Notch pathway inhibition using DAPT, a \( \gamma \)-secretase inhibitor (GSI), enhances the antitumor effect of cisplatin in resistant osteosarcoma

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Overcoming platinum drug resistance represents a major clinical challenge in osteosarcoma (OS) treatment. The high rates and patterns of therapeutic failure seen in patients are consistent with a steady accumulation of drug-resistant cancer stem cells (CSCs). Notch signaling is implicated in regulating CSCs and tumor resistance to platinum. Thus, we attempt to investigate whether inhibiting of Notch pathway could sensitize cisplatin (CDDP) to CDDP-resistant OS cells and the underlying molecular mechanisms. OS cell lines resistant to CDDP were treated with DAPT, CDDP or combination, we present evidences that DAPT enhances the cytotoxic effect of CDDP in resistant OS by inhibiting proliferation, resulting in G0/G1 cell-cycle arrest, inducing apoptosis, and reducing motility. In addition, DAPT targeting depletes OS stem cells (OSCs), thus increasing tumor sensitivity to platinum, which indicating that a dual combination targeting both OSCs and the bulk of tumor cells are needed for tumor eradication. We also found that the combination of CDDP and DAPT exhibit additive suppression on phosphorylated AKT and ERK, contributing to the anti-cancer effects. In animal model, this combination therapy inhibits the growth and metastasis of CDDP resistant tumor xenografts in nude mice to a greater extent than treatment with either reagent alone. Based on these results, we conclude that CDDP plus DAPT was able to sensitize CDDP-resistant human OS cells to CDDP by downregulation of Notch signaling. CDDP and DAPT combination treatment may be effective and promising for advanced OS.

**KEYWORDS**
cancer stem cell, chemoresistance, combination therapy, Notch signaling pathway, osteosarcoma (OS)

**Abbreviations:** ANTTS, adjacent non-tumor tissues; CDDP, cisplatin; CI, combination index; CSCs, cancer stem cells; DAPT, N-[N-(3,5-Difluorophenacyl)-L-alanyl]-S-phenylglycine \( t \)-butyl ester; DIL, delta-like; DRI, drug reduce index; DSL, Delta/Serrate/LAG-2; FCM, flow cytometer; GSI, gamma-secretase inhibitor; GSIs, gamma-secretase inhibitors; IC50, half-inhibitory concentration; IHC, immunohistochemistry; LV, lentivirus; MAPK, mitogen-activated protein kinase; MDR1, multi-drug resistance gene1; NC, negative control; NICD1, Notch1 intracellular domain; OS, osteosarcoma; OSCs, OS stem cells; p-AKT, phosphor-AKT; p-ERK, phosphor-ERK; qRT-RCR, quantitative real-time polymerase chain reaction.

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1 | INTRODUCTION

Osteogenic sarcoma, also referred to osteosarcoma (OS), is the most common primary malignant bone tumor. OS affects patients of all ages but shows a substantially higher incidence in children and early adulthood, which is the leading cause of disabling for childhood and adolescence. Platinum compounds-based therapies are the current global standard for neoadjuvant chemotherapy. The platinum-based anticancer drugs, including cisplatin (CDDP) (Supplementary Figure S1A), carboplatin and oxaliplatin, are currently among the most potent and widely used chemotherapeutic agents in clinical. They are also used for treating a variety of solid cancers, such as breast, ovarian, cervical, testicular, colorectal, bladder, non-small-cell lung, and head and neck cancers. As the leading anticancer drug, CDDP has been used for nearly four decades in standard chemotherapy regimens. The effect of platinum-based anticancer chemotherapy is positively with dose, but high doses of CDDP are accompanied by severe side effects as manifested by renal impairment, ototoxicity, neurotoxicity, and vomiting on the patients during the treatment. In addition, prolonged usage of CDDP in chemotherapy also induces drug resistance from intrinsic and acquired via multiple mechanisms.

Platinum resistance is the single most important factor after stage in determining prognosis. Development of CDDP resistance is often associated with multidrug resistant phenotype, including cancer stem cells (CSCs) and disorder of signaling pathway. It has been postulated that within a tumor, a minor subpopulation of cells, named CSCs, drive the self-renewal and differentiation that account for the initiation, metastasis, proliferation, therapeutic resistance, and recurrence of cancer. Recently, studies have also presented that CSCs may be involved in the mechanisms of multidrug resistance. OS stem cells (OSCs) can also evade senescence and apoptosis through the Notch pathway to resist toxicity of chemotherapy.

Notch signaling is one of the most important signaling cascades involved in drug resistance in tumor cells. A previous study demonstrated that activation of the Notch signaling was linked to chemoresistance of urothelial carcinoma to CDDP and inhibition of Notch signaling with gamma-secretase inhibitors (GSIs) could target the leukemia-initiating cells, sensitize acute myeloid leukemia to chemotherapy, and be synergistic with some antineoplastic agents. Notch genes encode transmembrane receptors that are highly conserved from invertebrates to mammals. These receptors interact with ligands expressed by adjacent cells to regulate cell fate specification, proliferation, differentiation, and survival. The Notch system in vertebrates is comprised of four receptors (Notch1-4) and at least five ligands from the families Delta/Serrate/LAG-2 (DSL): Delta-like (Dll)-1, Dll-3, Dll-4, Jagged-1, and Jagged-2. In ovarian cancer patients who received CDDP treatment, the activity of Notch signaling in tumor tissue correlates with drug resistance and poor prognosis. Also, in a mouse model, the actived Notch pathway promotes acquired resistance to CDDP in serially passaged OS xenografts. Similar drug resistance to Doxorubicin, Methotrexate, and Cyclophosphamide were reported in OS cells and lymphoblastic leukemia cells, both due to intracellular Notch signaling. Additionally, treating mice with a Notch inhibitor restores CDDP sensitivity. Most interestingly, other groups found that inhibition of Notch signaling result in down-regulation of phosphor-AKT (p-AKT) and phosphor-ERK (p-ERK) expression, which indicates that Notch signaling occurs upstream of AKT and ERK signaling, and Notch may positively regulate p-AKT and p-ERK expression directly or indirectly.

