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Disruption of SSBs repair to combat platinum resistance via the JWA-targeted Pt(IV) prodrug conjugated with a wogonin derivative

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An octahedral Pt (IV) prodrug, Cis-wog, containing a wogonin derivative as a bioactive axial ligand was designed and prepared to suppress DDR (DNA damage repair)-related proteins. *In vitro* biological studies indicated that a Pt (IV) prodrug with axially functional groups (Cis-wog) showed cytotoxicity superior to cisplatin and reversed its resistance against two pairs of cisplatin sensitive and resistant cell lines. Further mechanistic research revealed that the powerful antitumor activity of Cis-wog resulted from its suppression of JWA and its multi-interaction with XRCC1 to repair DNA single strand breaks (SSBs) caused by the introduction of wogonin. It is concluded that Cis-wog is a promising cytotoxic agent, which could be used for enhancing the antitumor activity of its corresponding Pt(II)-based drugs and reversing cisplatin resistance via decaying JWA-mediated SSBs repair pathways and inducing apoptosis.

1. Introduction

DNA-targeted chemotherapeutics (Hurley 2002; Sternberg et al. 1989; Baruah 2004), the most clinically effective anticancer chemotherapeutics, are diffusely used for treating human cancers as the essential components. It has demonstrated that DNA-targeted agents such as conventional chemotherapy drugs cisplatin, carboplatin and oxaliplatin exert their function *via* activating DNA damage to drive cells into death (Roos and Kaina 2006; Surova and Zhivotovsky 2012; Roos and Kaina 2013). However, such DNA-targeted chemotherapeutic drugs commonly generate acquired resistances (Longley and Johnston 2005; Brabec and Kasparkova 2005) through activating specific DNA damage repair pathways, thus limiting its clinical application. Based on that, an effective strategy is to combine platinum-based chemotherapeutic drugs with various DNA repair inhibitors (Brown et al. 2017) to improve the sensitivity of tumor cells to reverse cisplatin-induced drug resistance (Sun et al. 2018).

Zhou et al. (1999) have previously reported that the JWA gene, a cytoskeleton-like gene involved in cell differentiation and regulation, was initially isolated and cloned by all-trans retinoic acid (RA). Additionally, Wang et al. (2009) have demonstrated that as a structurally neoteric microtubule-associated protein, JWA, participates in DNA strand break repair under oxidative stress condition as evidenced by comet assay. The reason is that inhibition of JWA expression can suppress DNA strand repolymerization and increase the sensitivity of cells to oxidative stress. JWA can also activate the MAPK cascades by adjusting the expression of E2F1 to participate in the regulation of cancer cell migration. Moreover, other studies have found that JWA is important to protect cancer cells against DNA damage induced by oxidative stress (Zhang et al. 2019; Wang et al. 2012). As we know, XRCC1 is the major protein for DNA single strand break repair (Huang et al. 2002; Tang et al. 2011). In turn, cell survival and DNA replication are dependent on the JWA gene. Further mechanistic investigations (Ren et al. 2011) have demonstrated that JWA has been translocated from the cytoplasm to the nucleus and co-localizes with its foci upon oxidative DNA damage.

Wogonin (5,7-dihydroxy-8-methoxyflavone) is an active ingredient of the traditional Chinese herbal medicine *Scutellaria baicalensis* (Tai 2005). Due to its unique role in the killing of tumor cells, it is considered as an attractive drug candidate. Importantly, multiple lines of evidence suggest that wogonin has moderate cell growth inhibitory and cellulotoxic activities against various types of cancer cell (Zhong et al. 2013; Ejendal and Hrycyna 2002). It exerts no cytotoxicity to HUVEC cells and peripheral blood cells (Jaiswal 2004; Chen et al. 2017). According to the above advantages, wogonin is now being used alone and in combination with other drugs in Phase I clinical trials as an antitumor agent. However, the exact mechanism by which wogonin overcomes chemical resistance remains to be further elucidated.

A large number of research results have currently shown that the octahedral Pt (IV) prodrugs with two additional coordination sites have less toxic side effects on normal cells due to their relatively stable structure and in contrast to having shown significant antitumor activity in many malignancies (Caldecott et al. 1994). Introduction of small molecule groups acting on different targets as axial groups into the platinum (IV) complex cannot only improve the antitumor activity of platinum complexes through synergistic effects but also to some extent overcome side effects of corresponding Pt(II)-based drugs (Parson et al. 2010). It is reasonable to design a heterozygous compound to increase the anti-inflammatory and antitumor activity of wogonin and reduce the toxicity of Pt (II) complex through its synergistic effect owing to the various biological functions of wogonin (Li et al. 2015). Interestingly, although the activation of specific DNA single strands damage repair pathways is closely related to drug resistance, the underlying mechanism remains to be explored. Based on that, we sought to investigate its effects of a novel Pt(IV) prodrug (Cis-wog) by conjugating the axial group wogonin on cisplatin-resistant non-small cell lung cancer (A549/cDDP cell) through preventing multiple DNA damage response-mediated pathways. It has been concluded that the new compound could improve antitumor properties and overcome the cisplatin-induced resistance *via* suppressing SSBs repair response essential for aberrant JWA activity.

