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LETTERS

DNA-protein crosslinks are key to platinum-based chemotherapeutic cytotoxicity

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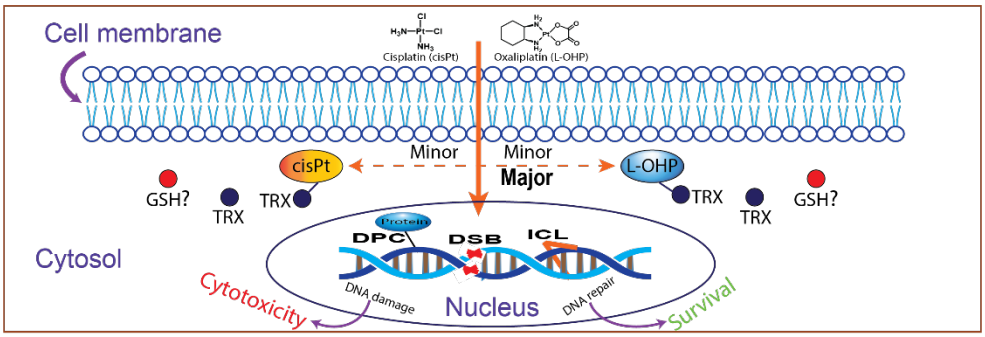
Article



Abstract

Platinum-based chemotherapeutics inflict a spectrum of DNA damage, including DNA adducts, DNA-protein crosslinks (DPCs), and interstrand crosslinks (ICLs) to variable extents. These diverse lesions may contribute to the overall toxicity of these therapeutic agents. Nonetheless, a gap exists in comparative studies elucidating the specific DNA damage responsible for their toxicity. Therefore, we exposed MRC5SV cells to equitoxic doses (LD_{10}) of cisplatin (cisPt) and oxaliplatin (L-OHP) and systematically examined the induction of DPCs, ICL, and protein damage. Our findings suggest that DPCs emerge as the crucial cytotoxic DNA damage for both cisPt and L-OHP, highlighting their central role in the mechanism of action driving the cytotoxicity of platinum-based therapeutics. Both drugs show induction of ICLs as computed by the unique sensitivity of Fanconi anemia cells to the drugs. Additionally, both cisPt and L-OHP didn't show protein damage as indicated by the absence of TRX1 oxidation post-treatment. Overall, our results underscore the critical involvement of DPCs in the toxicity of platinum-based drugs, emphasizing the importance of DPCs as potential cancer therapeutic targets.

Keywords: Platinum-based chemotherapeutics, DNA damage, DNA-protein crosslinks, Interstrand crosslinks, Lethal Dose

INTRODUCTION

Cancer is one of the major causes of mortality around the world, accounting for approximately 9.5 million deaths in 2020.[1] Chemotherapeutic drugs are effectively used for the treatment of several types of cancers.[2] Platinum-based chemotherapeutics are the most clinically used drugs for the elimination of cancer including colorectal, breast, and ovarian cancers.[3,4] cisPt and L-OHP are platinum drugs that possess two reactive groups which facilitate the direct interaction with DNA purine bases generating adducts at the same DNA strand forming intrastrand crosslinks or between two opposite strands forming ICLs together with monoadducts.[5] Platinum drugs have been regarded as the predominant cause of monoadducts and intrastrand crosslinks.[6–8] Moreover, platinum drugs can also lead to the formation of DPCs.[9,10] Therefore, DPCs may cause deleterious and severe damage to cells. Previous studies found that DPCs hinder DNA replication by blocking the progression of replicative helicases and DNA polymerases.[11–13] In addition, DPCs on the transcribed strand may also impede transcription.[14] Notably, one study found that cisPt inhibits thioredoxin reductase (TRXR) in cultured cells, which coincided with its cytotoxicity.[15] These studies demonstrated that platinum drugs can cause DNA and protein damage, potentially contributing to their cytotoxicity. However, no comparative studies have been reported about which damage has more effect on cell viability. Moreover, previous reports used very high doses to analyze the potency of these drugs in inducing DNA damage in cultured cells. In this study, we performed our experiments using equitoxic physiologically relevant doses to circumvent potential artifacts from overdosage.

