, the requirement of CSB was specific to cisplatin ICLs, as CSB- and XPC-deficient cells did not display reduced repair activity of the 1,3-GTG cisplatin intrastrand crosslink (Figure 3B), suggesting that the GG-NER and TC-NER pathways are redundant for this lesion. Therefore, the repair of cisplatin inter-, but not intrastrand crosslinks is specifically dependent on TC-NER in our assay conditions.

Reactivation of transcription of cisplatin ICL plasmids is independent of proteins involved in homologous recombination, the FA pathway or MMR

Factors involved in homologous recombination (such as BRCA1/2 or the RAD51 paralogs XRCC2/3, RAD51B-D) and the FA pathway play a major role in the metabolism of damage induced by crosslinking agents (7,45). These factors have been clearly implicated in replication-dependent ICL repair, but we wished to test whether they contributed to the removal of ICLs in our system. We measured levels of ICL repair in the *XRCC2*- (irs1) and *XRCC3*- (irs1SF) mutant hamster cells and found that the repair of cisplatin ICLs in these *RAD51* paralog mutants was comparable with their corresponding parental cell lines V79B and AA8, respectively (Supplementary Figure S2A). We also tested the involvement of three FA proteins involved at different stages of the pathway; FANCA is part of the FA core complex, FANCD2 protein is monoubiquitinated by the FA core and regulates incision and translesion synthesis steps in replication-dependent ICL repair, whereas FANCJ is a helicase that acts downstream of FANCD2 (7). We found that the levels of NER-dependent ICL repair of cisplatin ICLs were the same in FANCA-, FANCD2- and FANCJ-deficient cells and their complemented counterparts (Supplementary Figure S2B). These studies show that the repair of the plasmid-based cisplatin ICLs is independent of homologous recombination and the FA pathway in this experimental model.

The role of MMR proteins in the recognition and processing of ICLs has been controversial. Some studies have reported that MMR proteins are involved in the processing of ICLs (46–48), in some cases in conjunction with NER proteins (49), whereas other studies have suggested

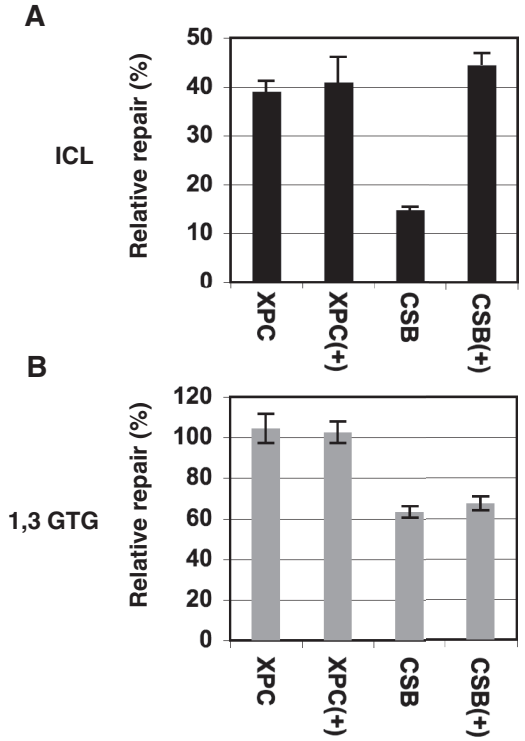

Figure 3. The repair of cisplatin ICLs depends on TC-NER, but not GG-NER. ICL- (**A**) or 1,3-GTG-*intra*strand crosslink– (**B**) containing plasmids were transfected into XP-C (XPC), XP-C corrected with WT-*XPC* [XPC(+)], CS-B (CSB) and CS-B corrected with WT-*CSB* [CSB(+)] fibroblast cell lines and the relative repair activity measured as in Figure 2.

that MMR has only a minor effect on ICL repair (50). Therefore, we tested whether the lack of MMR proteins influenced the repair of cisplatin ICLs in our assay. However, neither the lack of MSH2 in MEFs nor the lack of MLH1 in an isogenic human cell system (293T $L\alpha^+$ and 293T $L\alpha^-$) (31,50) had any significant influence on the repair of cisplatin ICL repair in our system (Supplementary Figure S2C).

