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RESEARCH ARTICLE

Molecular insights into the repair mechanism of enzymatic DNA-protein cross-link damage induced by a DNMT inhibitor

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ABSTRACT

Background: DNA-protein cross-links (DPCs) are a special type of DNA damage that is formed when a protein that participates in DNA transactions is irreversibly and covalently linked to DNA bases. DPCs are formed after exposure to chemicals, anticancer drugs, and ionizing radiation resulting in enzymatic and non-enzymatic DPCs. Up to now, the exact repair mechanism of DPCs has not been fully identified. **Aim:** The outputs of the current study provide molecular insights about the repair mechanism of enzymatic DPCs formed by DNA cytosine methyltransferase (DNMT). **Materials & Methods:** Cells were treated with 5-aza-2'-deoxycytidine (5-azadC) and the cytotoxicity, DNA damage and the role of essential DNA repair genes were examined. **Results:** A clear cytotoxic effect of 5-azadC was observed with LD_{20} ranging from 0.4 to 5 µM. The analysis of DPCs by fluorescence labeling reveals that 5-azadC induces DPCs in a dose-dependent manner. Moreover, cells that are deficient in homologous recombination (HR) pathway (*RAD51D and XRCC3*) were 2-4 folds sensitive to 5-azadC compared to wild type. In contrast, cells deficient in nucleotide excision repair (NER) pathway (*XPD and XPF*) and Fanconi anemia (FANC) pathway (*FancA*, *B*, and *C*) were not sensitive.. Unexpectedly, mutation in nonhomologous end-joining (NHEJ) gene (*DNA-PKcs*) gives cells a great survival. Furthermore, double-strand breaks (DSBs) were significantly detected in HR mutant (*RAD51D*) compared with a wild type indicating that the replication fork stalled at the trapped DNMT site generating DSBs. **Conclusion:** HR pathway genes (*RAD51D* and *XRCC3*) are essentially required for the repair of enzymatic-DPCs induced by 5-azadC and NER pathway genes have neglected roles. Further investigations are required to understand the accurate mechanism by which cells can repair DPCs that will provide good knowledge regarding the targeting of DPCs in cancer treatment.

Keywords: 5-aza-2'-deoxycytidine (5-azadC), Cell sensitivity, DNA cytosine methyltransferase (DNMT), DNA-protein cross-links (DPCs), DNA repair

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INTRODUCTION

Various types of the currently used anticancer drugs target DNA bases and induce disparate DNA damages such as monoadducts, DNA single-strand breaks, DNA double-strand breaks (DSBs), interstrand cross-links (ICLs), and DNA-protein cross-links (DPCs) (Helleday et al., 2008). Among these damages, DPCs are less characterized; they are formed when a protein is covalently and irreversibly linked into DNA bases. DPCs are bulky-damaged and then obstruct the progression of replicative helicases (Nakano et al., 2013), DNA polymerases (Chvalova et al., 2007; Novakova et al., 2003) and also inhibit transcription when existing in the transcribed strand (Nakano et al., 2012). Based on the nature of the trapped protein, DPCs can be classified into enzymatic and non-enzymatic DPCs (Zhang et al., 2020). Enzymatic

DPCs are produced by several anticancer drugs including topoisomerase (Topo) inhibitors such as camptothecin and etoposide that cause trapping of Topo I and Topo II, respectively, via tyrosinyl–phosphodiester bonds (Ide et al., 2011; Nitiss, 2009; Pommier 2009). 5-aza-2'-deoxycytidine (5-azadC), a DNA cytosine methyltransferases (DNMTs) inhibitor when enters the cells, is metabolized, and incorporated into DNA bases during the replication process. When DNMT comes to methylate the CpG sequence, covalently traps the enzyme, resulting in enzymatic DPC formation (Figure 1A,B) (Gowher et al., 2004; Liu et al., 2003; Santi et al., 1984). Although cells have accurate mechanisms to repair various types of DNA damages such as DSBs and ICLs (Helleday et al., 2008), the exact repair mechanism of DPCs still not fully clarified. Previous studies show that DPCs induced by formaldehyde are not repaired by nucleotide excision repair (NER) that is the main repair pathway of bulky DNA adducts (Nakano et al., 2009). Besides, cells treated with formaldehyde have an increase in sister chromatid exchange (Shoulkamy et al., 2012), and have accumulated RAD51 foci (Nakano et al., 2009) suggesting a role of HR pathway in the repair of DPCs. Regarding the direct repair of DPCs, previous studies have been reported that cross-linked proteins in DPCs can be targeted by specific proteases Wss1 (yeast) and SPRTN (human) and allow its degradation process (Stingle et al., 2014; 2016). Moreover, some enzymes such as tyrosyl-DNA phosphodiesterase 1(TDP1) and TDP2 were involved in the repair process of DPCs (Nakano et al., 2020; Pommier et al., 2014). To this end, it would be interesting and informative to investigate the possible repair mechanisms of DPCs. Accordingly, we examined the role of essential DNA repair genes in the repair of enzymatic DPCs induced by a DNMT inhibitor (5-azadC). HR pathway genes (RAD51D and XRCC3) are critical DNA repair genes that are essentially required for the repair of enzymatic-DPCs induced by 5-azadC and NER pathway genes (XPA and XPD) have neglected role. Further investigations are required to understand the accurate mechanism by which cells can repair DPCs that provide good knowledge regarding the targeting of DPCs in cancer treatment.