The undesirable properties of the CDDP have triggered intense research interest to design a new pattern for drug application, such as combination therapy. In this study, we sought to identify therapeutic agents to enhance the sensitivity of CDDP in resistant OS. Here, we reported that the activity of CDDP can be pharmacologically enhanced by γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (Supplementary Figure S1B). Unexpectedly, we found that DAPT suppressed the levels of stem cell-like properties in resistant OS and reduced the chemotherapy resistance. These results indicate that Notch signaling play an important role in the maintenance of OSCs and platinum chemoresistance and hint an important clinical application of combining CDDP and DAPT for advance OS.

2 | MATERIALS AND METHODS

2.1 | Human OS specimens and cell lines

Formalin-fixed, paraffin-embedded primary human OS tissue samples were collected from Peking University Cancer Hospital and Renmin Hospital of Wuhan University, from January 2014 to December 2016, from patients aged 8-35 years (median, 19 years). There were 36 adjacent non-tumor tissues and 43 OS samples. We obtained written informed consent from all patients. The Ethics Committee of Peking University Cancer Hospital and Renmin Hospital of Wuhan University approved the study protocol.

The human OS cell lines U2OS and MG-63 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and 143B was purchased from China Centre for Type Culture Collection (CCTCC, Wuhan, China). The CDDP resistant cell lines U2OS, MG-63 and 143B were established by serial desensitization of these three cell lines for 9 months and partly according previous studies, and these resistant OS cell lines are named U2OS/R, MG63/R, and 143B/R, respectively. In parallel, parental U2OS, MG-63 and 143B cells were exposed to DMSO (vehicle solution) in the same dose-escalation manner. Generation of parental cells and resistant OS cells and growth conditions were described previously.

2.2 | Cell viability

Cell viability was assessed as described previously.

2.3 | Synergy analysis

The synergistic effect of CDDP and DAPT was determined based on the combination index (CI) using CalcuSyn, version 2.1 (Biosoft®). The CI indicated synergism at less than 1.0, antagonism at greater than 1.0, and an additive effect at 1.0.
2.4 | Reverse transcription quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was done according to standard techniques, as described previously. The gene specific primers used are listed in Supplementary Table S1.

2.5 | Western blot analysis

For western blot analysis, proteins were extracted with Protein Lysis Buffer (Roche, Rotkreuz, Switzerland). And detail protocol for western blotting is described previously and manufacturer’s instructions.

2.6 | Animal experiment

To determine the tumorigenicity of resistant model in vivo, 4-week-old male BALB/c-nu/nu nude mice were obtained from Center for Animal Experiment of Wuhan University and housed in laminar flow cabinets under specific pathogen-free conditions with food and water ad libitum. The study protocol was approved by the Experimental Animal Care Committee (IACUC) (approval number: S01315022l) of Renmin Hospital of Wuhan University. All surgeries were performed under sodium pentobarbital anesthesia (Sigma, St. Louis, MO), and all efforts were made to minimize suffering. Male nude mice were introduced to establish resistant xenograft tumor model of 143B cells as previously described and made a little improvement. Specific experimental methods and steps are described in Supplementary Materials and Methods.

2.7 | Statistics

Statistical analyses were performed using the SPSS 16.0 statistical software package. P value was calculated using chi-square in contingency table. All data are expressed as the mean ± SD of at least three independent experiments. The Student’s t-test was used to compare the means of two groups. Where more than three means were compared, one-way ANOVA followed by multiple comparisons among the means was used. Enhanced expression of NICD1 in OS versus ANTTs was determined by the Mann-Whitney U Test. Spearman’s correlation tests were used to analyze the association between Hes and Oct4 mRNA expression in OS tissues. The Kaplan-Meier method was used to calculate the survival curve, and Log-Rank test to determine statistical significance. Statistical significance is indicated by *, where \( P < 0.05 \), **, where \( P < 0.01 \), and ***, where \( P < 0.001 \).

Additional information is described in Supplementary Materials and Methods.

3 | RESULTS

3.1 | Notch signaling pathway is activated in resistant OS tissues and positively correlated with stem cell-like properties

Compared with normal samples, Notch signaling pathway is both overexpressed and downregulated in human tumors. In the present study, we investigated NICD1 (Notch1 intracellular domain, NICD1) expression in OS and the adjacent non-tumor tissues (ANTTs) using immunohistochemistry (IHC). We detected NICD1 expression which localized to the cytoplasm and nucleus in a total of 43 OS and 36 normal specimens. Figures 1A and 1B and Table 1 showed very low NICD1 expression in the ANTTs, but higher expression in the OS tissues. Intriguingly, NICD1 was gradually upregulated in the ANTTs (19.44%) and in OS chemosensitivity (72.41%) and chemoresistance (100.00%), and the strong positive rates of NICD1 also followed in that order (Figure 1B and Table 1). Among the 43 OS tissues, 35 samples (81.40%) had high NICD1 expression; but among the ANTTs, NICD1 expression was in only 7 samples (19.44%) (Table 1). Similarly, Western blot analysis of ANTTs, chemotherapy sensitive and chemotherapy resistant OS tissues displayed an incremental NICD1 expression (Figures 1C and 1D). The mRNA levels of Notch target genes (Hes1, Hes5, and HeyL) also elevated in tumor samples compared with ANTTs (Figure 1E). It is noteworthy that the whether NICD1 protein expression levels or Notch downstream target genes mRNA transcription levels exhibit features that they were higher in chemotherapysensitive group and chemotherapististant group than in chemotherapy-sensitive group (Figure 1A–E). These results indicate that NICD1 is upregulated in OS tissues and plays an important role in OS tumorigenesis and chemoresistance. In view of the fact that tumor resistance to chemotheraphy is closely related to stem cell-like properties, we examined the expression of stem cell related gene Oct4 in normal tissues, drug-sensitive, and drug-resistant OS tissues. qRT-PCR analysis showed a gradually increasing mRNA levels of Oct4 in ANTTs, chemosensitive, and chemoresistant OS tissues. And the difference was statistically significant (Figure 1F). Spearman’s correlation analysis disclosed a positive correlation between Hes1 expression and that of Oct4 (Figure 1G). Collectively, the Notch signaling pathway was activated in resistant osteosarcoma and the characteristics of stem cell-like properties were enhanced, and there was a positive correlation between them, it hints that combination of CDDP and Notch inhibitor may have a better therapeutic effect in resistant OS, so we performed a further study in vitro and in vivo.