Polymerase ζ and Rev1 are required for the repair of cisplatin ICLs

Translesion synthesis (TLS) has been shown to be involved in the replication-dependent and -independent repair of ICLs (51). In particular, deficiencies in Polymerase ζ (composed of Rev3 and Rev7) and Rev1 confer sensitivity to crosslinking agents in avian and mammalian cells, and a role for these two factors in ICL repair has been shown in cellular studies (52–54). In addition to a role in replication-dependent ICL repair (8), Rev3 and Rev1 have been specifically implicated in the repair of ICLs in the G1 phase of the cell cycle (14,15).

We investigated the repair of our plasmid-based cisplatin ICLs in *Rev3*$^{-/-}$ and *Rev1*$^{-/-}$ MEFs, and found the repair efficiency to be ~4-fold lower than that of the wild-type cells (Figure 4A). The lack of Rev3 and Rev1 had no impact on the repair of cisplatin-1,3-GTG intrastrand adducts, as the *Rev3*$^{-/-}$ and *Rev1*$^{-/-}$ cells were as proficient as the wild-type MEFs (Figure 4B). The functions of Polζ and Rev1 are thus specifically required in the recombination-independent pathway of ICL repair, but not in a classical NER reaction of a lesion that affects only one DNA strand.

Polη, Polι and Polκ are not essential for the repair of plasmid-based cisplatin ICLs

TLS may require one polymerase or the sequential action of two different polymerases, one for nucleotide insertion opposite the damaged base, and the other for the extension from these termini (55). Furthermore, a number of TLS polymerases have the ability to bypass ICLs under certain conditions (56–58). We therefore aimed to determine whether Y family TLS polymerases are involved in the replication-independent pathway of cisplatin ICL repair. Polη was included, as it is able to bypass certain ICLs *in vitro* (56) and Polη-deficient XP-V cells show some sensitivity to cisplatin (34,59). Using isogenic pairs of XP-V and complemented fibroblasts, we found no difference in the relative repair of cisplatin ICLs (Figure 4C and Supplementary Figure S3).

Polι is another Y family DNA polymerase with broad substrate specificity that has been shown to bypass ICLs under certain conditions *in vitro* (55,56). In our reporter assay, no difference between Polι-deficient and -proficient MEFs was observed (Figure 4C), suggesting that Polι does not contribute to the TLS step in our system.

Polκ is believed to have a special role in the bypass of minor-groove lesions, including ICLs, and has additionally been shown to bypass major groove ICLs *in vitro* (56,57). *Polκ*$^{-/-}$ MEFs showed a small, but reproducible and statistically significant, reduction in the relative repair of cisplatin ICL compared with the parental Polκ-proficient MEFs (Figure 4C). It is therefore possible that Polκ plays a minor role in this repair pathway, which may be partially redundant with Polζ. This activity may be related to the recently described activity of Polκ in NER (60). However, the contribution of Polκ to repair was specific to cisplatin ICL, as the repair of 1,3-GTG intrastrand crosslink was not affected in its absence (Figure 4D).

The replication-independent cisplatin ICL repair is error-free

ICLs are potentially mutagenic lesions and it has been directly shown that repair of psoralen- and MMC-induced ICLs can be mutagenic (16,17,61). We therefore investigated the fidelity of the repair of cisplatin ICLs in our plasmid-based assay. The crosslinked bases (GpC) are located within a *BsrB*I recognition site that is inactivated by the cisplatin lesion or by mutagenic repair. Cisplatin ICL containing plasmids were transfected into various mammalian cell lines, recovered after 20 h and analysed after transformation into a NER- and recombination-deficient bacterial strain (AB2480) (38). Since the replication efficiency of cisplatin ICL-containing plasmid transformed directly to AB2480 was only 1.7% compared with the undamaged control plasmid, the clones obtained with the DNA extracted from mammalian