MATERIALS AND METHODS

Cell culture and drug preparation

DNA Repair-proficient and – deficient human and Chinese hamster ovary (CHO) cells (Gene Chemistry laboratory, Hiroshima University, Japan) were used for the current experiments and their mutations were listed in Table 1 (For details see Nakano et al., 2009; Xie et al., 2016). Human and CHO cells were cultured in DMEM (Nissui, Japan) and Eagle's MEM (Wako, Japan), respectively, supplemented with 10% FBS. Cells were cultivated in a humidified incubator at 37°C with 5% CO_2 atmosphere, and recovered by 0.05% trypsin-EDTA. 5-azadC (Wako, Japan) was prepared in 1% acetic acid at 1 mM initial concentration.

Induction and repair kinetics of DPCs in 5-azadC treated cells

The amount of DPCs induced by 5-azadC was measured by the fluorescence labeling method as described in previous reports with some modifications (Nakano et al., 2015; Shoulkamy et al., 2012). Briefly, human fetal lung fibroblast cell line (MRC5) was cultured in 150-mm plates until cells reach to midlogarithmic phase and then treated with 0.5, 1 and 2 µM from 5-azadC for 24 hours. For analysis of DPC induction, cells (~2 x 10^6) were collected immediately after treatment. However, for analysis of repair kinetics of DPCs cells were treated with 5-azadC (LD_{20}) and collected at 0,6,12 hours after treatment. All samples were incubated in lysis buffer (10 mM phosphate buffer (PB) containing 1% sarkosyl) and subjected to CsCl density gradient ultracentrifugation (500,000×g at 20°C for 4 h) for DNA separation and complete removal of RNA and proteins. For fluorescence labeling of cross-linked proteins, purified DNA (30µg) was incubated at room temperature for 1 hour in 20 mM borate buffer (pH 8.0, 100 µl) containing FITC (Dojindo, final concentration 0.1 mM) in the dark. DNA was precipitated by ethanol and the pellet (DNA and cross-linked proteins) was washed twice with 70% ethanol, dried, dissolved in MilliQ water and concentration of DNA was detected on a UV spectrophotometer. The fluorescence of FITC-labeled DNA (20µg) was detected on a Hitachi F-2500 fluorescence spectrophotometer and used as a measure of

the amount of DPCs. The excitation and emission wavelengths for FITC measurement were (490 and 520 nm) respectively.

Analysis of DSB formation in 5-azadC-treated cells

The formation of DSB was measured by static-field gel electrophoresis (SFGE) as reported previously (Shoulkamy et al., 2012; Xie et al., 2016). Cells were cultured in 100-mm plates until the midlogarithmic phase. Cells were treated with 0.5, 1 and 2 µM from 5-azadC for 24-hours, collected immediately after treatment and suspended in cold phosphate-buffered saline (PBS). Cells were mixed with 1% InCert agarose plugs (LONZA, USA) and 10^4 cells/plug in 50 µl total volume were prepared for each sample using a plug mold. Cells in agarose plugs were incubated for 1 hour in lysis buffer (1% sarkosyl, 0.5 M EDTA, and 0.5 mg/ml proteinase K) followed by 24-hour incubation at 50°C at the same lysis buffer. The plugs were equilibrated in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) for 1 hour at room temperature. The plugs containing the same number of cells were embedded into the wells of 0.6% Seakem Gold agarose gel (LONZA) and electrophoresis was done for 36 hours at a constant field strength of 0.6 V/cm in 0.5× TBE buffer (40 mM Tris, 40 mM boric acid, and 1 mM EDTA). The gel was stained with ethidium bromide (2 µg/ml) for 30 min and destained for 3 hours in 0.5× TBE buffer. The gel was imaged on FAS-III (TOYOBO) and the band intensity was analyzed densitometrically by ImageJ software version 1.33. The amount of DSBs was calculated by measuring the fraction of DNA released from the agarose plug relative to the summation of DNA fraction released and retained in the plug.