3.2 | Anti-proliferative activity of CDDP and DAPT in resistant OS cells

Initially, to study the phenomenon of drug-resistance, relapse and metastasis following CDDP therapy, we generated in vitro chemoresistance models using three widely used OS cell lines U2OS, MG63, and 143B. Drug-resistant cells were established by exposure to increasing concentrations of CDDP, and resistant cells were validated by qRT-PCR, Western blot, and CCK-8 assay (Figure 2A–C and Supplementary Figure S2A and Table S2). The expression of MDR1 (multi-drug resistance gene1) and its corresponding expression protein P-gp (P-glycoprotein) in resistant OS were significantly increased, which fully demonstrated the generation of drug resistance. The resistance index of these three resistant OS cell lines increased by 2.56-, 2.03-, and 2.26-folds, respectively, according to the 24 h IC50 (half-inhibitory concentration) (Figure 2C and Supplementary Figure S2A and Table S2).
Subsequently, to assess the effect of CDDP and DAPT alone on the viability of resistant OS cells in vitro, U2OS/R, MG63/R, and 143B/R cells were treated with different concentrations of CDDP and DAPT respectively. First, we demonstrated growth-inhibitory efficacy of CDDP (0-28 µM) in resistant OS cells after 24, 48, and 72 h. CDDP (16 µM) inhibited ∼45% to ∼55% proliferation of these cells (Figure 2D and Supplementary Figure S2B) and IC50 values were 17.9 ± 0.7, 15.5 ± 0.6, and 18.3 ± 0.3 µM respectively after 24 h treatment (Supplementary Table S3). We also verified anti-proliferative effect of DAPT (0-50 µM) in resistant OS cells after 24, 48, and 72 h (Figure 2E and Supplementary Figure S2C), their IC50 values were 42.4 ± 1.4, 48.2 ± 2.1, and 38.5 ± 1.2 µM, respectively, after 24 h treatment.

**TABLE 1**  IHC detection of NICD1 expression in resistant OS and ANTTs

| Sample     | Case | Negative | Positive | Strong positive | P-value |
|------------|------|----------|----------|-----------------|---------|
| ANTTs      | 36   | 29       | 6        | 1               |         |
| OS         | 43   | 8        | 18       | 17              | <0.01a  |
| Chemosensitivity | 29   | 8        | 15       | 6               | <0.01a  |
| Chemoresistance | 14   | 0        | 3        | 11              | <0.01b  |

ANTTs, adjacent non-tumor tissues; IHC, immunohistochemistry; OS, osteosarcoma.

aWhen compared with ANTTs.

bWhen compared with ANTTs and OS chemosensitivity.
FIGURE 2  DAPT synergistically enhanced cytotoxicity of CDDP in resistant OS cells in vitro. A, qRT-PCR analysis of MDR1 (multi-drug resistance gene1) in parental cell and resistant cell of three OS cell lines. B, Level of P-gp (P-glycoprotein), MDR1 corresponding expression protein, was detected by immunoblotting in parental cell and resistant cell of three OS cell lines. C, Different concentrations of CDDP were applied to U2OS cells and U2OS/R cells for 24 h, and the cell viability was assessed by CCK-8 assay. D and E, Resistant U2OS cells were treated with CDDP and DAPT alone at different concentrations for 24, 48, and 72 h, and the cell viability was assessed by CCK-8 assay. F, Either CDDP (1–12 µM) or DAPT (2.5–30 µM) alone or in combination at 1:2.5 (CDDP:DAPT) fixed molar ratio treatment for 24 h. Cell proliferation was determined by CCK-8 assay. G, Clonogenic assay shows effect in resistant U2OS/R cells treated with CDDP (6 µM) alone, DAPT (15 µM) alone or in combination for 48 h. Cells were cultured in fresh medium for 14 days to form colonies. H, Percentage of colonies of resistant OS cells. I, Combination index (CI) analysis of resistant OS cells treated with CDDP and DAPT. A CI of 1.0 (dashed line) reflects additive effects, whereas values greater than and less than 1.0 indicate antagonism and synergy, respectively. J, Isobologram analysis of cytotoxicity of CDDP and DAPT treatments alone or in combination. The diagonal line represents the isoeffect line of additivity. Points above this line indicate antagonism between drugs, and points below this line indicate synergy. Data are shown by means ± SD from at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Our results showed that the growth-inhibitory effects of CDDP and DAPT is different in U2OS/R, MG63/R, and 143B/R cells whereas all are in the vicinity of 16 and 40 µM, respectively. Both CDDP and DAPT are used in a concentration- as well as time- dependent manner to perform an anticancer effect.

### 3.3 DAPT synergistically increased CDDP-mediated cytotoxicity in resistant OS

The treatment of OS with CDDP is linked with its concentration-limiting toxicity. Therefore, we aimed to improve the cytotoxic
efficacy of CDDP by reducing its concentration in presence of DAPT. Firstly, we examined whether the combination of relatively low concentrations of CDDP and DAPT additively or synergistically inhibited resistant OS cells proliferation. To assess the effects of CDDP and DAPT in combination on the viability of resistant OS cells in vitro and based on the IC50 values of CDDP and DAPT respectively, we selected 1:2.5 (CDDP:DAPT) molar ratio for combination therapy among these cells. U2OS/R, MG63/R, and 143B/R cells were treated with different concentrations of CDDP (1-20 µM), DAPT (2.5-30 µM), or both agents for 24 h. When combination of these two drugs, the CDDP inhibited cell viability dose-dependently with an IC50 ∼6 µM in U2OS/R, MG63/R, and 143B/R cells and DAPT also reduced cell viability in a dose-dependent manner with an IC50 ∼15 µM in resistant OS cells (Figure 2F and Supplementary Figure S2D). The combinatorial treatment resulted in a significantly reduce of IC50 values for CDDP and DAPT respectively when compared to application of either agent alone (Supplementary Figure S2E), indicating that treatment with CDDP in combination with DAPT, with lower doses respectively, was more cytotoxic than the monotherapy groups. There were no significant difference been identified between the CDDP and DAPT monotherapy according the selected 1:2.5 (CDDP:DAPT) molar ratio to suppress cell viability (Figure 2F and Supplementary Figure S2D). And in order to evaluate the synergistic effect of CDDP and DAPT, we subjected resistant OS cells to a DAPT dose equal to half its IC50 (15 µM) in combination with a lower concentration (6 µM) of CDDP for subsequent studies.