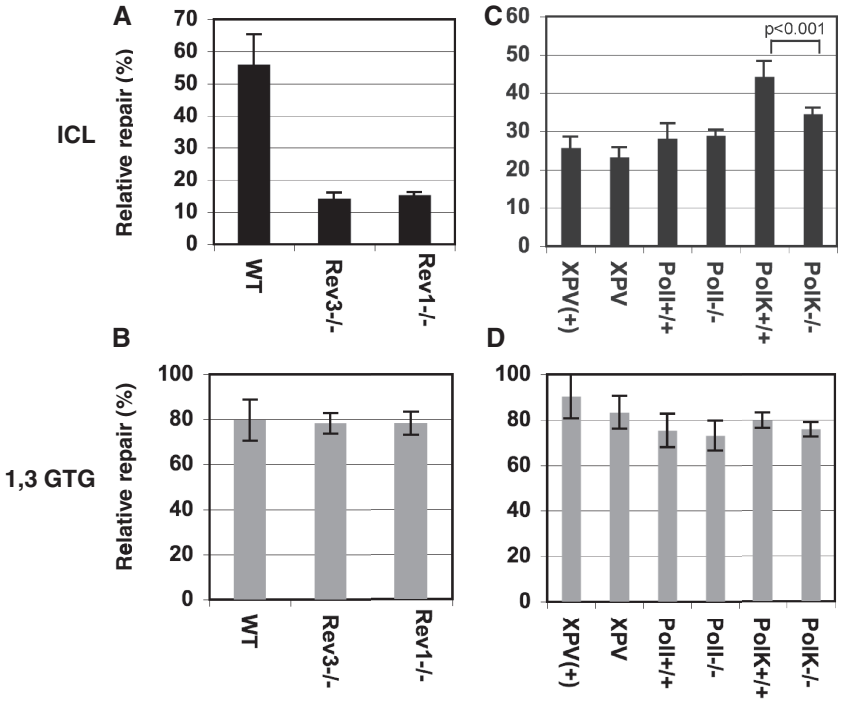

Figure 4. The repair of cisplatin ICLs is defective in *Rev1*$^{-/-}$ and *Rev3*$^{-/-}$ cells, to a minor degree in *Polκ*$^{-/-}$ cells, but not in XP-V and *Polι*$^{-/-}$ cells. ICL- (**A**) or 1,3 GTG-*intra*strand crosslink– (**B**) containing plasmids were transfected into WT, *Rev3*$^{-/-}$ and *Rev1*$^{-/-}$ mouse ES cells and the reactivation of luciferase gene expression measured. The same plasmids were transfected into *Polη*-deficient (XPV) and -complemented human XP-V [XPV(+)] cells and *Polι*- and *Polκ*-positive and deficient mouse ES cells, and levels of reporter gene expression determined (**C**, **D**).

cells represent almost exclusively repair events that have occurred in the transfected cells. The repair of cisplatin ICLs was highly accurate, as *BsrB*I was able to digest the DNA of 124 clones recovered from wild-type human fibroblasts (Table 1). This observation suggests that the TLS step in the bypass of ICLs, likely mediated by Rev1 and Polζ, is accurate. Interestingly, we also failed to detect any mutations in the cells with defects in NER, represented by XP-A cells, and in Rev1 and Rev3 mutant cells, suggesting that the pathway operating in the absence of NER and TLS is also accurate. The same high fidelity of repair was observed in cells with deficiencies in Polη or Polι. Only in *Polκ*$^{-/-}$ MEFs did we find a very low percentage of point mutations (1.1%) within the *BsrB*I site, but a similar frequency of mutations (1.0%) occurred in the Polκ proficient MEFs, so we cannot make a definite statement that this is due to a deficiency of this enzyme. As previous published studies using similar reporter systems (13,16,17) and the same bacterial strain as well as our own studies with a nitrogen-mustard like lesion (62) (Enoiu M, Angelov T, Schärer OD, unpublished observations) yielded significant mutation rates, we are confident that the lack of mutations at cisplatin ICLs reflects error-free repair.