Cell sensitivity measurement

Cell sensitivity was measured by analyzing the ability of cells to form a colony (Xie et al., 2016). Cells were plated at a fixed number in culture plates and incubated overnight to allow cell attachment. Cell culture medium was changed and a fresh medium containing the indicated doses of the 5-azadC drug was added and allowed for 24 hours incubation at 37°C with 5% CO_2 atmosphere in which 5-azadC is intercalated into DNA during DNA synthesis. After treatment, cells were washed twice by fresh medium and incubated for a week to allow cells to form colonies. Finally, colonies in untreated and treated plates were fixed, stained with crystal violet, scored and the survival rates were calculated.

Statistical analysis

Results were shown as mean ± standard deviation (STDEV) and statistical significance was done using Student t-test. Differences were considered significant when $p < 0.05$.

RESULTS

Formation and repair kinetics of DPCs induced by 5-azadC

Human cells (MRC5) were treated with different doses of 5-azadC and the amount of DPCs was measured by FITC-labeling. The amount of DPCs increased significantly in a dose-dependent manner (Figure 2A). To ensure that the fluorescence signal is related to DPCs, the fluorescence signal of DNA (30µg) was measured before and after proteinase K digestion. A clear reduction in the fluorescence signal was observed after proteinase K digestion confirming that the fluorescence signal is due to DPC induction (Fig. 2 B). To understand whether cells can eliminate the formed DPCs by 5-azadC, the repair kinetics of DPC (trapped DNMT) was analyzed at 6 and 12 hours after treatment. A significant reduction in the amount of DPCs was observed at 6 hours after drug removal and the remaining DPCs were about 40 and 20% at 6 and 12 hours respectively (Figure 2C), indicating that the cells can eliminate most of the DPCs formed by the trapped DNMT from the genome.

Detection of DSBs in 5-azadC treated cells

The analysis of DSBs in human MRC5 cells treated with 5-azadC shows that no significant increase in the fraction of DNA released from the plug relative to total DNA (Fig. 3). It is well established that X-rays induce DSBs (Hirayama et al., 2005; 2011). Accordingly, human MRC5 cells irradiated with X-rays (10Gy) show significant induction of DSB and were used as a positive control during 5-azadC treatment.

Analysis of cell sensitivity after 5-azadC treatment

The possible DNA repair genes that are essential for the repair of DPCs induced by 5-azadC were examined by cell survival assay (Fig. 4). The cell sensitivity results showed that the NER deficient cells (XPA) exhibited a mild sensitivity to 5-azadC, especially at 0.25 µM. Conversely, no significant sensitivity was observed in NER deficient cells (XPF) relative to wild type (MRC5) and XPF cells can form more colonies after 5-azadC treatment (Figure 4A). Additionally, no sensitivity was observed with CHO cells deficient in the NER pathway (XPD and XPF) treated with 5-azadC (Figure 4A). The data obtained with human and CHO cells suggesting that the NER pathway has no role in the repair of DPCs induced by 5-azadC. Conversely, both HR pathway genes (RAD51D and XRCC3) show significant sensitivity to 5-azadC doses compared with AA8 (WT) cells (Figure 4B). Unexpectedly, CHO cells deficient in DNA-PKcs, an NHEJ pathway component significantly show resistance to 5-azadC treatment (Fig. 4 C). Furthermore, Human and CHO cells deficient in FANC pathway components including FANCA, C and G did not show significant sensitivity to 5-azadC doses (Fig. 4 D).

DSBs accumulate in RAD51D deficient cells after 5-azadC treatment

To give evidence about the involvement of HR in the repair of DPCs induced by 5-azadC, we compared the DSBs induction in HR -deficient 51D1 (RAD51D) and proficient AA8 (WT) cells (Fig. 5). The results showed that treatment with 5-azadC (1 and 2 µM) didn't induce a significant amount of prompt DSBs in AA8 (WT) cells. Interestingly, the agarose gel clearly shows a significant increase in the DNA released from the plug in 51D1 (RAD51D) cells relative to AA8 (WT) (Fig. 5 upper panel). The percentage of eluted DNA was used as a measure of DSBs induction (Fig. 5 lower panel). The cells were treated with Camptothecin (CPT), an anticancer drug that generates TOPO1-DPCs and X-rays (mainly induce prompt DSBs) and analyzed at the same time with 5-azadC as a positive control.