2. Investigations, results and discussion

2.1. Interaction of Pt(IV) complexes with DNA reduced by AsA

As shown in Fig. 1B, no distinct fluorescence intensity change was observed after treatment with Cis-wog. In contrast, cisplatin containing divalent Pt (II) species could strongly cause a decrease

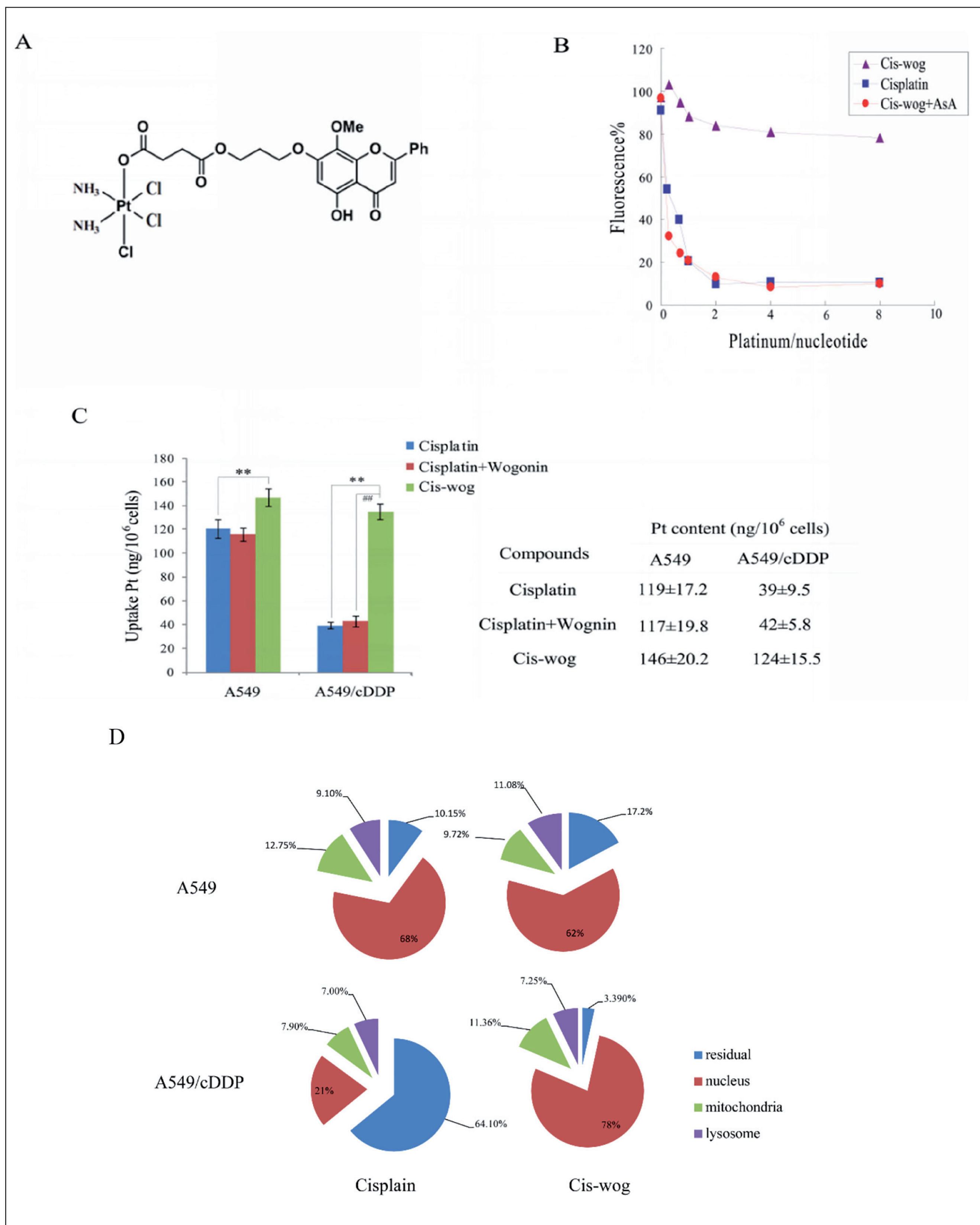


Fig. 1: Reactions of Cis-wog with DNA by reduction. A) Molecular structure of Cis-wog ($C_{23}H_{27}Cl_3N_2O_9Pt$, MW=776.5). B) The different Pt/nucleotide ratios of DNA platination. All the reactions were conducted with 0.01mg/mL DNA in 10 mM $NaClO_4$ in 10 mM phosphate buffer (pH=7.4) at 37 °C for 24 h and then add 0.04 mg/mL EtBr before the fluorescence measurements with the excitation wavelength of 546 nm and the emission wavelength of 590 nm. C) The cellular uptake of cisplatin, an equivalence mixture, Cis-wog in A549 and A549/cDDP cells. The levels of Pt in cancer cells were detected by ICP-MS after 12 h incubation with the treatments of evaluated complexes at 30 μM. D) The Pt accumulation of cisplatin and Cis-wog in lysosomes, mitochondria, nucleus and residual were determined by ICP-MS. Results are representative of at least three independent experiments and shown as the mean±S.D. *$P<0.05$*, **$P<0.01$* compared with cisplatin-treated cancer cells. $P<0.01$ compared with an equivalence mixture-treated cancer cells.

of fluorescence intensity. Interestingly, after the addition of reductant ascorbic acid (AsA), Cis-wog was reduced to platinum (II) complex, and the fluorescence intensity decreased to be comparable to that of cisplatin. Collaboratively, the series of data suggested that in the presence of reductant ascorbic acid AsA, Cis-wog might play a central role in the cancer therapy process as the prodrug of cisplatin to exert its antitumor potency.

2.2. Cellular uptake assay

To assess whether the enhanced cytotoxic activity caused by Cis-wog due to the high uptake of platinum, the cellular uptake was determined by ICP technique following treatment with Cis-wog and cisplatin in A549 and A549/cDDP cancer cells. As shown in Fig. 1C, when investigating Cis-wog, significant increases in cellular uptake were observed in all tested cell lines relative to cisplatin alone in A549/cDDP cancer cells. The Pt accumulations in three subcellular compartments were detected in Fig. 2D. The results showed that 68% of the intracellular Pt was accumulated in the nucleus of A549 cells, while only 21% in A549/cDDP cells. By the contrary, 62% and 78% for Cis-wog in A549 cells and A549/cDDP cells, respectively. These data indicated that Cis-wog led to higher platinum accumulation and exhibited higher cytotoxic activity than cisplatin in A549/cDDP cancer cells. This suggests that the drugs circumvent resistance by increasing cellular uptake.