Cells with defects in TC-NER are sensitive to interstrand crosslinking agents

The NER pathway is believed to play a minor role in ICL repair, since defects in NER genes (other than *ERCC1-XPF*) result in only moderate sensitivity to ICL-forming agents (63,64). In light of our cisplatin ICL reporter assay results, we wanted to investigate whether the TC-NER-dependent pathway contributes to the survival of cells treated with cisplatin in clonogenic assays. We tested three isogenic pairs of human fibroblasts (XP-A, XP-C, CS-B and the corrected counterparts) after treatment with cisplatin. The XP-A and CS-B cells showed a significant 3–4-fold increased sensitivity to cisplatin compared with the respective corrected cells (18% versus 70% survival for XPA deficient and complemented cell lines, and 11% versus 56% for CSB-deficient and complemented cell lines at 0.2 μg/ml cisplatin, respectively, Figure 5A and C), in agreement with the defect in the reactivation of transcription in the reporter plasmid (Figures 2A and 3A) and with previous studies (65,66). In contrast, XP-C fibroblasts were not hypersensitive to cisplatin (Figure 5B), again in agreement with the results from the reporter assay (Figure 3A). Indeed, XP-C cells were even slightly more resistant to cisplatin than the corrected clone.

To establish the generality of this observation and to rule out that the observed sensitivity was not due to toxicity of cisplatin DNA intrastrand crosslinks, we tested the sensitivity of the same cell lines to MMC. XPA- and CSB-deficient cells were sensitive to MMC (Supplementary Figure S4A and C), albeit to a lesser extent than to cisplatin, while XPC-deficient cells were slightly resistant (Supplementary Figure S4B). Since the sensitivity of CS-B cells to MMC was rather small in cycling cells, we wanted to address the impact of CSB

Table 1. Analysis of fidelity of cisplatin ICL repair

Cell line, genotype	Clones analyzed	Mutations identified
Human, wild type	124	0
Human, XP-A	99	0
MEFs, wild type	120	0
MEFs, *Rev3*$^{-/-}$	96	0
MEFs, *Rev1*$^{-/-}$	120	0
Human, XP-V+*Polη*	100	0
Human, XP-V	120	0
MEFs, *Polk*$^{+/+}$	197	2
MEFs, *Polk*$^{-/-}$	258	3
MEFs, *Poli*$^{+/+}$	103	0
MEFs, *Poli*$^{-/-}$	125	0

DNA extracted from transfected cells was transformed to AB2480 *Escherichia coli* strain, and individual clones were analyzed by *BsrB*I digest. The restoration of *BsrB*I site is indicative of accurate repair at the ICL site.

deficiency on ICL removal in the absence of replication. Cells were synchronized in G0/G1 by contact inhibition and serum starvation, treated with a low dose of MMC and allowed to repair the damage in G0/G1 before being released into S-phase. We chose these experimental settings to mimic a G1 checkpoint arrest, which is defective in the SV40-transformed fibroblasts because of p53 inactivation (67). At 24 h after release, both CSB-deficient and -corrected cells showed a similar G2/M arrest (Figure 6). At 48 h after release, the corrected CS-B cells overcame the arrest (41.2% versus 34.9% G2/M arrested cells at 24 h and 48 h, respectively). By contrast, CSB-deficient cells continued to accumulate in G2/M (43.6% versus 60.2% G2/M arrested cells at 24 h and 48 h, respectively). The difference in the recovery from the G2/M arrest after MMC treatment and repair in G0/G1 suggests that CS-B cells are entering S-phase with a higher number of unrepaired ICLs, allowing us to unmask the contribution of the G0/G1 pathway to overall ICL repair. Indeed, the difference between CSB-deficient and -corrected cells was masked by the replication-dependent pathway in cycling cells (data not shown).

Taken together, our data suggest that TC-NER, but not GG-NER, is an important determinant of sensitivity to cisplatin and other ICL-forming agents in human fibroblasts. The correlation with our reporter assay data suggests that the sensitivity of TCR mutants is because of the defect in the removal of ICLs, rather than of the other adducts formed by cisplatin.