DISCUSSION

DPCs are superbulky damage that is generated when a particular protein is covalently trapped into DNA bases. DPCs are formed after exposure of cells to DNA damaging agents including, chemicals (Formaldehyde), anticancer drugs (alkylating agents and platinum compounds), and ionizing radiation (X-rays and C-ions) (Ide et al., 2011; 2018). Recently, DPCs are classified into two types enzymatic and non-enzymatic (Zhang et al., 2020). Enzymes that associated with DNA actions can be covalently cross-linked to DNA forming DPCs, of these enzymes, topoisomerases (TOPO I and II), DNA methyltransferases (DNMT 1 and 2), DNA polymerases (Polβ) and DNA glycosylases are the most abundant enzymes that form a reaction intermediate with DNA (Ide et al., 2011; Nakano et al., 2020; Zhang et al., 2020). Under certain conditions, specific proteins located around DNA bases can be trapped into DNA when cells are exposed to DNA damaging agents such as aldehydes and ionizing radiation forming non-enzymatic DPCs (Nakano et al., 2015; Shoulkamy et al., 2012). DPCs are superbulky damage that prevents several processes in cells such as replication and transcription, if DPCs are not repaired by cells thereby genome instability, diseases, and cell death can be generated (Barker et al., 2005; Ide et al., 2011). Up to date, the exact repair mechanisms of enzymatic and non-enzymatic DPCs have not been fully understood. For this reason, the outputs of the current study provide molecular insights about the repair mechanism of a specific type of enzymatic DPCs formed by the DNA trapped DNMT. In the current study, all types of cells treated with 5-azadC show a clear cytotoxic effect as measured by colony formation with LD_{20} ranging from 0.4 to 5 µM. The dose range is close to that recently reported in malignant meningioma (IOMM-Lee) cells in which 5-azadC (1 to 10µM) significantly decreases cell proliferation and viability (Stögbauer et al., 2020). The analysis of DPCs by fluorescence labeling reveals that exposure of human cells (MRC5) to 5-azadC induces DPCs in a dose-dependent manner and the signal was confirmed by proteinase K digestion.

Table 1. DNA repair-deficient and proficient cells used in the current study.

Cell	Mutation	Repair defect
CHO cells		
AA8	wild type	none
UV5	ERCC2 (XPD)	NER
UV41	ERCC4 (XPF)	NER
51D1	RAD51D	HR
irs1SF	XRCC3	HR
V3	DNA-PKcs	NHEJ
40BP6	FANCG+ wt FANCG	complemented
KO40	FANCG	ICL
Human cells		
HeLa	wild type	none
MRC5-SV	wild type	none
XP12ROSV	XPA	NER
XP2YOSV	XPF	NER
PD20 RV: D2	FANCD2+wt FANCD2	complemented
PD220	FANCA	ICL
PD331	FANCC	ICL

ERCC2: Excision Repair Cross-Complementation group 2, XPD: Xeroderma Pigmentosum Group D, ERCC4: Excision Repair Cross-Complementation group 4, XPF: Xeroderma Pigmentosum Group F, XRCC3: X-ray Repair Cross- Complementing 3, DNA-PKcs: DNA-dependent Protein Kinase, XPA: Xeroderma Pigmentosum Group A, FANCA: Fanconi Anemia, Complementation group A, FANCC: Fanconi Anemia, Complementation group C.

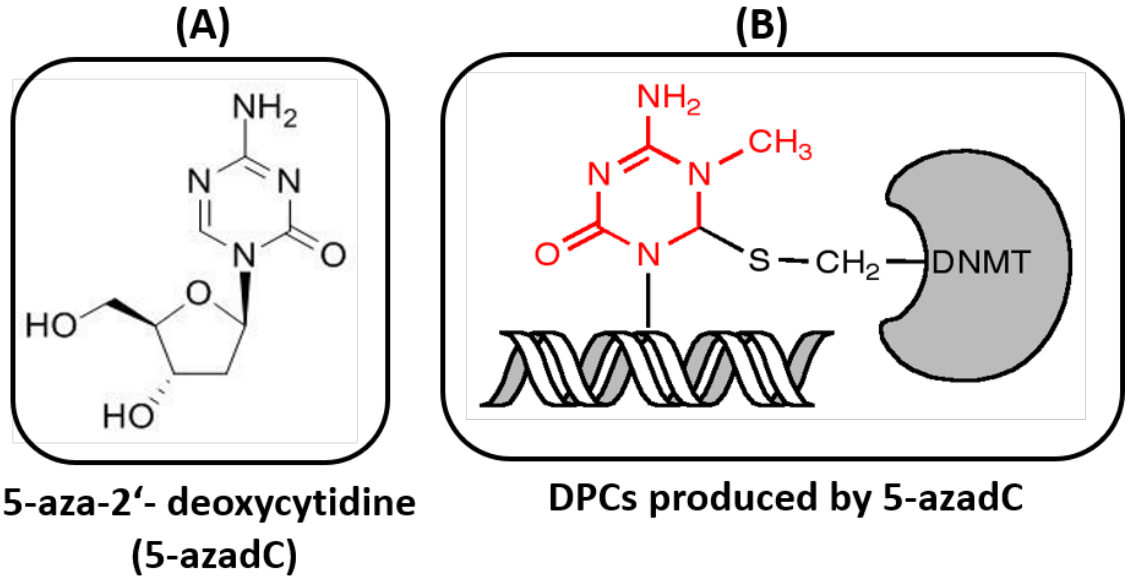

Figure 1. Reaction of 5-azadC with a DNA base forming DPC. (A) Structure formula of 5-azadC (B) DPC formation by 5-azadC. When 5-azadC enters the cell, it is metabolized and incorporated into DNA nucleotides during DNA synthesis in place of cytosine. 5-azadC traps the reaction intermediate of DNMT and SAM (S-adenosyl methionine) is a methyl-donating cofactor of DNMT.