2.3. In vitro cytotoxicity assay

To evaluate the effect of the tested compound on the antiproliferative capacity of cancer cell lines *in vitro*, A549 (cisplatin-susceptive lung cancer) and A549/cDDP (cisplatin-resistant lung cancer) cells were treated with cisplatin, wogonin, co-administration and Cis-wog. As shown in the Table, Cis-wog following 48 h treatments showed stronger cytotoxic activity than cisplatin against A549/cDDP cancer cells. Notably, the drug resistance factor (RF) was reduced from 5.10 to 0.89. These data highlighted that Cis-wog was promising to reverse cisplatin-induced resistance. Taken together, these data support our hypothesis that Cis-wog has superior anticancer activity than cisplatin or wogonin alone, and wogonin may be the main reason for its anticancer potency by disrupting DNA repair response mechanisms.

Table: IC_{50} values of the measured drugs against A549 and A549/cDDP cancer cells for 48 h

IC_{50} (μM)				
Drug	A549	A549/cDDP	LO2	FI[a]
Cisplatin	1.24	6.324	11.34	5.1
Wogonin	72.75	87.49	118.63	1.2
Cis+Wog	7.31	14.58	36.51	1.99
Cis-Wog	4.48	3.98	42.45	0.89

a. Fold Increase, IC_{50}(A549/cDDP)/ IC_{50}(A549)

2.4. Cellular responses

To further determine the cytotoxicity effect of Cis-wog *in vitro*, the inhibitory activity of Cis-wog was evaluated by cell cycle arrest, cell apoptosis and comet assays in A549 and A549/CDDP cancer cell lines. Treatment two cancer cells with wogonin (30 μM), cisplatin (30 μM), Cis-wog (30 μM) and an equimolar mixture of cisplatin and wogonin for 24 h, the results are shown in Fig. 2A. As expected, wogonin alone had little effect on the two cancer cell lines in triggering cell apoptosis, whereas Cis-wog could not only remarkably increase the percent of cell apoptosis compared to cisplatin. It was also observed to have a slight enrichment than the mixture, showing a high antiproliferation effect of Cis-wog on facilitating apoptosis.

Given that the DNA replication was effectively blocked by antitumor platinum drugs to cease cell apoptosis in cancer cells, we next examined the effect of Cis-wog on cell cycle arrest. To this end, we selected A549 and A549/cDDP cancer cells by treatment with 30 μM wogonin, cisplatin, an equimolar mixture of cisplatin and wogonin and Cis-wog for 24 h. Subsequently, distribution of cell cycle was detected. Previous work has suggested that the cells were arrested at the S phase by cisplatin (Kristoffer and Arun 1995). Strikingly, there was a profound enrichment in the number of A549 and A549/cDDP cells of the S phase following cisplatin treatment, compared with the untreated cells (Fig. 2B). And wogonin also blocked the cells at the S phase, slightly weaker than cisplatin. Whereas the amount of cells arrested at the S phase were more variable against A549/cDDP cancer cells following Cis-wog treatment, which has been shown the best. Correspondingly, the findings indicated that treatment with Cis-wog still targeted DNA to arrest the cycle of A549/cDDP cells. Notably, the introduction of wogonin had a pronounced effect towards the ability of the Pt (IV) complex to arrest the cell cycle. Furthermore, DNA-targeted platinum complexes are commonly inducing apoptotic cells and produce DNA damage.

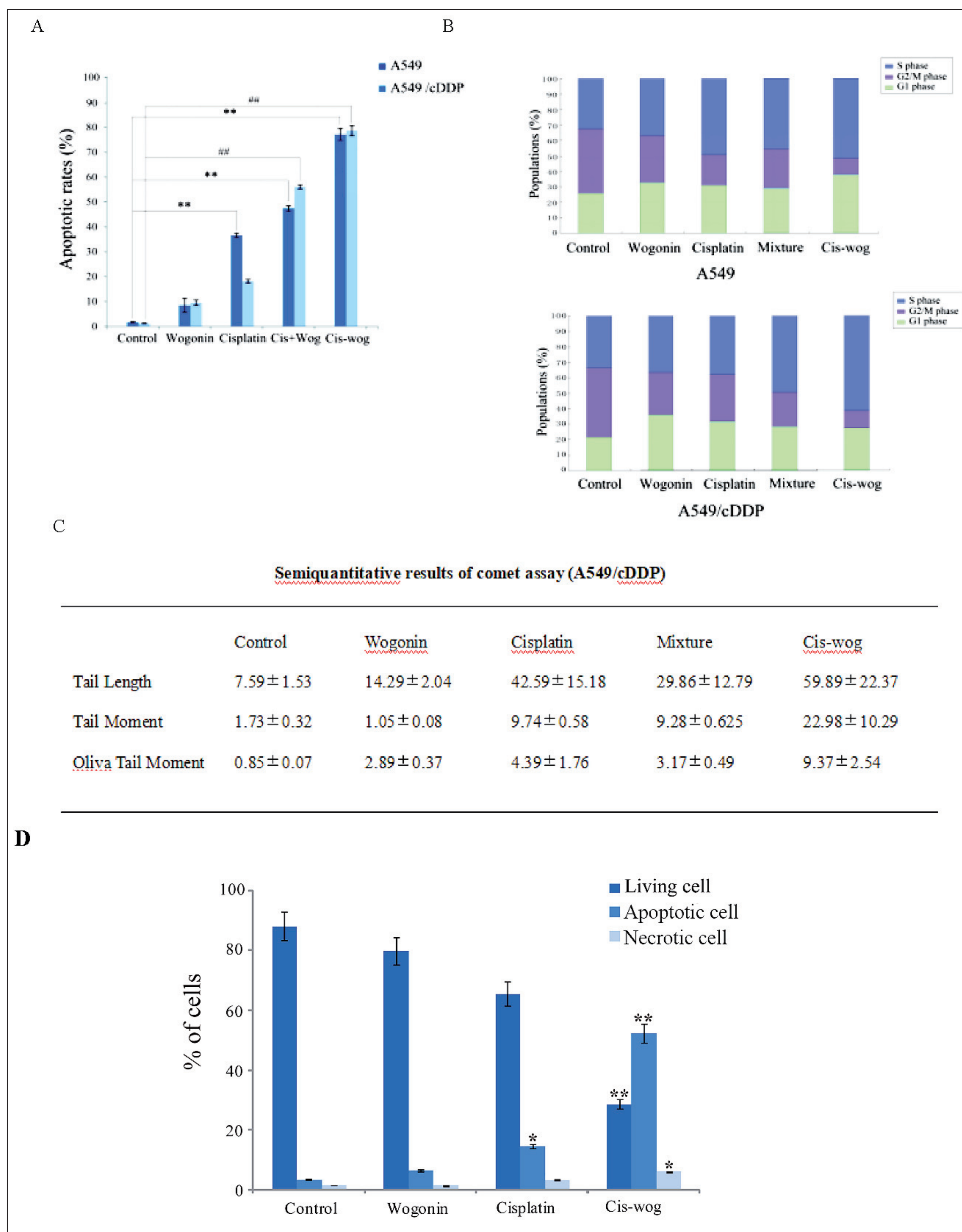
Finally, we analyzed the extent of DNA damage in therapeutic response progress by comet assay (Sorenson et al. 1990). Intriguingly, cancer cells treatment with Cis-wog showed a significantly better DNA damage response than other groups in three parameters (tail length, tail moment, olive tail moment, Fig. 2C). Collectively, these series of experiments demonstrated that when treated with Cis-wog, A549 and A549/cDDP cells lines exhibited a marked DNA damage compared to cisplatin alone, as evidenced by an increase in cancer cell apoptosis. The data in Fig. 2D proved that Cis-wog exerted a higher apoptotic rate than cisplatin in A549/cDDP cells, indicating that Cis-wog could induce a high amount of apoptosis in the cisplatin resistant cells.