MATERIALS AND METHODS

Cell culture and drugs

SV40-transformed human fetal lung fibroblast cell line (MRC5SV) and human Fanconi anemia (FANC) cells were used

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for the experiments. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Nisus) supplemented with10% fetal bovine serum (FBS), 1% L-glutamine, penicillin (10,000U/ml) and streptomycin sulfate (10,000µg/ml). The cells were cultivated in a humidified incubator with 5 % CO2 and 37°C. cisPt, L-OHP and auranofin (AUR) were obtained from Sigma-Aldrich.

Cytotoxicity assay

Colony formation assay was used for the measurement of drug-induced cytotoxicity. The cells were seeded into culture dishes (10^3 cells/plate) and maintained for 12 hours. The culture medium was then replaced by a fresh medium containing different doses of cisPt and L-OHP for 2 hours. The effective concentration range for cisPt was observed to be between 5 and 15 µM, whereas for L-OHP it was between 10 and 30 µM. After treatment, drugs were withdrawn, cells were washed twice with fresh culture media, and cultured for a week to produce colonies. The survival fraction compared to untreated control cells was computed by using a threshold of at least 50 cells per colony and used to determine the physiologically relevant dose (LD_{10}).

Measurement of ICLs in the drug-treated cells

Genomic ICLs cannot be directly quantified yet. So, we assessed a genetic approach to measure the induction of ICLs through the analysis of the drug sensitivity of FANC cells with complementation groups A (FANCA) and C (FANCC) relative to wild-type cells using above mentioned colony formation assay.

DPC measurement in drug-treated cells

Exponentially growing cells were exposed to the drug doses specified above. Subsequently, the cells were collected and DPCs were examined using the fluorescein isothiocyanate (FITC) labeling method, with adaptations from a previously reported protocol.[16] Briefly, recovered cells were lysed and centrifuged using CsCl density gradient ultracentrifugation for DNA isolation. For FITC labeling, crosslinked proteins in 30 µg of DNA were incubated at room temperature for 1 hour in 20 mM borate buffer (pH 8.0, 100 µl) combined with FITC (Dojindo) at a final concentration of 0.1 mM. DNA precipitation was achieved through ethanol, and the resulting air-dried DNA pellet was reconstituted in MilliQ water. DNA concentration was determined, and fluorescence signals of FITC-labeled DNA (20 µg) for both treated and untreated samples were quantified using a Hitachi F-2500 fluorescence spectrophotometer.

***In vivo* eradication of DPCs**

To investigate the cellular response to DPCs produced by cisPt and L-OHP, we employed MRC5SV as a cell-line model to track the kinetics of DPC clearance.[16,17] LD_{10} was applied to MRC5SV cells for a duration of two hours to induce DPCs. The medications were removed, and the cells were rinsed with new culture fluid and permitted to proliferate in fresh media. The cells were collected at various time intervals following the treatment, specifically at 0, 6, and 12 hours. DNA was extracted and DPCs were quantified by FITC labeling. The proportion of residual DPCs was determined at 6 and 12 hours in comparison to the initial measurement at 0 hours.

Thioredoxin 1 (TRX1) redox analysis

The redox status of TRX1 was used to indicate protein damage by the drugs cisPt and L-OHP. TRX1 was detected with a modified western blotting approach.[18] Briefly, the drug-treated cells were lysed by urea buffer containing 30 mM iodoacetic acid (IAA). Subsequently, cells were collected and incubated at 37 °C for 15 min. The cell lysate was precipitated with 10 volumes of cold acetone-1 M HCl (98:2, v/v) and washed with cold acetone-1 M HCl-H2O (98:2:10, v/v/v) to obtain the protein. The protein pellet was dissociated in urea buffer containing 3.5 mM DTT and incubated for 30 min at 37 °C. IAM (10 mM) was added to the protein extract and incubated at 37 °C for 15 min. The amount of protein was determined by BCA protein assay kit (Thermo Scientific). For positive control, gel mobility standards were prepared by harvesting cells in 300 µl of urea buffer containing 3.5 mM DTT and incubating for 30 min at 37 °C. Protein extracts were treated with either 10 mM IAM, 30 mM IAA, or 15 mM IAA+15 mM iodoacetamide (IAM) and incubated at 37°C for 15 min. Auranofin-treated cells were processed at the same time and used as a positive control for the oxidation of TRX1.