 Colony formation assay is an effective method to determine single cell proliferation capacity. Thus, we performed a clonogenic assay to further examine the anti-proliferation effects of CDDP and DAPT alone or in combination on U2OS/R, MG63/R, and 143B/R cells growth, respectively. Compared to the control group, colony number of tumor cells was significantly reduced in CDDP and DAPT alone or in the combinatorial groups. It is noteworthy that CDDP in combination with DAPT resulted in an even higher percentage of reduction colony number than the either drug alone group (Figures 2G and 2H). These results further indicate that CDDP and DAPT combination treatment could inhibit the proliferation of resistant OS cells.

 The combined drug effects were analyzed using CalcuSyn software to further confirm the synergistic effect of CDDP combined with DAPT. The combined effects of CDDP and DAPT at a concentration ratio of 1:2.5 were subjected to medium drug effect and combination index (CI) analyses were shown in Figure 2I. The combination of CDDP and DAPT exhibited synergistic effects (CI < 1) in resistant OS cells, which indicate that the cooperation of both agents increased the inhibitory effect on cell viability. Analysis of the enhanced efficacy obtained by combining CDDP and DAPT indicates synergism, as depicted in the isobologram: most of the data points are positioned below the line of additive effects (Figure 2J). These analysis reflect the observations shown in Figure 2F-H and Supplementary Figure S2D, where combined treatment with the two drugs yielded greater growth inhibition than either agent alone in U2OS/R, MG63/R, and 143B/R cells. In addition, combining CDDP and DAPT resulted in a favorable drug reduce index (DRI), ranging from a 2.7- to 4.1-fold doses reduction for CDDP in resistant OS (Supplementary Table S4).

3.4 | Enhanced cell cycle arrest by combination of CDDP and DAPT in resistant OS cells

To elucidate the mechanisms involved in CDDP or DAPT mediated cell proliferation inhibition, we detected and analyzed cell cycle distribution by flow cytometer (FCM). The percent of cells arrested in the G0/G1-phase of cells treated with 6 µM CDDP alone and 15 µM DAPT alone for 24 h was slightly higher than that of untreated control cells. However, cells subjected to combination treatment showed a significant increase in the percent of cells in G0/G1-phase than untreated control and cells treated with either agent alone (Figures 3A and 3B). These results indicated that these two drugs exert synergistic growth inhibitory effects, probably due to the cell cycle is blocking in the G0/G1-phase.

 To identify the specific regulatory proteins associated with the induction of cell cycle arrest in response to CDDP and/or DAPT treatment, we then examined the transcription of genes related to the cell cycle. qRT-PCR revealed that CDDP plus DAPT significantly prevented the transcription of accelerators of the cell cycle, including Cyclin D1, Cyclin E1, SKP2, and c-Myc (Figure 3C and Supplementary

**FIGURE 3** DAPT synergistically enhanced G0/G1-phase arrest and induced apoptosis of CDDP in resistant OS cells in vitro. A. Flow cytometry histograms of cell DNA content distribution in each phase after treatment with CDDP (6 µM) and DAPT (15 µM) alone or in combination after 24 h in MG63/R cells, showing G0/G1-phase arrest. B. Quantitative analysis percentage of cells distributed in each phase of the cell cycle. C. After treatment as in (A), total RNA were extracted from cultured resistant OS cells and probed with specific primers. Representative results of Cyclin D1, Cyclin E1, SKP2, and c-Myc mRNA levels were as determined by qRT-PCR analysis. D. After treatment as in (A), proteins were extracted from resistant OS cells and probed with appropriate dilutions of specific antibodies. Representative results of p21, Cyclin D1, CDK2, and pRb protein levels were as determined by a Western blot analysis; GAPDH was used as the internal control. (E) Flow cytometry histograms of cell apoptosis distribution after treatment with CDDP (6 µM) and DAPT (15 µM) alone or in combination after 24 h in U2OS/R cells. F. Quantitative analysis of apoptosis of resistant OS cells measured by flow cytometry. G. Hoechst 33258 staining of U2OS/R cells treated with CDDP (6 µM), DAPT (15 µM), or combination of both agents for 24 h. Apoptotic cells were identified by the presence of bright-blue fluorescent and highly condensed or fragmented nuclei (∗×200). H. After treatment as in (E), proteins were extracted from resistant OS cells and probed with appropriate dilutions of specific antibodies. Representative results of cleaved caspase-9, cleaved caspase-3, cleaved PARP, cytochrome c, and γ-H2AX protein levels were as determined by a Western blot analysis. All data are expressed as the mean ± SD of three independent experiments. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001
Figures S3A and S3B). Furthermore, we examined p21, CyclinD1, CDK2, and pRb proteins expression by Western blotting. CDDP and DAPT combination treatment markedly increased the level of p21 and decreased the expression of CyclinD1, CDK2, and pRb (Figure 3D). These findings suggested that CDDP combined with DAPT promoted G0/G1-phase arrest by inhibition of G1-S phase progression.

### 3.5 Enhanced apoptosis by combination of CDDP and DAPT in resistant OS cells

To investigate whether CDDP and DAPT alone or in combination induced apoptosis, cell apoptosis assay was performed by FCM. FCM revealed that treatment with CDDP and DAPT alone or in combination for 24 h led to a markedly increase in apoptotic cells compared to the control group (P < 0.05) as shown in Figures 3E and 3F. In addition, treatment with the combination of CDDP and DAPT resulted in an even significantly increase in apoptotic cells compared to the monotherapy groups.

In order to observe the morphological changes of CDDP and DAPT in resistant OS cells, we performed Hoechst 33258 staining assay. The resistant OS cells were treated with 6 µM CDDP, 15 µM DAPT, or a combination therapy, respectively, for 24 h and determined by inverted fluorescence microscope. Both CDDP and DAPT were effective in initiating nuclear damage after 24 h. Interestingly, cells exposure to the CDDP/DAPT combination therapy increased the bright-blue fluorescent and condensed nuclei compared with either monotherapy (Figure 3G).