2.5. Biomarkers of DNA damage

DNA single-strand break (SSBs) assay (Hartmann and Speit 1997) is an effective method to study DNA damage levels. Thus, to further corroborate DNA single-strand break damage caused by Cis-wog and cisplatin in lung cancer, we next used an alkaline elution assay to determine the extent of DNA SSBs in cancer cells. In Fig. 3A, the DNA fragment content treated with the tested compounds against cancer cells at different time points is shown. The results showed that there was an obvious upregulation in the extent of SSBs following Cis-wog treatment, which was mirrored by an increase of the numbers of SSBs especially in the A549/cDDP cells. DNA damage has many different forms, among which DSBs are considered to be the most severe DNA damage (Higo et al. 2017). The phosphorylated histone H2AX (γH2AX) is also considered to be an indicator for detecting DSBs in cells. Accordingly, we assessed whether the measured samples caused DSBs and determined the extent of DNA fragmentation by immunofluorescence assay. After 8 h and 12 h, Cis-wog caused more γH2AX foci than cisplatin in A549 but little in A549/cDDP cancer cells (Fig. 3B). In line with these experiments, the DNA damage mechanism covered the possibility that Cis-wog induced much higher levels of SSBs in both cancer cells. Overall, the result highlighted a crucial role for the deadliest attack of SSBs in Cis-wog-induced cell apoptosis which probably was linked with introduction of wogonin.

2.6. Blocks with SSBs repair response

Previous comet assay studies showed that compared to vector control cells, DNA damage in JWA knockdown cells is more severe (Sancar et al. 2004). The DNA damage repair frequency was reduced by JWA knockdown, which resulted from oxidative stress. Therefore, the damaged DNA can be visually observed under a fluorescence microscope by the alkaline comet assay. However, it has also been reported that alkylating agents and oxidizing agents induce alkaline unstable base lesions or AP sites, causing SSBs (Chen et al. 2015). Studies were undertaken to examine the effect of endogenous JWA on the repair of DNA lesions induced *via* platinum-based prodrugs. First, in LUC^{con} plasmid, the DRC of the plasmids damaged by cisplatin was determined. We next



Semiquantitative results of comet assay (A549/cDDP)

	Control	Wogonin	Cisplatin	Mixture	Cis-wog
Tail Length	7.59±1.53	14.29±2.04	42.59±15.18	29.86±12.79	59.89±22.37
Tail Moment	1.73±0.32	1.05±0.08	9.74±0.58	9.28±0.625	22.98±10.29
Oliva Tail Moment	0.85±0.07	2.89±0.37	4.39±1.76	3.17±0.49	9.37±2.54

Fig. 2: Cellular responses of cisplatin, wogonin, their equimolar mixture, Cis-wog in A549 and A549/cDDP cells. A) Apoptosis inducing property of the measured samples by Annexin V-FITC/PI staining of cancer cells. The Y-axis shows the PI-labeled population and the X-axis shows FITC-labeled Annexin V-positive cells. B) Cell cycle analysis upon exposure to Cis-wog. A549 and A549/cDDP cancer cells exposed to the measured compounds were stained with propidium iodide and subjected to flow cytometry analysis. C) Comet assay revealing increased chromosomal DNA strand breaks including SSBs, DSBs and active excision repair of DNA cross-links triggered by the measured samples in A549 and A549/cDDP cancer cells. D) The dual staining of AO/EB in cisplatin resistant A549/cDDP cancer cells following the corresponding treatments for 24 h. The graph shows apoptotic and necrotic rates of A549/cDDP cells. Results are representative of at least three independent experiments and shown as the mean±S.D. *P<0.05, **P<0.01 compared with control group. P<0.01 compared with cisplatin-treated cancer cells. &P <0.05, &&P<0.01 compared with the combination group.