Cells with defective TC-NER are further sensitized to ICL-forming agents by inhibition of replication-dependent repair

Our studies strongly suggest that a TC-NER-dependent pathway contributes to the removal of cisplatin and MMC ICLs. We therefore asked the question whether downregulation of replication-dependent ICL repair and TC-NER-dependent ICL repair would lead to a synergistic sensitivity to crosslinking agents. FANCD2, a key player in replication-dependent ICL repair, was downregulated

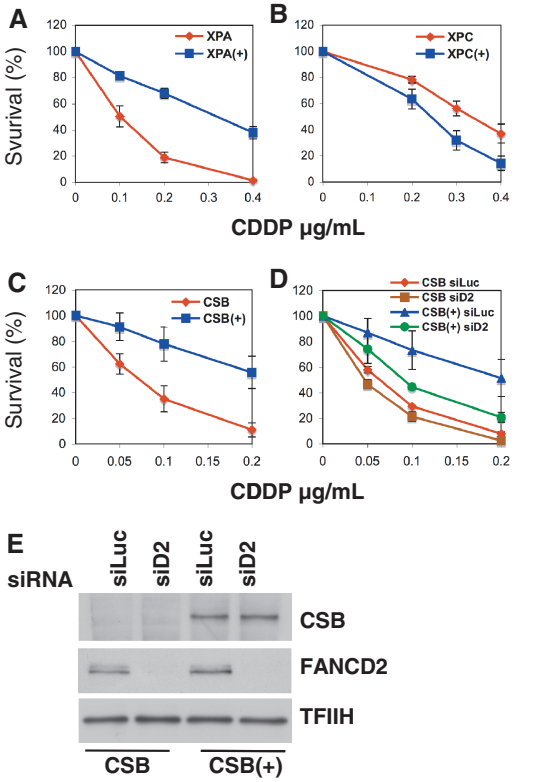

Figure 5. TC-NER–deficient, but not GG-NER deficient cells are sensitive to cisplatin, and the sensitivity is additive to FA pathway. XP-A (XPA) (**A**), XP-C (XPC) (**B**) and CS-B (CSB) (**C**) fibroblasts and the corresponding corrected cell lines [XPA(+), XPC(+), CSB(+)] were treated with the indicated doses of cisplatin and the surviving fraction determined using clonogenic assays. (**D**) CSB-deficient and -complemented cells were transfected with *FANCD2*-specific (siD2) and control (siLuc) siRNAs, treated with cisplatin and the surviving fraction determined using clonogenic assays. The data in A–D are plotted as the percentage of colonies that grew on the treated plates relative to untreated plates ± S.E. (error bars). (**E**) Western blot showing downregulation of FANCD2 in CS-B and corrected fibroblasts.

by siRNA in CSB-deficient and corrected cells (Figure 5E), and the cells were then exposed to cisplatin or MMC. We observed a strong increase in the sensitivity to cisplatin in the CSB-corrected cells on downregulation of FANCD2 (Figure 5D). The intrinsically high cisplatin sensitivity of the CSB-deficient cells was further enhanced after downregulation of FANCD2. Importantly, similar results were obtained when MMC was used as an ICL agent instead of cisplatin (Supplementary Figure S4D). We conclude that replication-dependent and TC-NER–dependent ICL repair act in parallel and are additive in mediating cell survival following treatment with crosslinking agents.