Western blot detection of TRX1

Urea-polyacrylamide gel electrophoresis (PAGE) was used for the detection of TRX1. Proteins were separated using stacking (2.5 %) and separating (12 %) gels at a constant current of 5 mA for 4 hours and probed with rabbit polyclonal anti-TRX1 (20000 X dilution, Abcam ab109385) at 4°C overnight. This was followed by incubating the primary antibody-bound proteins with HRP-conjugated goat anti-rabbit IgG (9000 X dilution, Abcam ab6721) for 1 hour at room temperature. TRX1 bands were then detected using ECL western blotting substrate (Promega) and visualized by chemiluminescence on a ChemiDoc XRS+ system (BIO-RAD).

Data analysis

Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA) were used for image preparation and statistical analyses. Differences among groups were examined using one-way ANOVA test. Error bars represent the mean ± standard deviation (s.d.) and significance levels are indicated by *P≤0.05, **P≤0.01, and ***P≤0.001. The final formal figure assembly was accomplished using Adobe Illustrator CC 2024 (Adobe Inc., San Jose, CA, USA).

RESULTS

Determination of cytotoxicity

Cancer continues to be a prominent cause of mortality on a global scale, and chemotherapy is a fundamental component in the management of different cancer forms. Platinum-based medications, such as cisPt and L-OHP, are widely employed as chemotherapeutic agents. Nevertheless, the effectiveness of these medications differs, and comprehending their ability to kill cancer cells is essential for optimizing treatment approaches. For our purpose here, we assessed and compared the cytotoxic effects of cisPt and L-OHP on MRC5SV cells. The MRC5SV cells were exposed to varying concentrations of cisPt and L-OHP, and the degree of cell death was assessed by measuring cell survival through colony formation. The cells displayed evident cytotoxicity when exposed to cisPt (Figure 1A) and L-OHP

(Figure 1B), as determined by the decrease in colony count following drug administration. The LD_{10}, which represents the doses at which only 10% of cells survive was determined. Under the given experimental conditions, the toxicity of cisPt was stronger (LD_{10} = 10.3 µM) than that of L-OHP (LD_{10} = 25 µM).

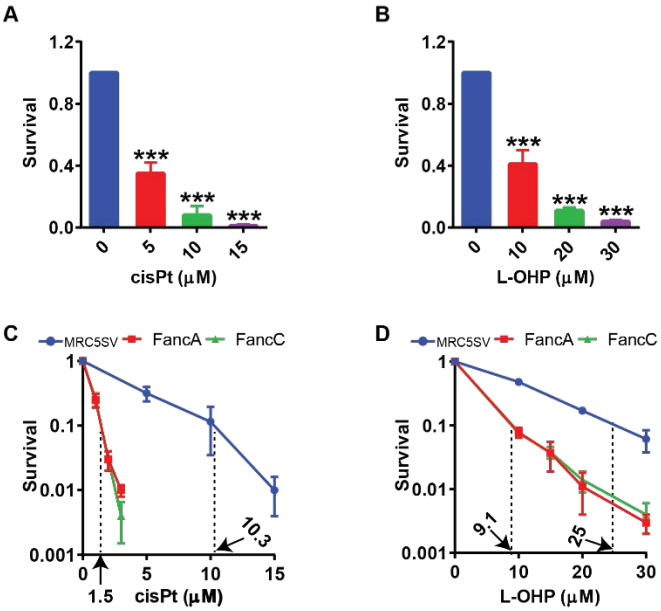

Figure 1. Cell survival assessment of MRC5SV and FANC cells treated with cisPt (A and C) and L-OHP (B and D). The cells were subjected to the indicated doses of the drugs for 2 hours, after which the drugs were removed. Cells were then grown in a medium without any drugs and observed for the development of colonies for one week. The dashed lines and the arrows mark the respective LD_{10} values. Data points represent the means from three independent experiments ± SD.

Analysis of ICL formation

Until recently, a well-established approach for directly detecting ICLs *in vivo* has not been available. In addition, it is well known that the FANC pathway serves as the primary mechanism for repairing ICLs.[19,20] With this consideration, we analyzed the sensitivities of human FANC cells to cisPt and L-OHP to assess the induction of ICLs. The sensitivities of human FANC cells with complementation group A (FANCA) and C (FANCC) to increasing dosages of cisPt and L-OHP were compared to those of FANC proficient wild-type cells (MRC5SV) and computed. The FANC cells exhibited a 6.6-fold sensitivity to cisPt compared to MRC5SV cells, as indicated by the LD_{10} values of 1.3 µM for FANC cells and 10.3 µM for MRC5SV cells (Figure 1C). Similarly, FANC cells exhibited a sensitivity to L-OHP that was about 2.7 times higher than that of MRC5SV cells, with values of 9.1 µM for FANC cells and 25 µM for MRC5SV cells (Figure 1D). These results suggest an essential role for ICLs in the cytotoxic effects induced by cisPt and L-OHP. Furthermore, the results showed that the ability to create ICLs is more effective with cisPt compared to L-OHP.