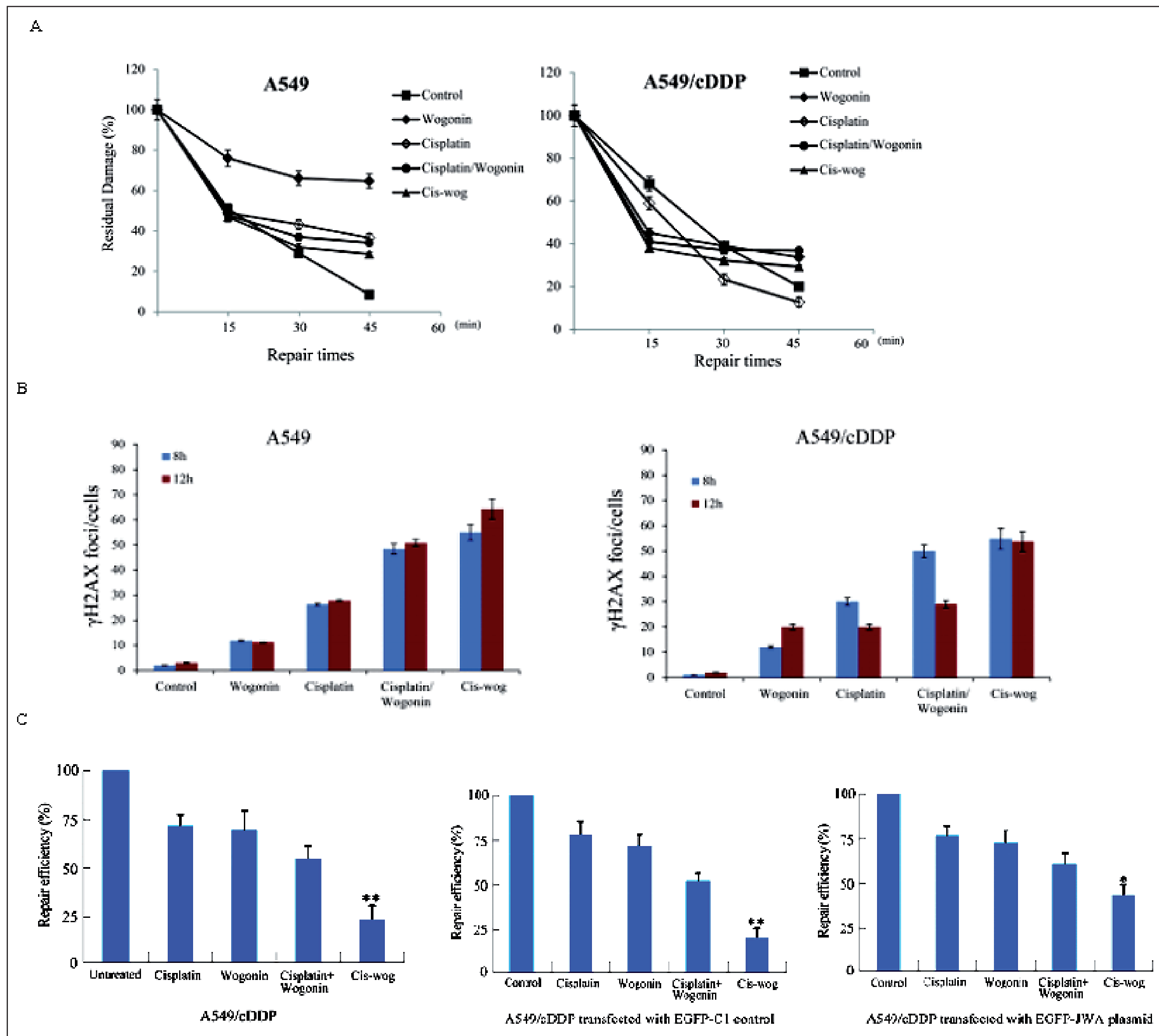


Fig. 3: DNA damage of the measured samples induced SSBs and DSBs. A) The number of SSB was determined by a neutral comet assay. B) γH2AX foci after treatments were counted in 50-60 individual cells per time points of 8 h and 12 h in A549 and A549/cDDP cancer cells. Graph represents average number of foci per cells ± SD. Results are representative of at least three independent experiments and shown as the mean ± S.D. *$P<0.05$, **$P<0.01$ compared with control group. C)A549/cDDP cells were transfected with control LUC plasmid DNA (pGL3-control) or the same plasmid damaged by measured samples. DNA repair rates were measured as the ratio of LUC activity in extracts from cells transfected with a damaged plasmid to the LUC activity in extracts from cells transfected with an undamaged plasmid. The experiment was done in triplicate. D) Overexpression of JWA increased DRC of damaged plasmids in A549/cDDP cells. A549/cDDP cells were transfected with either the EGFP-C1 control or EGFP-JWA plasmid together with undamaged and damaged LUC plasmids. Twenty-four hours after transfection, the DRC of the damaged LUC reporter was assayed as indicated in (C). The renilla luciferase reporter (internal control, Promega) was used to normalize the activity of the LUC reporter. Graph represents average number of foci per cells±SD. Results are representative of at least three independent experiments and shown as the mean±S.D. *$P<0.05$, **$P<0.01$ compared with control group.

assessed whether JWA plays a functional role in Cis-wog-treated A549/cDDP cancer cells by HCR assay. Indeed, Cis-wog, unlike cisplatin, was found to effectively inhibit the DRC in A549/cDDP cells, with the DRC of 75% (Fig. 3C). Conversely, Cis-wog treatment significantly increased DRC toward JWA overexpressed A549/cDDP cell (Fig. 3D).

2.7. In vivo antitumor effects and pharmacokinetics of Cis-wog in xenograft nude mice

An xenografts nude mice model of A549/cDDP cells was performed to detect the anti-tumor effect of Cis-wog *in vivo*. Cisplatin (i.v. 5 mg/kg once a week), wogonin (i.v. at 5 mg/kg once a week), the combination of cisplatin and wogonin, and Cis-wog (i.v. at 5 mg/kg once a week) were administered to four nude mice groups. The results indicated that the tumor volumes (Fig. 4A) in the Cis-wog-treated group were prominently decreased, and the tumor growth inhibition (TGI) rate of Cis-wog reached 71.15% (Fig. 4B). There is no obvious difference in TGI between Cis-wog and cisplatin/wogonin due to their different *in vivo* pharmacokinetic processes. The levels of Pt in liver, spleen, Lung and kidney (Fig. 4D) were significantly reduced after Cis-wog proving that Cis-wog could exert marked anticancer activity and be safe for the treated animals (Fig, 4C). The interaction owes to the preferable plasma stability of Cis-wog and the longer blood retention as compared to cisplatin (Figs. 5A and 5B).