DISCUSSION

In this study, we describe a pathway for the repair of cisplatin-induced ICLs that relies on TC-NER and TLS. We further show that this pathway appears to be the main pathway in the G0/G1 phase of the cell cycle, and that it occurs independently of proteins involved in homologous

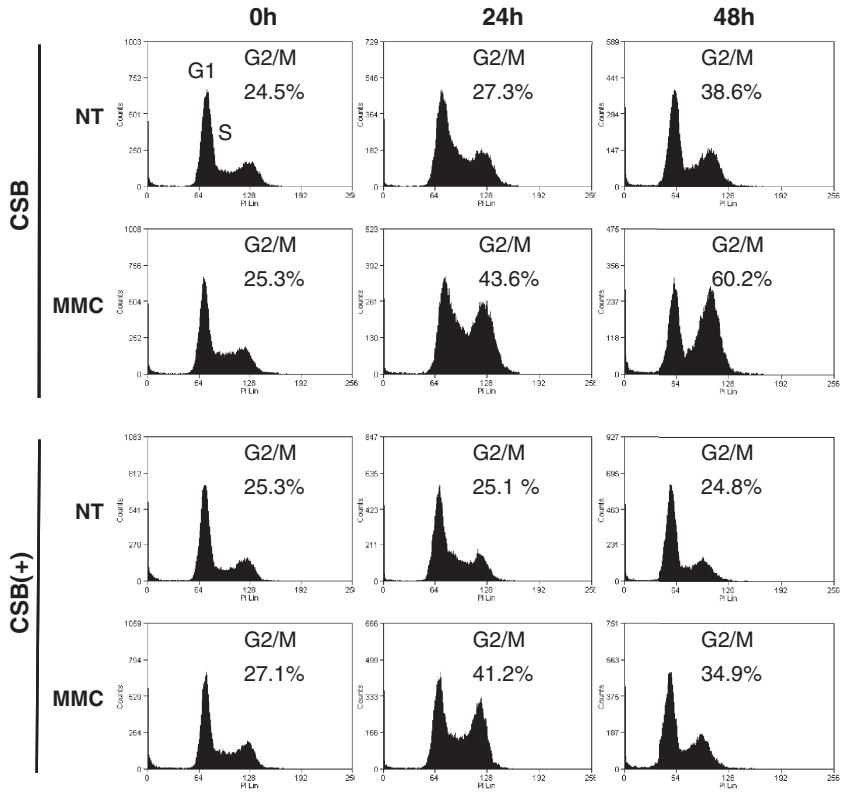

Figure 6. Defective repair of MMC lesions in G1/G0 causes a stronger G2/M arrest in CSB-deficient cells versus corrected cells. CS-B (CSB) and complemented [CSB(+)] cells were arrested in G0/G1 by contact inhibition followed by 48 h serum starvation (0.1% FBS). MMC (10 ng/ml) was added or not added (NT) to the media for the last 16 h. Cells were further incubated in 0.1% FBS media without drug for 8 h, then trypsinized and released in complete media (time 0). Samples collected at times 0, 24 and 48 h were analysed by FACS after propidium iodide staining of the DNA.

recombination or the FA pathway implicated in replication-dependent ICL repair. Importantly, this pathway contributes to the repair of ICLs with S-phase specific pathways, providing important evidence for a role of ICL repair in G0/G1 to reduce the burden of ICLs formed by antitumor agents.

TC-NER, but not GG-NER, is involved in the repair of cisplatin ICLs

Previous studies, including those using plasmid-based reporter assays similar to ours, have shown that NER is involved in the replication-independent repair of ICLs (13,16,17,68). While these studies implicated mainly GG-NER in the repair of psoralen- and MMC-induced ICLs, and a combination of TC-NER and GG-NER in the repair of alkyl ICLs, our studies show that only TC-NER is involved in the repair of cisplatin ICLs. We are confident that this difference is real, as the repair of cisplatin 1,3-intrastrand crosslinks was achieved by either TC-NER or GG-NER in our system as anticipated. A role for TC-NER, but not GG-NER, in the repair of cisplatin ICLs is also consistent with studies that monitored different end points. It has been shown that defects in genes involved in TC-NER (*CSA*, *CSB*), but not in GG-NER (*XPC*) were associated with cellular hypersensitivity to cisplatin exposure (65,66). Furthermore, *in vitro* studies have shown that cisplatin ICLs are not incised in an XPC-dependent manner by GG-NER proteins and that they do not induce "futile cycles" of incision and repair synthesis adjacent to the crosslink as has been observed for psoralen and minor groove alkyl ICLs (69,70). These studies suggest that XPC-RAD23B, the initial damage-recognition protein in NER, is unable to bind cisplatin ICLs, indicating that the unusual structure of this adduct, in which two C residues opposite the crosslinked G residues are evicted from the helical stack, are incompatible with the geometry of XPC-RAD23B binding to DNA lesions (71–73).

