Original Article
A novel camptothecin analogue FL118 reduces cisplatin resistance of non-small cell lung cancer cells

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Abstract: As the first-line drug therapy for non-small cell lung cancer (NSCLC), cisplatin has a wide range of applications. However, inherent and acquired resistance reduces its effectiveness in the treatment of NSCLC. Our study was to investigate the antitumor activity of a new camptothecin analogue FL118 on cisplatin-resistant NSCLC cells. Western blot assay showed that the expression of survivin protein down-regulated by FL118, Flow cytometry analysis displayed that A549 cells and its cisplatin-resistant counterparts (A549/DDP) cells were both arrested in S phase. MTT assay showed that the viability of A549/DDP cells were decreased, indicating that FL118 may effectively inhibit the growth of NSCLC cells regardless of the chemoresistance of tumor cells. Meanwhile, wound healing scratch assay and transwell-matrigel invasion assay were used to demonstrate that FL118 may inhibit the migratory and invasive capabilities of A549 and A549/DDP cells. To further explore the mechanism of antitumor activity of FL118, the expression of ERCC1, P-gp, E-cadherin and Vimentin proteins were examined by immunohistochemistry and western blot. The resistance-associated proteins, P-gp and ERCC1, were both inhibited by the treatment of FL118, suggesting that FL118 may promote the chemosensitivity of tumor cells through inhibiting their expression. Consistent with the change in chemosensitivity, epithelial-mesenchymal transition (EMT) of A549/DDP cells was reversed by FL118, implying that the antitumor efficacy of FL118 is at least partly, dependent on regulating the process of EMT. These findings contribute to the understanding of the molecular mechanism by which FL118 reverses cisplatin-resistance of NSCLC cells and demonstrates that FL118 is a promising candidate for further clinical lung cancer treatment.

Keywords: FL118, anticancer activity, cisplatin resistance, NSCLC, survivin, EMT

Introduction
Currently, lung cancer is the most frequent cancer and the leading cause of cancer death in the world [1]. Approximately 80% of newly diagnosed lung cancers are non-small cell lung cancer (NSCLC). Despite efforts to improve efficacy of therapy for NSCLC patients, the 5-year overall survival rate remains approximately 15% [2, 3]. Cisplatin is used in combination with other antitumor agents as a first-line therapy for NSCLC, but the chemoresistance to cisplatin often leads to the failure of therapy [3] and cancer recurrence. Therefore, a better understanding of the underlying mechanism by which the chemoresistance arises has great clinical significance for the successful treatment of patients with NSCLC, and it is urgent to uncover more novel anticancer drugs to effectively inhibit or overcome the resistances.

The molecular mechanisms of chemoresistance are complicated. Growing evidence indicates that survivin protein is a central molecule involved in drug resistance [4-6], because survivin may act as a functional molecule in cancer stem cells (CSCs) biology [7, 8], which result in inherent drug and radiation resistance cancer relapse and metastasis. Therefore, survivin appears to be a pivotal protein involved in the drug resistance of cancer cells by different of mechanisms, and the development of novel survivin inhibitors may overcome the challenging issues of drug/radiation resistance and cancer relapse.

Dr. Fengzhi Li and his team (Roswell Park Cancer Institute-RPCI, Buffalo, New York) has reported that an exceptional antitumor compound, designated as FL118, was identified through high throughput screening (HTS) of compound libraries using genetically modified...
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cancer cell models in which the survivin gene is the biomarker and target [9]. More recent studies have further showed that FL118 also exerted strong anticancer activity on human colon and head-and-neck tumors in vitro and in vivo [10, 11]. Although FL118 is structurally similar to camptothecin analogs, such as irinotecan, a known topoisomerase 1 (Top1) inhibitor, FL118 possesses weaker Top1 inhibitor activity compared to irinotecan. However, FL118 is able to selectively inhibit the expression of several members of the inhibitor of Apoptosis family (Survivin, XIAP, and cIAP2) and the Bcl-2 family (Mcl-1), which was demonstrated to contribute to FL118 function and anticancer activity [12, 14]. In addition, FL118 may effectively inhibit cancer cell growth regardless of P53 status (wile type, mutant or null) [11]. Furthermore, the antitumor activity of FL118 is almost not impaired by camptothecin-resistance in human colon and head-and-neck cancer cells [10], indicating that FL118 is effective for human tumors that acquire irinotecan and topotecan resistance due to its ability to bypass the drug resistance induced by multiple ABC transporter efflux protein [15, 16]. Briefly as an inhibitor of survivin, FL118 has been demonstrated to be a novel camptothecin analogue and shows superb antitumor activity on colon or head-and neck tumors with or without irinotecan-resistance. Based on the previous studies and the properties of FL118 using survivin as a target, we hypothesize that FL118 could effectively inhibit wider ranges of malignant tumors with chemoresistance.