The DNA damage repair pathway is a potential target for tumor therapy. Radiotherapy and some of the main chemotherapeutic drugs used clinically (Luke et al. 2010) have achieved anti-tumor effects by causing DNA damage in tumor cells, and activation of the tumor cell repair system is an important reason of radiotherapy and chemotherapy tolerance. Therefore, the inhibition of the repair

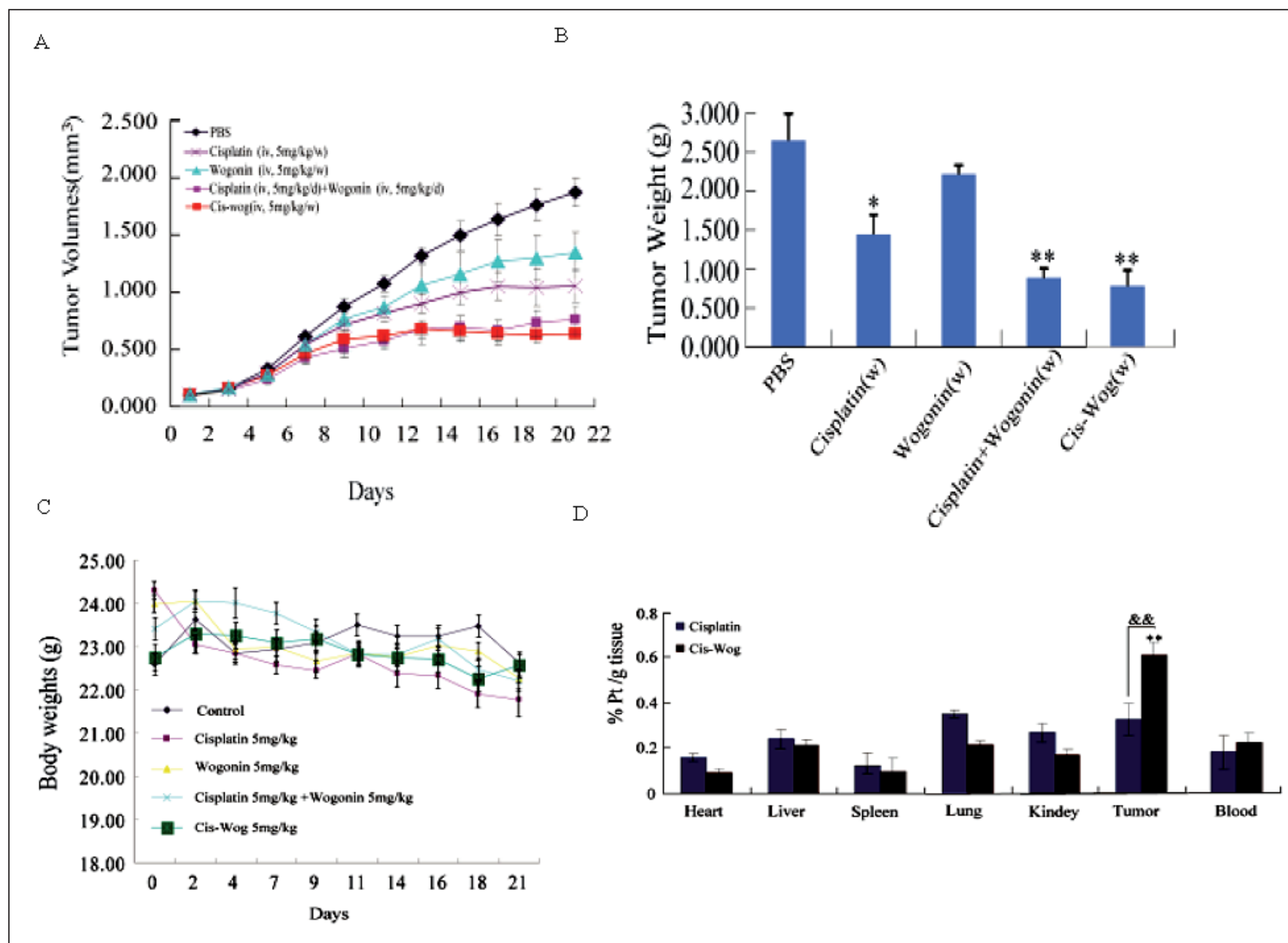


Fig. 4: *In vivo* antitumor activity of cisplatin, wogonin, cisplatin+wogonin and Cis-wog in cisplatin resistant A549/cDDP xenograft tumors. Mean tumor volumes, tumor and body weights are presented. Cisplatin (dosed intravenously at 5 mg/kg), wogonin (dosed intravenously at 5 mg/kg), combination of cisplatin and wogonin, Cis-wog (dosed intravenously at 5 mg/kg). A) The tumor volumes in xenograft mouse models at the administration of the tested groups. B) The tumor weight in each group at the end of the *in vivo* experiment. C) Measured body weight loss of mice during the treatments. D) Normal tissues and tumor distribution of cisplatin and Cis-wog in mice bearing cisplatin resistant A549/cDDP tumors after the corresponding administration. Results are representative of at least three independent experiments and shown as the mean±S.D. *P < 0.05, **P < 0.01 compared with control group. #P < 0.05,   P < 0.01 compared with cisplatin-treated group.