ICLs are repaired through replication-dependent and -independent pathways

Currently, it is believed that ICLs are mainly repaired in the S-phase of the cell cycle in a pathway that depends on replication and recombination (7,8,45,74). This notion is based on the observation that cells with defects in genes that contribute to the S-phase-dependent repair of ICLs display marked sensitivity to crosslinking agents (45,54). However, there are an increasing number of reports describing replication-independent ICL repair (14–17,68,75–77). Our studies show that inactivation of genes involved in replication-dependent and -independent ICL repair have an additive effect on cellular sensitivity to cisplatin and MMC (Figure 5 and Supplementary Figure S4), suggesting that repair in both the S/G2 and G0/G1 phases of the cell cycle contribute to cellular survival following treatment with ICL-forming agents.

To the best of our knowledge, this has not been previously demonstrated. It is likely that the importance of replication-independent ICL repair has been overlooked because the acute toxicity caused by ICLs is much more pronounced during S-phase. Here, even a single ICL constitutes an absolute block to replication, therefore preventing completion of genome duplication and S-phase in the absence of repair. By contrast, ICLs are more readily tolerated in the absence of replication, where most of the genome is not actively utilized. Our data showing that CSB-deficient cells emerge from G1 with a higher damage load following treatment with MMC than CSB-proficient cells (Figure 6) clearly demonstrates the importance of TC-NER in repairing ICLs outside of S-phase. We note that genes with involvement in replication-dependent and -independent ICL repair, such as *ERCC1-XPF* or *REV3*, are especially sensitive to crosslinking agents (54). While the relative contributions of G0/G1- and S/G2-dependent removal of ICLs remain to be determined, our studies clearly demonstrate the importance of TC-NER, TLS-dependent ICL repair pathway.

What is the contribution of DNA intrastrand crosslinks versus ICLs to cisplatin toxicity?

Studies of cisplatin toxicity caused by DNA adducts have focussed mainly on intrastrand crosslinks, as they are formed ~20-fold more frequently that ICLs (78). The current thinking is that the 1,2-intrastrand crosslinks are the clinically most relevant adducts, as (i) they are formed with the highest frequency, (ii) their cellular adduct levels correlate with clinical efficacy, (iii) transplatin, which is clinically inactive, cannot form this adduct and (iv) their repair has been reported to be inhibited by binding of HMG proteins, which recognize the characteristic bend of this adduct, shielding them from repair (78,79). These reports suggest that cisplatin appears to differ from other crosslinking agents such as nitrogen mustards, MMC or chloroethyl nitrosoureas, for which it has been clearly shown that the ICLs are therapeutically most important adducts (reviewed in (1,2)).

Based on our and other recent studies, we believe that the importance of cisplatin ICL in mediating cellular toxicity should be reevaluated. Because of lower frequency of formation and increased difficulty of synthesis of defined adducts for functional studies, the biological responses of ICLs are less well studied than those of the intrastrand crosslinks. However, in functional studies, cisplatin behaves similarly to other crosslinking agents such as MMC or nitrogen mustards, and their ICL adducts are processed in similar ways (10). Furthermore, recent studies have shown a correlation of cisplatin ICL repair levels with the sensitivity of tumor cell lines (80). Our data, along with studies of cellular sensitivities (65,66), suggest that outside of replication, cisplatin ICLs can only be repaired by TC-NER, but not GG-NER. This property would lead to higher relative levels of ICLs versus intrastrand crosslink adducts, leading to a strong cytotoxic response to the cisplatin ICLs when the cells enter S-phase. We therefore suggest that the relative importance of cisplatin inter- versus intrastrand crosslinks deserves new consideration.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–4.

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