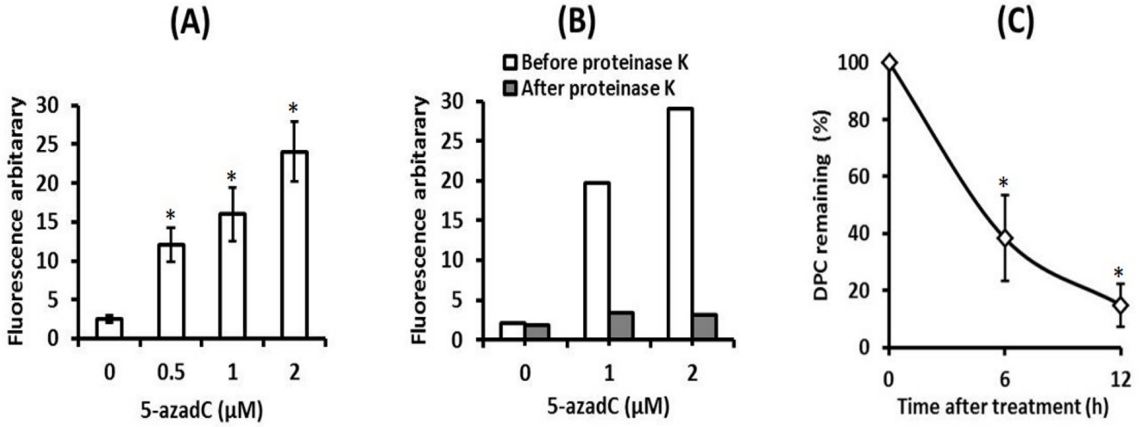

Figure 2. Induction and repair kinetics of DPCs induced by 5-azadC as measured by FITC-fluorescence labeling method. (A) Initial amounts of DPCs in the genomic DNA. Cells were treated with indicated doses of 5-azadC for 24 hours, chromosomal DNA was purified by ultracentrifugation and the CLPs in DNA (30µg) were labeled by FITC followed by fluorescence intensity measurement. Data points are means of four samples with standard deviation. Asterisks (*) indicate significant differences compared with the control when $p < 0.05$ (Student t-test). (B) Confirmation of DPC signal by proteinase K treatment. DNA (30µg) from control and 5-azadC treated cells was incubated with proteinase K for 30 min, dialyzed, and the FITC signal was measured (C) Repair kinetics of DPCs induced by 5-azadC. Cells were treated with 5-azadC at LD20 concentration, the drug was removed and allowed for post-treatment for 6 and 12 hours. Nearly 50% of DPCs were repaired after 6 hours, however, about 20% of DPCs remain in the genome after 12 hours from 5-azadC treatment. The values represent the means ± STDEV (n=3). Asterisks (*) indicate significant differences compared with the control when $p < 0.05$ (Student *t*-test).