DPC induction and *in vivo* elimination

Having shown the essential role of cisPt and L-OHP in ICL formation, we investigated the cytotoxic impacts of platinum-based medications. For this analysis, we focused on the induction and elimination of DPCs. We exposed MRC5SV cells to LD_{10} dosages of cisPt and L-OHP, the two platinum-based drugs. Immediately after drug treatment, cells were collected, DNA was purified, and DPC induction was analyzed by FITC-labeling (Figure 2A). The results revealed that both drugs induced DPCs, as demonstrated by the increased fluorescence intensity of labeled genomic-DPCs. Notably, the induction was about 5-fold higher compared to the untreated control, indicating a significant impact of these drugs on DPC formation. Moreover, cisPt showed a higher induction of DPCs compared to L-OHP, suggesting a more potent cytotoxic effect for cisPt compared to L-OHP. To further investigate the role of DPCs in the cytotoxicity observed with cisPt and L-OHP, we monitored the *in vivo* elimination of DPCs (Figure 2 B and C). For this, genomic-DPCs were isolated from drug-treated cells at 0-, 6-, and 12- hours post-treatment and the amounts of remaining DPCs were assessed. Our results showed a time-dependent decrease in the percentage of remaining DPCs, with an apparent half-life of 6.2 hours for cisPt and 4.7 hours for L-OHP. These results suggest that DNA adducts induced by platinum drugs are retained in the genetic material, potentially resulting in significant cytotoxic consequences. Taken together, our results highlight the potential role of DPCs in the cytotoxicity induced by platinum-based drugs and open up new avenues for understanding the clinical features of these drugs.

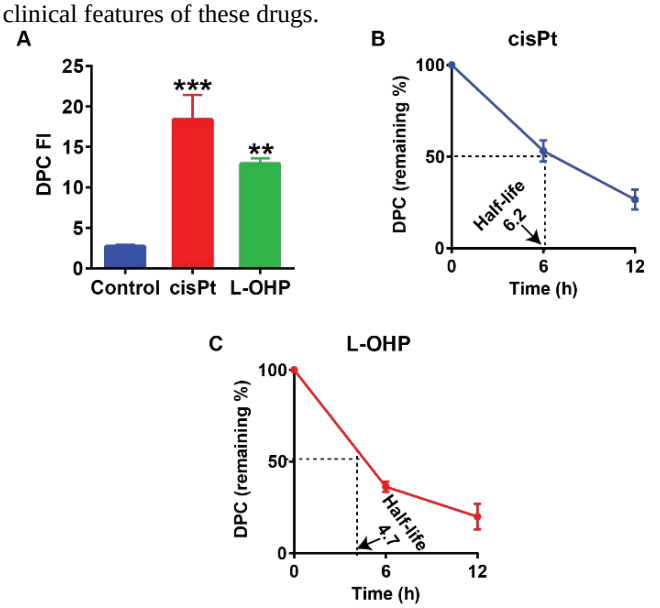

Figure 2. Measurement of DPCs in MRC5SV cells that were exposed to equitoxic doses of cisPt and L-OHP. (A) Evaluation of DPC levels in MRC5SV cells exposed to LD_{10} concentrations (10.3 µM for cisPt and 20 µM for L-OHP) over a duration of 2 hours. The DNA extracted from untreated (Control) and treated cells immediately after treatment was tagged with FITC for detection of DPCs and the fluorescence intensity (FI) was evaluated. (B, C) *In vivo* elimination kinetics of DPC were analyzed after treatment with LD_{10} doses. Genomic DNA was obtained from cells that were subjected to drug treatments at time points of 0, 6, and 12 hours. The recovered crosslinked proteins were then measured using FITC labeling. Data points represent the means of four independent experiments ± SD.