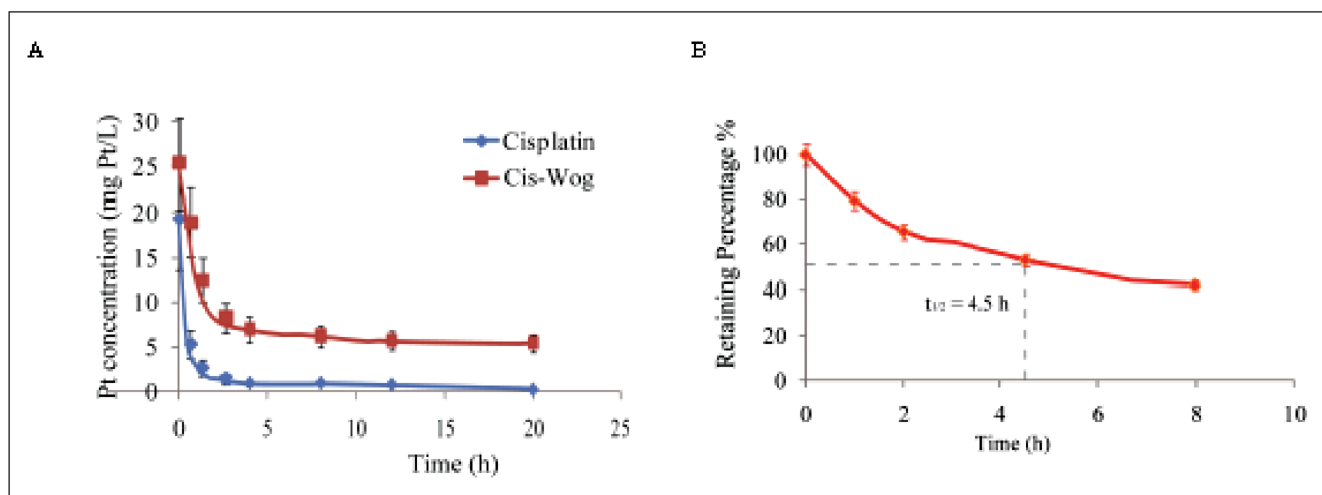


Fig. 4: *In vivo* antitumor activity of cisplatin, wogonin, cisplatin+wogonin and Cis-wog in cisplatin resistant A549/cDDP xenograft tumors. Mean tumor volumes, tumor and body weights are presented. Cisplatin (dosed intravenously at 5 mg/kg), wogonin (dosed intravenously at 5 mg/kg), combination of cisplatin and wogonin, Cis-wog (dosed intravenously at 5 mg/kg). A) The tumor volumes in xenograft mouse models at the administration of the tested groups. B) The tumor weight in each group at the end of the *in vivo* experiment. C) Measured body weight loss of mice during the treatments. D) Normal tissues and tumor distribution of cisplatin and Cis-wog in mice bearing cisplatin resistant A549/cDDP tumors after the corresponding administration. Results are representative of at least three independent experiments and shown as the mean±S.D. *P < 0.05, **P < 0.01 compared with control group. #P < 0.05,   P < 0.01 compared with cisplatin-treated group.

system can enhance the sensitivity to radiotherapy and chemotherapy, and improve the therapeutic effect. The antitumor efficacy of cisplatin has been widely investigated, with high levels of DNA damage, but this may be impaired by toxicity and the acquisition of resistance. Targeting newly potential proteins to highly reverse drug resistance and enhance combination radiotherapy and chemotherapy efficiency by disrupting more extensive DNA repair pathway.

JWA is a newly discovered and retinoic acid-induced cytoskeleton-like gene involved in the regulation of cell differentiation. Previous studies (Nataliya et al. 2016) pointed out that JWA gene is an environmental response gene regulated by a variety of induced differentiation agents and is involved in cell differentiation and apoptosis. It is widely involved in cellular responses to stress stimuli as well as in DNA damage and repair processes, thus indicating that JWA may be a functionally target gene. In addition, Myrna et al. (2018) confirmed that JWA inhibited the ubiquitination of XRCC1, regulated and stabilized the function of nuclear XRCC1, and participated in base excision repair BER and DNA single-strand damage repair.

Here, our study provided direct evidence that JWA has a notable impact on the repair of DNA SSBs (Mego et al. 2013). The HCR assay confirmed that it is necessary for endogenous JWA to remove the Cis-wog-induced DNA lesions. Greater DNA damage was observed in JWA-depleted A549/cDDP cancer cells. These phenomena indicate that JWA has a protective effect on cells and is involved in the proliferation and differentiation of cells in response to the Pt(IV) prodrug Cis-wog. Upon above all the study, JWA is a potentially active target of Cis-wog to elevate antitumor activity in contrast to cisplatin, with nearly no toxic effect. Moreover, it plays a key role in DNA repair signal transduction, which produces resistance induced by DNA-targeted agent in cancer cells. The content of cellular uptake of Pt and the extent of DNA platination were enhanced by Cis-wog, hence activating significant DNA damage repair pathways. Our data indicated increased SSBs in cancer cells and inhibition of the potent SSBs repair through Cis-wog treatment. Notably, suppressed interaction of JWA with XRCC1 was observed in Cis-wog due to its repression on SSBs repair.

2.8. Conclusions

Taken together, a series of biological experiments showed that Cis-wog has excellent antitumor activity and could overcome cisplatin-induced resistance through inhibiting JWA-mediated recruitment of SSBs repair proteins XRCC1. Meanwhile, it caused DNA strand break in both cancer cells (especially single-strand break) and hindered the repair of DNA damage. It is rational to conclude that Cis-wog is a potential antitumor candidate drug with research and development prospects.

3. Experimental

3.1. Chemical compounds

Cis-wog (MW: 776.5; Fig. 1A) was stored in DMF solvent with a purity not less than 98% for cell analysis. The compound was synthesized at Jiangsu Provincial Key Laboratory of Biomedical Research of Southeast University.

3.2. Reactions of Cis-wog with DNA

The DNA of herring sperm concentration was confirmed by UV-Vis Spectra using a 6600 M^{-1} extinction coefficient at 260 nm. The mixture of platinum compound and DNA was incubated in the dark at 37 °C. The fluorescence of the DNA solution added in 0.04 mg/mL EtBr was measured in 0.4 M NaCl. Cis-wog is reduced by adding ascorbic acid. The fluorescent meter was set to a scanning speed of 2000 nm·min^{-1}, an excitation slit width of 5 nm, an emission slit width of 10 nm. The fluorescence was determined under the conditions of an excitation wavelength of 530 nm and an emission wavelength of 592 nm.