We further examined the expression of proteins related to apoptosis and nuclear damage that could be affected by treatment with CDDP and/or DAPT in resistant OS cells. The expression of cleaved caspase-9, cleaved caspase-3, cleaved PARP, cytochrome c, and γ-H2AX were higher in cells subjected to combination treatment than in untreated control and treated with either agent alone (Figure 3H and Supplementary Figure S3C). To examine the possible mechanism of the pro-apoptotic effects of combination with CDDP and DAPT, caspase-3, -8, and -9 activity was detected using ELISA. The results showed that caspase-3, -8, and -9 activity was markedly increased in the CDDP and DAPT alone or combination treatment groups compared to the control group. However, compared to the single-drug treatment groups, the combinational treatment significantly increased greater caspase-3, -8 and -9 activity (Supplement Figure S3D-F). These results indicate that the caspase family members involved apoptotic pathway and DNA-damage are major mechanisms by which CDDP and DAPT combination treatment exerted the synergistic cytotoxicity effect in resistant OS cells.

### 3.6 Inhibited migration and invasion by combination of CDDP and DAPT in resistant OS cells

To ascertain the inhibitory effects of CDDP and DAPT as a single or combined treatment on resistant OS cells migration, a wound-healing assay was performed to investigate the effects on the migration potential of U2OS/R, MG63/R, and 143B/R cells respectively. After 48 h observation, cells in the CDDP and DAPT alone or combinatorial group migrated less than those in the control group. And combination treated cells were migrated significant less compared to either single drug application (Figures 4A and 4B).

The ability of CDDP and DAPT alone or in combination reduced the invasiveness of resistant OS cells which were further investigated by the Transwell system assay. It was found that invasion capability was decreased significantly with CDDP and DAPT alone or in the combination treatment groups compared to the control group (Figures 4C). However, compared with the results with either agent alone, the combination of CDDP and DAPT greatly inhibited the invasion ability of U2OS/R, MG63/R, and 143B/R cells. The results of the cell invasiveness assay showed that there were no significant difference in the number of cells that had passed through the simulated basement membrane between the CDDP and DAPT groups.

To determine the potential mechanism of CDDP in combination with DAPT inhibits cell migration and invasion in vitro, the migration and invasion associated with VEGF and MMPs proteins expression were detected by immunoblotting analysis. Results of the analysis revealed a significant decrease in VEGF, MMP-2, and MMP-9 proteins in the CDDP and DAPT alone or combinational group compared to the control group. And the combinational group obviously decreased the VEGF, MMP-2, and MMP-9 proteins expression compared to the either monotherapy (Figure 4D and Supplementary Figure S4A). Then, we explored whether combination-treated could lead to a decrease in MMPs activities. Gelatin zymography analysis demonstrated that both MMP-2 and MMP-9 activities were reduced in combination-treated cells compared with control and single application group (Figure 4E). In additional, ELISA assay showed that combination-treated could lead to a decrease in the levels of VEGF secreted in the culture medium (Figure 4F). All these data indicated that combined application of CDDP and DAPT could effectively inhibited the expression and activities of VEGF, MMP-2, and MMP-9 in resistant OS cells compared to the monotherapy groups and control group.

### 3.7 Inhibition of Notch signaling pathway decreases the CSCs phenotype in resistant OS cells

We have confirmed that CDDP-resistant OS cells could enrich CSCs previously. And in this study we also showed that the expression of stem cell-related genes Oct4 and Sox2 were upregulated in CDDP-resistant cells (Figure 5A), and CDDP-resistant cells were able to generate more tumor spheres than parent cells during primary and secondary sphere assay (Figure 5B). Interestingly, when we add the DAPT, the volume of the tumor spheres is not only become significantly smaller, but also the number becomes less (Figure 5C-E). Collectively, these results indicate that CDDP-resistant cells display stem cell-like properties in vitro, but DAPT could decrease the CSCs phenotype in resistant OS cells.

In view of the fact that the Notch signaling pathway plays a key role in stem cell generation and maintenance, And Notch inhibitor, DAPT, could eliminate tumor stem cell properties. Thus, we hypothesized that suppression of the Notch signaling pathway may
reduce the phenotype of resistant OS stem cells. In order to further prove the hypothesis, we employed recombinant lentivirus (LV) to construct the negative control (NC), downregulation of Notch signaling pathway (LV-shRNA-Notch1) of resistant OS cells. All these transfected OS cells were confirmed by both qRT-PCR and western blot (Figures 5F and 5G, Supplementary Figures S5A and S5B). After obtaining stable transfected resistant OS cell lines of U2OS/R, MG63/R and 143B/R, we performed a number of functional experiments. As shown in Figure 5H and Supplementary Figure S5C, the expression of stem cell related genes Oct4 and Sox2 was significantly decreased in the Notch signaling pathway downregulated group. Spheroid formation has been widely used to assess the in vitro self-renewal potential of stem cell-like cells, so we compared the ability of Notch pathway down-regulated and control cells to form spherospheres. The LV-shRNA-Notch1 cells formed not only less in number but also smaller in volume spherospheres than the blank and NC cells in all three resistant OS cells after 24 h treatment (Figure 5I–K, Supplementary Figure S5D and S5E). Taken together, these findings suggested that inhibitory Notch signaling pathway decreases the CSCs phenotype in resistant OS cells.