The effects of cisPt and L-OHP on the level of TRX1 oxidation

Thioredoxin 1 (TRX1) is a protein that plays a crucial role in regulating the redox state of cells. The redox state of TRX1 is associated with essential cellular processes such as DNA synthesis, enzyme activity, and regulation of transcription factors that are involved in cell growth, differentiation, and apoptosis.[21–23] We thus aimed to explore whether the redox state of TRX1 is implicated in the cytotoxic effects of cisPt and L-OHP. To evaluate the effects of cisPt and L-OHP on TRX1 redox state, we treated MRC5SV cells with LD_{10} for both drugs. We then assayed the state of cytosolic TRX1 using a modified western blot analysis protocol.[18] The results showed that TRX1 was separated into six bands, indicating different redox states of TRX1. The oxidized TRX1 can be identified through an upward shift in band migration (Figure 3). The pattern of bands was similar between drug-treated cells and untreated cells, indicating the absence of oxidation of TRX1 with both cisPt and L-OHP. This result indicates the absence of oxidation of TRX1 with both cisPt and L-OHP. Cells treated with AUR were assayed at the same time as a positive control for TRX1 oxidation. Overall, these findings suggest that TRX1 oxidation is not involved in the cytotoxicity of cisPt and L-OHP under the tested experimental conditions where LD_{10} doses were used.

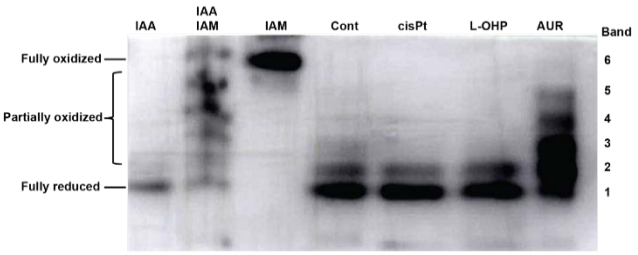

Figure 3. Western blot analysis illustrating the TRX1 protein status after exposure to cisPt and L-OHP at LD_{10} doses. MRC5SV cells were exposed to the drugs for 2 hours. Subsequently, the redox state of TRX1 was examined following the procedures outlined in the Materials and Methods section. The bands, ranging from 1 (completely reduced TRX1) to 6 (completely oxidized Trx1), were isolated and analyzed utilizing IAA, IAM, and IAA+IAM as markers. Auranofin was used as a positive control and was tested at the same time as the cisPt and L-OHP samples.

DISCUSSION

Chemotherapeutic drugs target DNA and induce a spectrum of lesions such as intrastrand crosslinks, ICLs, DPCs, DNA single-strand breaks, DNA double-strand breaks (DSBs), altered bases, and monoadducts.[24] Among these lesions, ICLs and DSBs are the most potent forms and effectively halt both replication and transcription processes when their protein machinery are encountered by ICLs or DSBs.[25] Cells have mechanisms to repair DSBs and ICLs, if these damages are not repaired, it can hinder the proliferation of cancer cells. In addition to DSBs and ICLs, DPCs can potentially cause major impacts on cancer cells.[26] DPCs hinder the advancement of replicative helicases and DNA polymerase, causing the replication process to stop.[11–13] Moreover, DPCs inhibit the transcription process *in vitro*.[14] Intriguingly, it is proposed that DSBs may arise at DPC-stalled replication forks.[27] Notably, DPCs evade repair by nucleotide excision repair, the primary pathway for repairing bulky DNA damage.[28] Also, DPCs are induced by several types of anticancer drugs.[26,29–31] The precise repair mechanism of DPC has not yet been defined. SPARTAN, a newly identified DNA-dependent protease, has been demonstrated to play a role in the restoration of DPCs.[32,33] Moreover, a role of SPARTAN in facilitating the effectiveness of cisPt in both cells and *C. elegans* has been reported.[34]This accumulating evidence underscores the significance of DPCs as bulky lesions contributing to drug cytotoxicity. Nevertheless, the specific impact of DPCs on the killing effects of chemotherapeutic drugs remains insufficiently explored.

Platinum-based chemotherapeutics, specifically cisPt and L-OHP, have been widely recognized for their efficacy in treating various cancers. Nevertheless, the precise DNA damage that causes their cytotoxic effects has been a topic of significant research and debate.[35] This study examined the cytotoxic processes of cisPt and L-OHP, with a specific emphasis on DPCs in relation to ICLs and protein damage. The results of our study provide evidence that highlights the central role of DPCs in facilitating the cytotoxic effects of platinum-based medicines.