3.3. Cellular platinum uptake and DNA platination assay

The cells were incubated in a six-well plate with appropriate density, and cultivated overnight. After the cells were incubated with 30 μM complexes at 37 °C for 12 h, the harvested cells were concentrated and digested by nitric acid and then counted. The lysosomes, mitochondria and nucleus of cancer cells were isolated from A549 and A549/cDDP cancer cells. The Pt concentrations of cellular DNA in A549 and A549/cDDP cancer cells were detected by ICP-MS before the isolation of DNA using Genomic DNA Mini Preparation Kit (Beyotime, China).

3.4. In vitro cell viability assay

A549 and A549/cDDP cancer cells were seeded in 96-well plates at a density of 10^5 mL^{-1} overnight. Then the cells were treated with cisplatin, wogonin, and Cis-wog separately at gradient concentration for 48 h at 37 °C. Cytotoxicity profiles of cisplatin, wogonin, and Cis-wog against A549 and A549/cDDP cancer cell lines were determined by the MTT assay (Yang et al. 2018).

3.5. Apoptosis assessment

Apoptosis was quantitatively detected by flow cytometry employing double staining. The cells were incubated with 30 μM cisplatin, wogonin and Cis-wog for 24 h at 37 °C before cultured in a 6-well plate overnight. Briefly, the harvested cells by trypsinization were incubated with binding buffer (pH 7.5, 10 mM HEPES, 2.5 mM $CaCl_2$ and 140 mM NaCl) for connecting with Annexin V-FITC, and then PI was added to every group for 10 min in the dark. Cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, USA) and data were analyzed by Cell-Quest software (BD Biosciences, Franklin Lakes, NJ, USA).

3.6. Cell-cycle analysis

Cell cycle (Kumar 2014) data were collected from the flow cytometry mentioned earlier and analyzed by FlowJo software (TreeStar, Inc.).

3.7. Comet assay

The extent of DNA damage in the cells was demonstrated by comet experiments. The A549 and A549/cDDP cells were incubated with 30 cisplatin, wogonin and Cis-wog for 24 h, and then collected. 1×10^5 cells were removed after centrifugation to mix with molten low melting Agarose (Trevigen) of 10 times the volume. The mixed solution is placed on a glass slide that has been coated with NM Agarose (Trevigen). The prepared samples were soaked in the precanceled lysis buffer at 4 °C for 30 min. The slides were immersed in alkaline electrophoresis solution (200 mM NaOH, 1 mM EDTA) at room temperature for 20 min, and then operated for 30 min at 25 V in the horizontal electrophoresis tank. The samples were washed three times in Tris-HCl (pH 7.4) for 10 min every time. Then the slides were observing by microscopy. The imagines were analyzed by applying Comet Assay Software Project (CASP).

3.8. Analysis of SSB repair

The cancer cells were treated with 30 μM cisplatin, wogonin, and Cis-wog for SSB detection at 37 °C, respectively, for 15 min, 30 min and 60 min. The numbers of SSB were detected by an alkaline elution assay after washing with PBSCMF (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1 mM KH_2PO_4) three times (Parodi et al. 1982). The SSB numbers in untreated cancer cells were subtracted in all cases.

3.9. γH2AX foci repair kinetics

Analysis of γH2AX repair foci kinetics was performed as described above and numbers of γH2AX foci were counted in 50-60 nuclei and plotted over time as previously described (Nikolova et al. 2017).

3.10. Luciferase reporter gene assays and host-cell-reactivation (HCR) assay

Cells incubated in 24-well plates were co-transfected with 2.25 mg of pGL3-XRCC1 (-881 to +158) or pGL3-XRCC1 (-766 to +158) luciferase-reporter plasmid, together with the JWA shRNA or control shRNA plasmid with Lipofectamine 2000. All the plasmids were co-transfected with 10 ng of pRL-SV40 containing the Renilla luciferase gene. After the treatment with 30 μM cisplatin, wogonin and Cis-wog for 24 min, cell lysates were prepared in line with Promega's instruction manual 48 h later. Luciferase activity was detected by a dual-luciferase reporter assay system (Promega, Madison, WI, USA) and the activity was normalized against the Renilla luciferase gene. The DNA repair capacity (DRC) was measured with the HCR assay (Valerie and Singhal 1995). SV40 promoter and enhancer sequences are included in the pGL3-control luciferase vector (LUC^{con}) (Promega, Madison, WI, USA) backbone used. H_2O_2 (v/v) of the indicated concentration was used to oxide the LUC^{con} DNA (50 mg/mL) at 24 °C for 1 h. Control plasmids undamaged were treated with vehicle solutions. All the DNA was re-suspended in TE buffer (pH 7.8, 500 mg/mL) after purifying by ethanol precipitation. A549/cDDP cells were then transfected with the indicated LUC^{con} plasmids. DRC (%) was calculated as the ratio of the damaged plasmid luciferase activity to the undamaged plasmid luciferase activity, multiplied by 100%.

3.11. In vivo antitumor efficacy

The A549/cDDP single-cell suspension in PBS was injected subcutaneously into the right oxter of 35 nude mice that were divided into 5 groups randomly. The mice with a size of 80-150 mm^3 tumor received cisplatin (i.v. at 5 mg/kg once a week), wogonin (i.v. at 5 mg/kg once a week), a combination of cisplatin and wogonin, and Cis-wog

(i.v. at 5 mg/kg once a week). The tumor growth was monitored and calculated based on the formula:

$$\text{Tumor volume (mm}^3) = 0.5 \times \text{length} \times \text{width}^2$$

Tumor weight was assessed as the antitumor activity. Systemic toxicity was evaluated as the body weight and physical state of the mice.

3.12. Statistical analysis

The data were represented as means±S.D. from at least three independent experiments. Statistical analyses were executed using Student's t-test. All comparisons with respect to untreated controls. $*P<0.05$ and $**P<0.01$ indicate significant difference.

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