In this study, we found that FL118 can not only effectively inhibit the growth of NSCLC with or without cisplatin resistance, but also decrease the movement capability and chemoresistance of cancer cells. More importantly, we observed that antitumor effectiveness of FL118 may be related to epithelial-mesenchymal transition (EMT), which plays an important role in a variety of tumors with chemotherapy drug resistance and metastasis. These observations expanded our knowledge of the antitumor efficacy of FL118, and at least partially, demonstrated FL118 as an attractive therapeutic option for NSCLC patients with cisplatin resistance.

Materials and methods

Chemicals and reagents

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma Chemical Company (St Louis, MO, USA). FL118, the chemical name is 10,11-methylene-dioxy-20 (S)-camptothecin, was supplied by our cooperating lab American Roswell Park Cancer Institute (RPCI). The secondary antibodies were purchased from ABGENT (San Diego, USA).

Cell culture and treatment

The human cisplatin resistance NSCLC cell line, A549/DDP, was from Sun Yat-sen University as a gift. Its parental, A549 cell line was conserved in our institute. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (HyClone SV30010) at 37°C in a 5% CO2 humidified atmosphere. To maintain the acquired resistance to cisplatin, A549/DDP cells were incubated with 1.0 μg/μL cisplatin and cultured in drug-free medium at least 7 days before use. In this study, FL118 concentrations were the same as those in previous reports made by Dr. Li research group. A549 and A549/DDP cells were treated with FL118 (1, 10, 100 or 300 nM) for 24 h, 48 h, 72 h.

Drug resistance assay

The logarithmic growth phase of A549 and A549/DDP cells was digested by trypsin, then cells (5 x 10⁴/well) were seeded into 96-well plates, cultured for overnight, and treated with cisplatin (DDP) at final concentrations of 0, 1, 5, 10, 15, 20 μg/mL for 48 h. After treatment, 20 μL of MTT solution (5 mg/mL) was added to each well and the cells were incubated at 37°C for 4 h. The culture media were then replaced with 100 μL DMSO. Absorbance of each well at 490 nm was measured with a Microplate Reader (Bio-Tek, Winooski, VT, USA). The results were represented with the average of five parallel samples. The percentage of cell survival was calculated using the following formula: Cell survival rate (%) = (the absorbance of the drug group-absorbance of the control group)/(the absorbance of the control group-absorbance of the blank control group) ×100%. The half maximal inhibitory concentration (IC₅₀) of each drug concentration was calculated according to the linear regression equation between the concentrations of the drug and the survival rate of the cells, and the resistance index (RI) was calculated according to resistant cell IC₅₀/parenatal cell IC₅₀.
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Cell growth/viability assay

The MTT assay was used to measure cell growth/viability. A549 and A549/DDP cells (5×10^3/well) were seeded into 96-well plates, cultured for 24 h, and treated with FL118 at final concentrations of 0, 1, 10, 100, and 300 nM for 24 h, 48 h, 72 h and 96 h. After treatment, following method would be same to drug resistance assay.

Wound-healing assay

Cells were seeded into 6-well plates at a density of 10^5 cells/well and cultured with RPMI-1640 medium containing 10% FBS overnight at 37°C in a humidified atmosphere of 5% CO_2, after which, the medium was changed to RPMI-1640 without FBS and the cells were cultured for a further 24 h until >90% confluence. Then the artificial scratch wound was created by a plastic 200 μL tip. Cell debris was removed by washing with PBS. The medium was changed to serum-free RPMI-1640 with 100 nM of FL118 and the cells were continuously cultured for 24 h. Cell scratch wound was photographed and the width of the scratch wound was measured at 0h and 24 h. Each assay was carried out in triplicate and repeated in three independent experiments. Micrographs were taken using an inverted phase contrast microscope (Olympus; magnification, 40x).