Exposing MRC5SV cells to LD_{10} dosages of cisPt and L-OHP caused a notable increase in DPCs, as seen by the higher intensity of fluorescence from FITC-labeled genomic DPCs. Notably, the level of DPC induction occurred even at equitoxic doses (LD_{10}), indicating that DPCs play a significant role in the observed cytotoxic effects of these platinum compounds. The comparative examination of cytotoxicity demonstrated that both cisPt and L-OHP caused DPCs, but cisPt exhibited a more pronounced cytotoxic effect than L-OHP. The difference in cytotoxicity between the two platinum medications corresponds to the greater production of DPCs by cisPt. The temporal elimination of DPCs further emphasized their importance in the cytotoxic mechanism, as cisPt and L-OHP exhibited differing half-lives for the removal of DPCs. Furthermore, our findings suggest that ICLs, although caused by both cisPt and L-OHP, were not proportional at LD_{10} dosages and so may have a restricted impact on the observed cell-killing effect. This finding challenges the current belief that ICLs are the primary cause of DNA damage caused by platinum-based drugs, emphasizing the importance of DPCs as the principal drivers of cellular damage.

Our investigation into protein damage, as indicated by the redox status of TRX1, revealed no significant oxidation in response to cisPt and L-OHP treatment. This suggests that under our experimental conditions, TRX1 oxidation may not be a primary contributor to the observed cytotoxicity of these platinum drugs, aligning with a more primary role for DPCs. Including equitoxic physiologically realistic dosages in our experimental design enhances the credibility of our findings by minimizing the possibility of side effects caused by excessive dosage. While the oxidation of TRX1 was not observed in our work in response to cisPt and L-OHP therapy, it is plausible that platinum medications may impact alternative redox-sensitive proteins and pathways, hence adding to their detrimental effects.

Glutathione, metallothioneins, and a range of antioxidant enzymes are among the potential alternatives.[36] Further investigation is warranted to investigate the relationship between platinum medications and redox signaling pathways to elucidate other mechanisms that contribute to their cytotoxicity.

The induction of DPCs has been extensively documented through the utilization of several anticancer drugs and ionizing radiation and the size and nature of the crosslinked proteins that attach to DNA to form DPCs vary depending on the type of anticancer drug.[37] This variability poses challenges in identifying these proteins and understanding their repair mechanism, making it difficult to monitor DPCs in tumors for clinical relevance. Nevertheless, previous studies have demonstrated that tumors exposed to C-ion and X-ray radiation elicit two distinct forms of DPCs: stable and unstable DPCs. It is noteworthy that the stable DPCs persist longer within the tumor exhibiting half-lives between 63–70 h whereas the unstable DPCs exhibit half-lives between 0.65–0.98 h.[38] This evidence reveals that we can observe the DPCs in a tumor *in vivo* model, which is our next course of action. The presence of persistent DPCs resulting from incomplete repair can impede the replication fork and transcription processes. Upon the resumption of DNA synthesis, the collapsed fork has the potential to be reactivated through the process of homologous recombination (HR), which inadvertently leads to uncorrected recombination.[39] Moreover, the presence of transcriptional errors might potentially impact the expression of crucial proteins necessary for cellular function, hence influencing cellular growth. In summary, the *in vivo* monitoring of DPCs and the characterization of individual proteins involved in DPC repair are crucial aspects to consider. Targeting or inhibiting this repair protein will result in the buildup of DPCs, leading to the development of new and more effective anticancer drugs or sensitizers. These sensitizers enhance the clinical effectiveness of anticancer drugs by interfering with the repair of significant cytotoxic DNA lesions. This increases the clinical significance and introduces new approaches for using platinum drugs. The recognition of DPCs as a major source of DNA damage leading to cell death is, therefore, crucial for the development of targeted therapies. This emphasizes the potential of DPCs as novel targets for cancer treatment.

In conclusion, our findings emphasize the importance of DPCs and enhance our understanding of the complex mechanisms that cause the cytotoxic effects of cisPt and L-OHP. These findings present unresolved inquiries that warrant additional investigation into platinum-based anticancer drugs, ultimately providing more efficient and targeted treatment approaches for various types of tumors.

CONFLICT OF INTERESTS

The authors have declared that no conflict of interests exists.

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