## 3.8 CDDP combined with DAPT inhibits Notch signaling, p-AKT, and p-ERK activation

As the combination of CDDP and DAPT showed the greater inhibition of cell proliferation, induction apoptosis and reduction mobility as synthetic way, insight was gained with regard to the mechanisms.
FIGURE 5 Effect of CDDP and DAPT alone or in combination on stem cell-like characteristics, Notch, PI3K/AKT and MEK/ERK signaling in resistant OS cell lines. A, Stem cell-related genes were upregulated in resistant cells compared to parental cells when assessed by qRT-PCR. B, In vitro sphere forming self-renewal ability was enhanced in resistant cells. Secondary spheres also demonstrated enhanced serial sphere-forming capacity in resistant cells. C, Representative tumor sphere formation with or without CDDP and DAPT or in combination. Scale bar, 50 µm. D and E, Quantification of in vitro sphere size and number of control, CDDP and DAPT alone and in combination as showing in (C). F and G, U2OS/R cells were transfected with negative control lentivirus (Negative control-LV), Notch1-shRNA lentivirus (LV-shRNA-Notch1) respectively for 48 h. The downregulation of Notch signaling pathway in LV-shRNA-Notch1 cells were confirmed by qRT-PCR and Western blot respectively. H, Stem cell-related genes Oct4 and Sox2 were assessed by qRT-PCR in blank control, negative control-LV, and LV-shRNA-Notch1 cells. I, Representative images of tumor sphere formation among blank control, negative control-LV, and LV-shRNA-Notch1 cells. Scale bar, 50 µm. J and K, The ability to form sarcospheres was lower in LV-shRNA-Notch1 group compared with negative control-LV and blank control group in U2OS/R cells. L, The cellular NICD1, Hes1, and cell survival related protein of resistant OS cells were detected by Western blotting. GAPDH was used as loading control. All data are expressed as the mean ± SD of three independent experiments. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001
Thus, Notch and survival signaling pathway PI3K/AKT and MEK/ERK were investigated. As Notch activates the PI3K/AKT and MEK/ERK pathway and since AKT and ERK activation is necessary for Notch-conferred resistance to apoptosis,28,29 we assessed the effect of combining CDDP with DAPT on Notch signaling, AKT and ERK phosphorylation in resistant OS cells. Firstly, U2OS/R, MG63/R, and 143B/R cells express 1.5-3-fold higher levels of NICD1 and Hes1 protein in response to CDDP treatment compared to control. And the increase in NICD1 and Hes1 expression were prevented by treatment with DAPT (Figure 5L).

We also determine whether AKT and ERK activities were affected by combination treatment. As shown in Figure 5L, AKT and ERK phosphorylation levels were attenuated in cells treated with CDDP and DAPT alone or in combination respectively, in the combined treatment group, however, the decrease was more pronounced. These results show that the CDDP and DAPT alone or in combination suppress cell proliferation may by reducing AKT and ERK phosphorylation. Taken together, these data suggest that the combination of CDDP and DAPT inhibits Notch signaling, p-AKT and p-ERK, and Notch-stimulated AKT and ERK activation is indirectly.

3.9 CDDP plus DAPT inhibits tumor growth and metastasis and prolongs survival time in chemoresistant xenograft mouse model

To further determine the role of CDDP and DAPT in resistant OS, the antitumor effects of CDDP and DAPT alone or in combination in closely mimic the physiology human chemoresistant OS xenografts model were evaluated. The male BALB/c-nu/nu nude mice were inoculated subcutaneous with 5 mm diameter (62.5 mm³) tissue from xenografts tumor of chemoresistant model at the forelimb according our previous study.8 When the tumors reached a size of about 50–100 mm³ (1 week after transplantation), mice were randomly divided into vehicle, CDDP, DAPT, and CDDP plus DAPT group, respectively. We found that chemoresistant tumors administered combination-treated formed substantially smaller tumors in nude mice compared with the vehicle and either drug alone as shown in Figure 6A. Both CDDP and DAPT significantly inhibited tumor growth compared with vehicle, and combined CDDP + DAPT treatment led to a significantly greater inhibitory effect than either compound alone (Figure 6A–C). Combined CDDP and DAPT administration did not significantly affect mice body weight compared with health mice which without tumor burdens, indicating the relative safety of this regimen with relatively low doses of CDDP and DAPT. However, relatively high doses of CDDP and DAPT alone affect the weight of nude mice (Figure 6D), indicating that high doses of either drug have a certain side effects. Furthermore, combination administration improved mice survival as shown in Figure 6E. In addition, tumor metastasis were found in the lungs according to the HE staining sections (Figure 6F), and we found that combined CDDP and DAPT compared to the vehicle and either agent alone, regardless of the number or volume of metastases were significantly less or smaller (Figures 6F and 6G).

In addition, apoptosis related mRNA and protein levels were detected from xenografts mice of tumor sections. We tested the expressions of Bax, Bcl-2, Caspase-3, and Caspase-9, respectively. qRT-PCR analysis demonstrated alterations in the levels of expression of Bax (proapoptotic) and Bcl-2 (antiapoptotic), resulting in a significant increase in Bax after treatment of the resistant xenografts with CDDP plus DAPT, and the Bcl-2 shown opposite result (Figure 6H). The relative mRNA expression of Caspase-3 and Caspase-9 of mice tumor tissues were highest in CDDP + DAPT group followed by CDDP alone, DAPT alone, and vehicle in that order (Figure 6H). Subsequently, Western blotting were performed and the results were similar to qRT-PCR as shown in Figure 6I. Hence, this combination regimen may provide a relatively safe and effective therapeutic option for the treatment of chemoresistant OS as demonstrated in the animal model.

We then further test whether treatment with DAPT affects the stem cell-like properties in vivo, we performed immunoblotting experiment and quantitative analysis. Western blotting analysis revealed that downregulation of stem cell-like properties were observed in xenografts treated with DAPT alone or in combination with CDDP in comparison with vehicle or CDDP alone (Figures 6J). Taken together, these data provide a possibility that inhibition of Notch with DAPT effectively decrease the stem cell-like properties in resistant OS tissues and cause a significant downregulation of chemosistance similar in vitro, thus enhancing the activity of CDDP to exert its anticancer effects.

We also examined expression levels of NICD1, p-AKT, p-ERK, and TUNEL in sectioned mice tumors after CDDP and DAPT alone or in combination administration (Figure 6K). TUNEL expression significantly increased in combination treatment, whereas NICD1 expression decreased by ~45% (Figure 6L). In addition, combination treatment resulted in significant downregulation of p-AKT and p-ERK compared to the control group (Figures 6K and 6L). However, both the expression p-AKT and p-ERK decreased in the CDDP treatment group, which indicated that CDDP could inhibit the activities of PI3K/AKT and MAPK signaling pathway (Figures 6K and 6L).

To further confirm the results mentioned above, Notch, PI3K/AKT, and MAPK signaling pathway in resistant xenografts mice tumor tissues were analysis. Consistent with the in vitro and above data, there was a reduction of NICD1, p-AKT, and p-ERK protein levels after combined treatment compared with the vehicle (Figures 6M and 6N). Thus, all these results indicated that CDDP in combination with DAPT treatment could markedly inhibit 143B chemoresistant xenograft growth in vivo and improves nude mice survival.

4 DISCUSSION

Chemotherapy resistance of OS is a difficult problem in clinical. Herein we demonstrate that Notch signaling plays a key role for OS stem cell maintain and underlies mechanism for resistance to chemotherapy. We present evidence of human specimen that Notch signaling is much greater in the chemoresistance OS and drives a CSCs phenotype. We
demonstrate that Notch pathway is critical for these OSCs and thus targeting the Notch signaling pathway may be an effective therapeutic strategy.