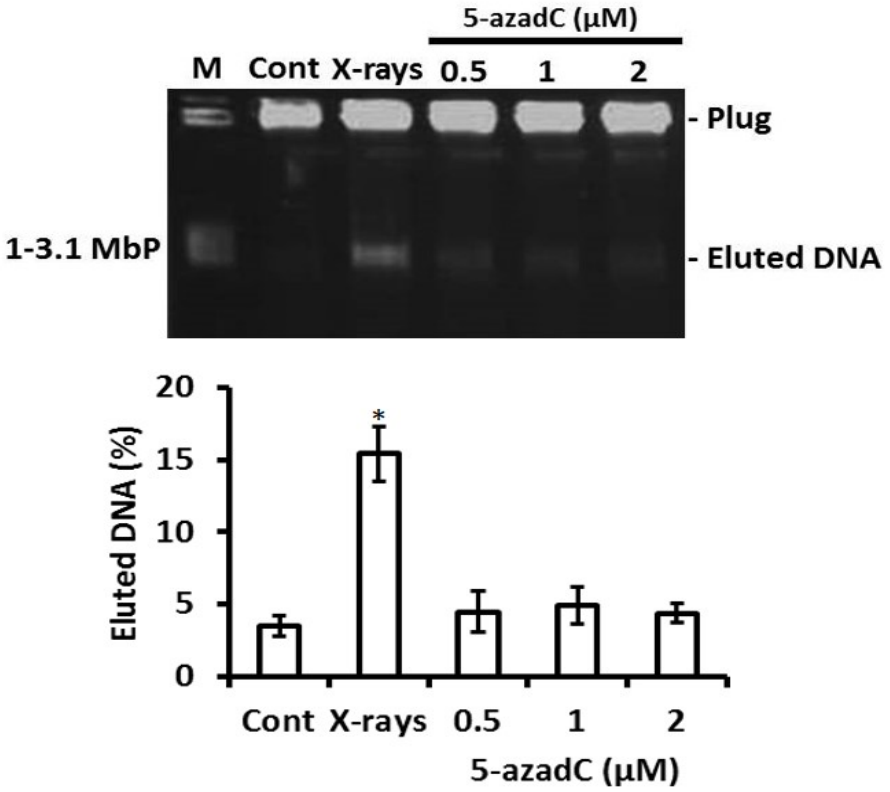

Figure 3. Analysis of DSB formation in human cells treated with different doses of 5-azadC as measured by SFGE. Quantitative analysis of prompt DSBs as measured from band intensity. Cells were collected immediately after 5-azadC treatment or X-rays (10Gy) which were used as a positive induction of DSBs. DSBs were analyzed by SFGE (upper panel) as described in materials and methods. The fraction of eluted DNA relative to total DNA (i.e., eluted and retained in the plug) was used as a measure of DSBs (lower panel). Data points are means of three replicates with standard deviation. Asterisks (*) indicate significant differences compared with the control when $p < 0.05$ (Student *t*-test).