Transwell-matrigel invasion assay

The invasiveness of cells was evaluated by a Boyden chamber assay. The polycarbonate filters (8 μm pore size, Corning) were precoated with Matrigel Matrix (Corning Incorporated, New York, USA). The A549 and A549/DDP cells were seeded at a density of 1×10^5 cells/well and treated with FL118 (10 nM) in the upper chamber with 200 μL serum free RPMI-1640 medium, while 650 μL RPMI 1640 medium with 15% fetal bovine serum was added to the lower chamber. After incubation for 48 h, the bottom of the inserts were fixed in methanol for 20 min, stained with 0.1% crystal violet and counted under phase contrast microscope (five fields per chamber were collected).

Immunocytochemistry (ICC) assays

A549 and A549/CDDP cells (10^5/well) were cultured on chamber slides for 24 h and then treated in RPMI 1640 medium with 10% FBS with or without 10 nM FL118 treatment. After 24 h incubation, the cells were washed three times with phosphate saline buffer (PBS), fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 for 10 min. Then the cells were incubation with 3% H_2O_2 for 10 min at 37°C. After incubation with primary antibodies against survivin, E-cadherin, vimentin (Santa Cruz, CA, USA) and P-gp, ERCC1 (ABCAM, Cambridge, MA, USA) overnight at 4°C, the cells were washed with ice-cold PBS and incubated for 1 h at room temperature with the appropriate secondary antibodies (ABGENT, San Diego, USA). Next, the cells were stained with 100 ul DAB for 3-5 min, followed by nuclear staining with hematoxylin. The cover slips were observed by microscopy (Eclipse E-800, Nikon, Japan).

Western blot analysis

The cells were washed twice in ice-cold PBS and lysed in 200 μL Radio Immunoprecipitation Assay (RIPA) lysis buffer with protease inhibitors Phenylnmethanesulfonyl fluoride (PMSF). A BCA Protein Kit (Beyotime Biotechnology, Shanghai, China) were used to quantify the protein concentrations. Equal amounts of protein sample was separated by 10-12% SDS-PAGE gels, transferred to polyvinyl diflouride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked with 5% skim milk in TBST for 2 h at room temperature. The membranes were incubated with anti-survivin (1:500), anti-E-cadherin (1:1000), anti-vimentin (1:1000) (Santa Cruz Biotechnology, USA) or anti-ERCC1 (1:1000), anti-P-gp (1:1000) (ABCAM, Cambridge, MA, USA) antibodies at 4°C overnight, washed three times with TBST and incubated with the appropriate HRP-conjugated secondary antibodies for 2 h at room temperature. The protein bands were detected by eECL western blot kit (CWBO, China) and visualized by autoradiography on X-Ray films (CWBO, China). And protein levels were normalized to GAPDH (1:2000, Santa Cruz Biotechnology, USA).

Quantitative real time-PCR

Total RNA was extracted from cells with Trizol (CWBO, China). Then, total RNA (1 μg) was reverse-transcribed into cDNA using FASTQuant RT Kit (TIANscript, Beijing, China) following the manufacturer’s instructions. Quantification of
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Each assay was repeated in three independent experiments.

Statistical analysis

Assay results were the average from at least 3 replicates in each of three independent experiments. All experimental data were shown as the mean ± SD. Differences between samples were analyzed using the Student's t test. Statistical significance was accepted at P<0.05 or P<0.01.

Results

FL118 significantly reduces the expression of resistance-associated proteins in A549/DDP

MTT assay (Figure 1A) showed that the IC_{50} of cisplatin on A549 cell proliferation was 1.59 μg/mL, while its IC_{50} on A549/DDP was 122.2 μg/mL. The resistance index was then 76.9, demonstrating A549/DDP cells possess the resistance against cisplatin. Consistent with the above results, Western blot and immunocytochemistry assay displayed that resistance-associated proteins, ERCC1 and P-gp were both highly expressed in A549/DDP cells compared with A549 (Figure 1B). After the treatment with 10 nM and 100 nM FL118 for 24, 48 and 72 h, the protein levels of ERCC1 and P-gp were significantly reduced in A549/DDP cells (Figure 1B), suggesting that FL118 can effectively inhibit the expression of resistance-associated proteins in A549/DDP cells. These observations implied that FL118 is not only able to eliminate irinotecan and topotecan resistant tumor cells [10], but also effective for at killing cisplatin-resistant tumor cells. Interestingly, although FL118 significantly inhibited the expression of ERCC1 and P-gp proteins in A549/DDP cells, qRT-PCR displayed that ERCC1 and MDR1 (P-gp) mRNA levels (Figure 1C) were not down-regulated. These data suggested that the inhibitory effect of FL118 on ERCC1, MDR1 protein expression is likely to be involved with both transcriptional and post-transcriptional mechanisms.