Defects in apoptosis are common phenomena in many types of cancer as well as critical steps in tumorigenesis and resistance to therapy. Thus, traditional cancer therapy mainly targets the cell apoptosis machinery or enhances apoptosis. CDDP is a platinum based, alkylating-like drug that crosslinks DNA to induce apoptosis. It is used to treat many types of solid cancer, such as ovary, testicular, bladder, lung, head and neck, thoracic, and colorectal cancers. Although CDDP has potent antitumor effects in various cancer models, it is limited in clinical practice due to its toxicities and intrinsic or acquired drug resistance. Many research teams have attempted to find ways to improve its efficacy and reduce its toxicity, such as...
-changing its structure or assessing combination therapy with other drugs.\(^4\) Researches have shown that combination therapy based on the synergistic effect between drugs owning multiple drug targets has been used to overcome drug resistance.\(^{16,19,32,33}\)

Recently many research groups have reported synergistic anticancer effects of treatment with a combination of a drug and a signaling pathway inhibitor in vitro and in vivo cancer models.\(^{34,35}\) Liu et al.\(^{34}\) noted synergistic action of RY-2f and CDDP on growth inhibition and induction of cell death in human ovarian via targeting the PI3K/AKT/mTOR signaling pathway in vitro and in vivo. Gurney et al.\(^{36}\) found that the application of Wnt pathway inhibition suppresses the growth of a range of tumor types, reduces tumor-initiating cell frequency, and exhibits synergistic activity with standard-of-care chemotherapeutic agents in xenograft studies with minimally passaged human tumors. In various experimental models several studies demonstrated the synergistic anticancer effect of Notch inhibitor combined with select chemotherapy agents such as CDDP, oxaliplatin, docetaxel, and 5-fluorouracil.\(^{16,19,29}\)

However, to the best of our knowledge, this is the first study to show that Notch inhibition DAPT synergistically enhances the antitumor effects of CDDP and resensitizes CDDP to resistant OS cells.

In the current study OS resistant cells were not affected by the CDDP dose up to a concentration of 4 µM. At a dose of greater than 4 µM CDDP resistant OS cells were decreased in a dose- and time-dependent manners. Exposure of resistant OS cells to 6 µM CDDP resulted in cell cycle arrest at the G0/G1-phase in agreement with other scholars studies.\(^{37}\) We also found that DAPT promoted G0/G1-phase arrest via reduction of Cyclin D1, Cyclin E1, CDK2, SKP2, c-Myc and increase p21, which were consistent with the findings of other earlier studies showed that DAPT induces G0/G1-phase arrest in human OS cells and other tumors.\(^{38,39}\) It is not surprising that the G0/G1-phase arrest induced by CDDP and DAPT combination treatment was significantly greater than monotherapy group. Cyclin D, cyclin E, and pRB have been reported to promote G0/G1-S phase progression.\(^{40}\) In addition, SKP2 has been reported to be a component of ubiquitin E3 ligase complex which regulates G0/G1-S transition by degradation of the CDK inhibitors p21.\(^{41}\) p21 can bind to various CDKs, including Cyclin D/CDK4, Cyclin E/CDK2, and Cyclin A, and inhibit their kinase activity.\(^{42}\) Thus, CDDP and DAPT combination treatment significantly inhibited U2OS/R, MG63/R and 143B/R cells proliferation, which was partially related to cell cycle arrest.

CDDP induces apoptosis via various proteins, likely through the extrinsic death receptor pathway or the intrinsic mitochondrial pathway. Resistance to CDDP might develop through decreased expression or loss of pro-apoptotic factors, or through increased expression of antiapoptotic proteins.\(^4\) Generally apoptosis is mediated by caspase activation, an event that is tightly regulated and involves two major pathways (the intrinsic and extrinsic pathways). The release of mitochondrial cytochrome c represents the main event for the intrinsic and the extrinsic pathways.\(^{43}\) Therefore, we analyzed the expression of the apoptosis regulated proteins cleaved caspase-3, 9, PARP, and cytochrome c. Our analysis revealed increased expression of the apoptosis related proteins associated with DAPT treatment in CDDP-resistant OS cells. Treatment with CDDP and DAPT alone resulted in increased expression of cleaved caspase-3, 9, cytochrome c, and cleaved PARP. However, DAPT combined with CDDP leading to a synergistically increase in the expression of apoptosis related proteins relative to the levels of these proteins in cells treated with DAPT or CDDP alone. Thus, the alteration in apoptosis related proteins expression are likely to have influenced the effect of CDDP and DAPT combination treatment on the inhibition of cancer cell growth. These results are consistent with those of previous studies demonstrating that DAPT could sensitize platinum resistant ovarian cells to CDDP by inducing apoptosis.\(^{16}\) Liu et al.\(^{44}\) also reported that the combination of DAPT and CDDP reduced cell viability and enhanced apoptosis more obvious compared to a single treatment in lung cancer cells.

Cancer invasion and metastasis is a complicated multi-step process involving numerous effector molecules. The degradation of extracellular matrix is an essential step in cancer invasion and metastasis. VEGF, MMP-2, and MMP-9 have been regarded as metastasis-related genes, which play a critical role in cancer invasion and metastasis. In the present study, we examined the expression of related proteins and found that CDDP and DAPT inhibited the expression and activities of VEGF and MMPs in resistant OS cells in vitro. VEGF and MMPs are crucial in the processes of tumor cell angiogenesis, invasion and metastasis, and VEGF, MMP-2 and MMP-9 are directly linked with angiogenesis and degradation of the basement membrane collagen leading to metastasis. Indeed, our in vitro results showed that combination CDDP and DAPT inhibited migration and invasion of resistant OS cells through the Matrigelᵀᴹ. The animal model employed in our study was a closely mimic of lung metastasis of human chemo resistant OS patients. This model was established by 143B OS cell line, which had highly metastatic