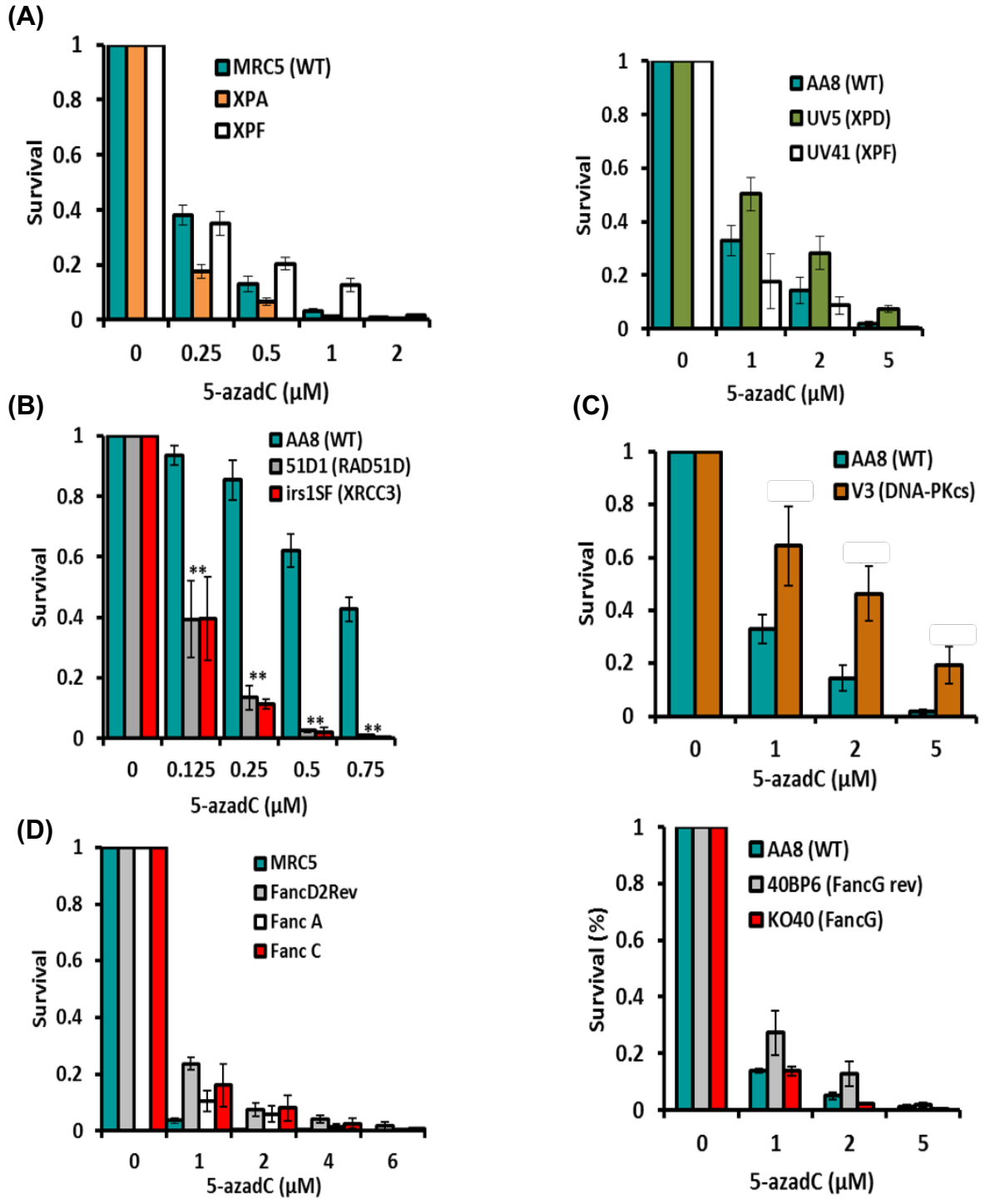

Figure 4. Cell sensitivity of DNA repair-deficient cells treated with 5-azadC. (A) Cell sensitivity of human (MRC5) and CHO (AA8) repair-proficient cells and those deficient in NER XPA and XPF (human) and UV5 (XPD) and UV41 (XPF) (CHO), HR (51D1 (RAD51D) and irs1SF (XRCC3) (CHO) (B), NHEJ (V3 (DNA-PKcs) (CHO) (C), and CHO cells deficient in Fanconi anemia group G (FancG) and human FancA and FancC (D). All cells were treated with the indicated doses of 5-azadC for 24 hours, washed twice with fresh medium, and cultivated for about a week to form colonies. The colonies were counted and the survival fraction was calculated. Data points are means of three to four independent experiments with SD. Asterisks (*) indicate significant differences compared with the wild type cells at each concentration when $p < 0.05$ (Student *t*-test).

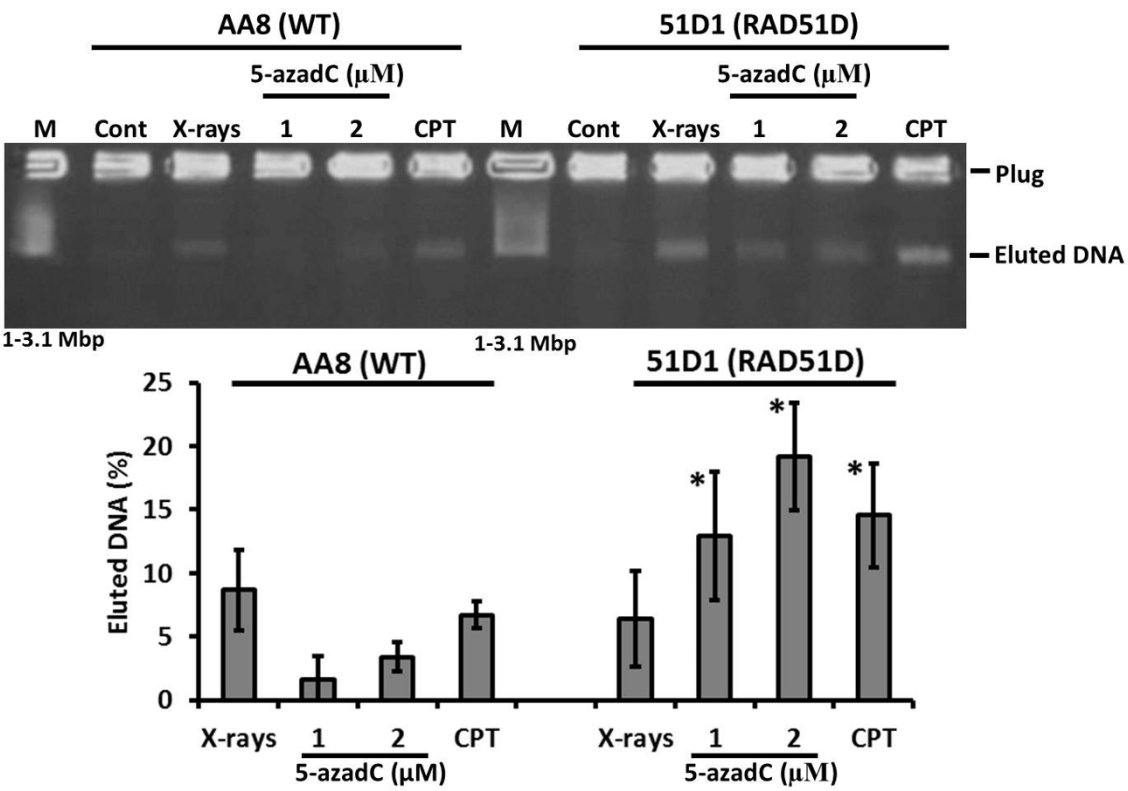

Figure 5. Comparison of DSB induction in CHO cells deficient in the HR pathway and wild type. AA8 (WT) and 51D1 (RAD51D) cells were treated with 5-azadC (1 and 2 μM) for 24 hours. CPT and X-rays were used as positive induction of DSBs. Cells were collected, lyzed, embedded in agarose plugs, and allowed for electrophoresis (Top panel). The percentage of eluted DNA was used as a measure of DSBs and calculated as mentioned in Material and Methods (lower panel). Data points are means of three independent experiments with SD. Asterisks (*) indicate significant differences compared between AA8 (WT) and 51D1 (RAD51D) cells when $p < 0.05$ (Student *t*-test).