FL118 effectively inhibits the proliferation of cisplatin-resistant NSCLC cells

In order to study the inhibitory effect of FL118 on cisplatin-resistant NSCLC cells, MTT assay was used to detect the inhibitory rates in both A549 and A549/DDP cells after the treatment.
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**FL118 shows antitumor efficacy through suppressing the expression of survivin in the NSCLC cells**

Several reports have demonstrated that survivin inhibitors, FL118 plays a key role in inhibiting cancer cell growth and apoptosis [7, 8, 10]. In our study, Western blot, ICC and qRT-PCR all displayed that both A549/DDP cells and A549 cells have aberrant expression of Survivin, especially in A549/DDP cells, there is a higher expression of Survivin compared to its parental cells (Figure 3A, 3B). After the treatment of FL118, survivin mRNA and protein were downregulated in A549/DDP cells. Therefore, the cells survival was significantly inhibited by FL118, even at a 1 nM level (Figure 2). Furthermore, A549/DDP cells with high Survivin expression are more sensitive to FL118.

**FL118 effectively inhibits the migratory and invasive capabilities of NSCLC cells and reverse EMT phenotype in A549/DDP cells**

Tumor cells with an aggressive phenotype should acquire migratory and invasive capability, which is characteristic of degradation of the basement membrane and extracellular matrix (ECM) to allow the dissemination of cells to distant secondary organs. In order to observe whether FL118 inhibits the migratory and invasive capability of A549 and A549/DDP cells, wound healing scratch assay and transwell invasion assays were performed, respectively. As shown in Figure 5A, wound healing scratch assay showed that A549/DDP cells have an obviously enhanced migratory capacity, compared with parental A549 cells. The migration distance of A549/DDP cells was increased by approximately 2 folds (Figure 5A). Transwell invasion assay also revealed that A549/DDP
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cells have an enhanced invasiveness than A549 cells (Figure 5B). Compared with A549 cells, the number of invasive A549/DDP cells was increased by 2 folds (Figure 5B). Next, the

Figure 4. Flow cytometry analysis of cell cycle distribution in both A549 and A549/DDP cells by FL118 treatment. The A549 (A) and A549/DDP (B) cells were both arrested in S phase after the treatment of FL118 (10 nM) for 12 and 24 h with a time dependent manner.
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Treatment of FL118 resulted in a significant decrease in the migration (Figure 5A) and invasiveness (Figure 5B) of both A549/DDP and A549 cells (P<0.05). Although the number of transwell cells in A549/DDP group was higher than that in A549 group (Figure 5B) after the treatment of FL118, the inhibitory effect of FL118 on invasive capability of both A549/DDP and A549 cells was similar. The above discoveries demonstrated FL118 suppressed the migratory and invasive capabilities of NSCLC cells.

Recent studies showed that EMT plays an essential role in the metastasis and chemoresistance of many types of tumors [17-20], and there are phenotypic and molecular associations between tumor progression and EMT [21-23]. Therefore, EMT phenotype markers were also detected by ICC and Western blot assay after FL118 treatment. As shown in Figure 6A, A549/DDP cells had a spindle-shaped morphology, a higher expression of vimentin and lower expression of E-cadherin, while A549 cells showed polygon-like shape and only expressed E-cadherin. After the treatment of FL118, the expression level of E-cadherin was dramatically increased in A549/DDP cells, whereas the expression level of vimentin was reduced (Figure 6B). These data suggested that the treatment of FL118 induced the reversal of EMT phenotype in A549/DDP cells, raising the possibility that the effect of FL118 on chemoresistance and movement capability of A549/DDP cells is likely to be related to EMT.