**FIGURE 6** Antitumor activity of CDPP combined with DAPT in BALB/c-nu mice bearing 143B cells. A, Representative images of 143B xenograft vehicle and treatment with CDDP and DAPT alone or in combination were shown. B, The tumor growth were recorded and compared (volume in mm³, recorded every week). C, Tumor weight was obtained at the end of the experiment. D, The body weight (in grams, recorded every week) were recorded and compared. E, Survival rate of mice after the indicated treatment. F and G, HE staining (magnification, ×100) indicated the lung metastases and its number and relative size were calculated. H and I, The mRNA and proteins expression of Bax, Bcl-2, Caspase-3, and Caspase-9 in xenograft tumor tissues of all treatment groups. J, Quantitative analysis of proteins. K, Immunohistochemical (IHC) analysis of NICD1, p-AKT, and p-ERK, and TUNEL staining for tumors of all treatment groups (magnification ×200). L, Quantitative analysis IHC and apoptotic cells as showing in (K), each group were counted on the basis of viewing eight random fields in each glide. M, Total proteins were extracted from the xenograft tumors and the expressions of NICD1, AKT, p-AKT, ERK, and p-ERK were analyzed by Western blotting. GAPDH was used as a loading control. N, Quantitative analysis of proteins by densitometric analysis NS, not significant. *P < 0.05, **P < 0.01
tendency to lung and used by many scholars working on the same field. In this study, with primary tumor resected at the end of the experimental design, all mice in vehicle group presented 100% lung metastasis, confirming the model to be ideal in studying tumor with lung metastasis. Whereas the combined treatment group of lung metastases is not only the smallest in volume but also the least in number, this result is consistent with the in vitro study. It might account for the underlying mechanism for the synergistic repression of primary OS and reduce metastatic potential by the combination of CDDP and DAPT.

We found that CDDP plus DAPT treatment significantly suppressed resistant OS cell growth and mobility that it is accompanied by restraining Notch signaling. The cytotoxic effect of CDDP plus DAPT seemed to be caused by cell cycle arrest and consequent apoptotic cell death and reduced the proteins related to invasion and metastasis. We tried to elucidate the mechanisms by which CDDP combined with DAPT affects resistant OS cells. It is well-known that Notch pathway interacts with many different signaling cascades to regulate critical cellular processes, and endogenous prosurvival molecules, PI3K/AKT and MEK/ERK pathway were investigated. DAPT impairs proliferative signaling whether used alone or in combination. AKT is a key regulator of survival and apoptosis. In particular caspase-9 is one of the main initiator caspasas in apoptosis and it is phosphorylated by AKT, leading to the inhibition of caspase-9 cleavage and apoptosis. Thus, inhibiting AKT activity may provide an avenue for cancer prevention and therapy. Cui et al. reported that γ-secretase inhibitor enhances the antitumor effect of docetaxel in prostate cancer, likely by including multiple cellular events such as activation of cleaved caspase-3 and Bax and inhibition Bcl-2, and suppressed AKT phosphorylation, leading to improve chemotherapeutic agents responsiveness. In the present study, the expression of cleaved caspase-3, 8 and 9, cleaved PARP and cytochrome c were increased and p-AKT were decreased by CDDP and DAPT treatment, especially in combination of these two drugs.

ERK is a major effect target of the mitogen-activated protein kinase (MAPK) pathway, and the MEK/ERK signaling pathway plays a critical role in proliferation, cell cycle, apoptosis and differentiation, and is an important downstream pathway of angiogenesis. Previous data obtained in in vitro cultured cells showed that the Notch pathway upregulates the levels of ERK phosphorylation and GST treatment could decrease p-ERK which presented therapeutic potential of targeting γ-secretase in non-small cell lung carcinoma. And p-ERK also can be suppresses by CDDP alone and then inhibit cell proliferation. In the present study, it was also found that p-ERK protein expression was downregulated by treatment with DAPT and CDDP alone or in combination.

So, it could be that CDDP plus DAPT inhibited the Notch, PI3K/AKT, and MEK/ERK pathway, which induced the cell apoptosis and cell cycle arrest and inhibited cellular motility, and then led to tumor arrest (Figure 7A).

The existence of OSCs was first demonstrated by Gibbs et al., who identified a subpopulation of cells in human OS tissue samples and cell lines that were capable of growing spherocites, or osteospheres, in serum-free conditions. Previously, our studies and other investigators have also demonstrated that low doses of CDDP could enrich CSCs in vitro and in vivo. In the present work, we also found that CDDP resistant OS cells exhibit stem cell-like properties. CSCs have been implicated in malignant tumor initiation, recurrence, drug resistance, invasion, and metastasis. Therefore, therapeutic targeting of OSCs offers a promising strategy for the treatment of OS. NICD1 are involved in the maintenance of stem cells. Notch-activated mutation acted as a second hit in a p53-loss-driven OS model. Mu et al. noted that Notch signaling may influence OSCs via suppression of ALDH and inhibited the Notch signaling pathway could reduce the OSCs properties. In this study, we also found that DAPT, Notch signaling pathway specific inhibitor, could reduce the number and volume of primary and secondary sphere assay. In addition, we used recombinant lentiviral specific blocking Notch1 signaling pathway, compared with the control group, it was found that: (1) the stem cell related gene expression was significantly decreased; and (2) the ability...
to form sarcospheres decreased significantly. Furthermore, we found that activation of the Notch signaling pathway is involved in osteosarcoma resistance, whereas Notch signaling pathway activity is positively related to stem cell-like phenotype (Figure 1). Taken together, all these results indicate that blocking the Notch pathway can be targeted to kill OSCs, therefore reducing the resistance of osteosarcoma. So the combination of CDDP and DAPT show a surprising therapeutic effect, the mechanism may be that CDDP/ DAPT combination is effectively eliminates both OCSs and the bulk of tumor cells, indicating that a dual combination targeting both populations is needed for tumor eradication (Figure 7B). Though the results are inspiring, the antitumor effects of this combination should be investigated in patients in future clinical studies.

In summary, this research identified that inhibition of Notch signaling using DAPT had an antitumor effect in vitro and in vivo in resistant human OS cell lines. Furthermore, DAPT had adjuvant activity with CDDP and importantly enhanced CDDP-mediated cytotoxicity on CDDP-resistant cells. We also provide evidence that inhibition of Notch signaling in resistant OS cells depletes a subpopulation of OCSs responsible for CDDP-resistance and tumor initiation, laying the foundation for a promising therapeutic strategy for therapy of OS and CDDP-resistant OS patients.

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