The detectable amount of DPCs is mainly due to the tapped DNMT to genomic DNA during the DNA methylation process as previously reported (Anders et al., 2020; Liu et al., 2003). Since 5-azadC induced DPCs, then we asked which DNA repair component is essential for cell survival. To clarify this issue sensitivity of cells deficient in various DNA repair pathways was examined. Among the examined mutants, CHO cells that are deficient in the HR pathway (RAD51D and XRCC3) were sensitive to 5-azadC about 2-4 folds relative to wild-type cells. This result is consistent with the reported finding with aldehydes treated cells in which non-enzymatic DPCs are abundantly formed (Xie et al., 2016). In contrast, cells that deficient in the NER pathway (XPD) and FANC pathway (FancA, B, and C) didn't show any sensitivity to 5-azadC doses. However, another NER mutant (XPF) exhibited a very mild sensitivity. It has been reported that both NER mutant (XPA) and wild-type cells share similar elimination kinetics of DPCs induced by formaldehyde and chloroacetaldehyde (Shoulkamy et al., 2012). This observation is also consistent with a recent study that reported that the loss of XPA in human cells has a minimal impact on DPC repair (HU et al., 2020). Since the NER genes are not intensively involved in the repair of DPCs in cells, the DNA synthesis will proceed and the replication fork will be stalled at the DPC site and become a substrate for HR pathway genes. Consistent with this notion, a previous study using a plasmid replication system in E. coli has reported that the DPCs induced by 5-azadC block DNA replication and generate RecA-dependent X-structures that can be intermediate structures of HR pathway (Kuo et al., 2007). Furthermore, 5-azadC generates the formation of RAD51 foci that is a marker for the activation of HR pathway (Nakano et al., 2009; Orta et al., 2013). Also, MRN complex (MRE11, RAD50, and NBS1), which is a nuclease complex involved in the initiation process of resection of

the HR pathway facilitates the removal of TopoII- adducts from DNA in mammalian cells (Hoa et al., 2016; Lee et al., 2012). These observations suggest that DPCs formed by 5-azadC generate DSBs through the stalled replication fork close to the DPC site and become a substrate for HR pathway gene. To investigate this hypothesis, we examined the DSB induction in HR deficient cells after 5-azadC treatment and compare the yields of DSBs with HR-proficient cells. Our results further demonstrated that treatment of cells with 5-azadC doses did not significantly induce prompt DSBs as measured by SFGE. However, DSBs were significantly detected in HR mutant (RAD51D) compared with a wild type cells indicating that the trapped DNMT by 5-azadC is possibly hampered the progression of stalled replication fork generating DSBs and reactivated by HR pathway. This hypothesis is consistent with previous studies which reported that HR genes maintain genomic DNA integrity by promoting repair and reactivation of collapsed replication forks by DPCs (Novakova et al., 2003; Nakano et al., 2009; Ridpath et al., 2007). In addition, DPCs induced by aldehydes, X-rays, and C-ions stay longer in the genome and are not repaired by the NER pathway (HU et al., 2020; Nakano et al., 2009; Shoulkamy et al., 2012) and further block the replication fork. To restore the collapsed replication fork by DPCs, proteases in yeast (Wss1) and humans (SPRTN) can directly target the cross-linked proteins in the genome and allow its degradation process (Stingle et al., 2014; 2016). Also, previous studies reported that tyrosyl-DNA phosphodiesterase 1(TDP1) and TDP2 were involved in the repair process of DPCs (Nakano et al., 2020; Pommier et al., 2014). Then the replication fork is likely reactivated by the HR pathway to proceeds synthesis of new DNA nucleotides as previously proposed (Nakano et al., 2007; 2009). Up to date, the exact repair mechanism and signaling pathways involved in DPCs are still not fully clarified and further research is required to demonstrate the impact of unrepaired DPCs on human diseases and cancer treatment.

CONCLUSION

DPCs are bulky DNA damage and eventually affect DNA transactions cause genomic instability and cell death. In the present study, we gave insights into the repair mechanism of DPCs induced by DNMT inhibitors. HR pathway genes (RAD51D and XRCC3) are critical DNA repair genes that are essentially required for the repair of enzymatic-DPCs induced by 5-azadC and NER pathway genes have neglected roles.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

Not applicable.

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