Discussion

Lung cancer is one of the most common malignant tumors, and the platinum drugs are widely used for the treatment of cancer. But the drug resistance of tumor cells frequently leads to the failure of treatment. Therefore, there is an urgent need to find new drugs used to overcome drug resistance. Due to FL118’s superior antitumor activity, favorable tolerability, and low toxicity, we believe that FL118 may become a good option for targeted lung cancer therapeutics to avoid drug failure by circumventing multiple mechanisms of drug resistance.

A series of initial discoveries have demonstrated that a novel small chemical molecule designated as FL118, exerts antitumor activity through selectively inhibiting survivin promoter activity [11]. Firstly, in our study, we verified that A549 and A549/DDP cells both have the expression of survivin protein and mRNA (Figure 3A, 3B). Especially in A549/DDP cells, there is a higher level of survivin, which was downregulated with treatment of FL118. These data imply that FL118 may effectively exert antitumor efficacy on A549/DDP cells. Secondly, we found that FL118 markedly inhibits NSCLC cell growth based on the results from MTT assay (Figure 2). Along with the concentration of FL118 was increased, the inhibitory rate was also increased. Furthermore, we found that FL118 inhibited the proliferation of NSCLC cells regardless of the existence of cisplatin resistance, and the inhibitory effects of FL118 on A549 and A549/DDP cells are significantly higher than cisplatin, revealing that FL118 not only inhibits the proliferation of NSCLC cells, but also has a superior antitumor activity compared to cisplatin.

This conclusion was further confirmed by flow cytometry analysis of cell cycle progression. As known, cell proliferation is correlated with the regulation of cell cycle progression. Our data (Tables 1, 2) showed that there was a time-dependent increase in the percentage of cells entering S phases after FL118 treatment. Blockade of DNA synthesis in S phase may prevent the replication of the damaged or mutated DNA, which allows cells to repair pre-mitotic

| Table 1. Cell cycle distribution (%) in A549 cells by flow cytometry analysis |
|-----------------------------|--------|-------|--------|
| Groups phase    | G0/G1 phase | S phase | G2/M  |
| Control        | 62.58±1.15  | 26.57±0.52 | 10.84±0.71  |
| 12 h           | 42.35±1.45a | 56.34±0.36b | 1.31±0.52  |
| 24 h           | 0        | 97.65±1.23a | 2.35±0.48  |

*aP<0.05; bP<0.01 vs. control group.

| Table 2. Cell cycle distribution (%) in A549/DDP cells by flow cytometry analysis |
|-----------------------------|--------|-------|--------|
| Groups phase    | G0/G1 phase | S phase | G2/M  |
| Control        | 46.57±1.72  | 30.91±0.59 | 22.52±0.73  |
| 12 h           | 62.97±1.40b | 32.82±0.46b | 4.21±0.33  |
| 24 h           | 2.52±0.12 | 96.95±1.32a | 0.83±0.78  |

*aP<0.05; bP<0.01 vs. control group.
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Figure 5. FL118 effectively inhibited the migratory and invasion abilities of A549 and A549/DDP. (A) The migration and (B) invasion capabilities are impaired in A549 and A549/DDP cells after FL118 treatment. (*P<0.05 vs. the negative group).

Figure 6. The changes in the morphology of A549 and A549/DDP cells and the expression of E-cadherin and vimentin. A. A549/DDP cells were incubated with 1.0 μg/mL of cisplatin and showed a spindle-shaped morphology, while A549 cells showed polygon shape. B. ICC and Western blot analysis of EMT markers.

DNA damage or undergo apoptosis [24]. Because survivin is involved in the inhibition of apoptosis, the regulation of mitotic cell division, and the promotion of G₁/S transition [25],
FL118 acts as an inhibitor of survivin and is capable of arresting A549 and A549/DDP cells in the S phase of the cell cycle, which explains the mechanism of inhibitory effect of FL118 on NSCLC cells.

It has been found that FL118 possessed superior function and distinct mechanism from those camptothecin analogues like irinotecan and topotecan [11, 14-16]. On one side, FL118 may effectively inhibit the proliferation of tumor cells as shown above. On the other side, FL118 may overcome irinotecan and topotecan resistance in human head-and-neck or colon tumors by bypassing multiple ATP-binding cassette (ABC) transporter efflux protein-induced drug resistance. ABC transporter (ABCG2/BCRP) and P-glycoprotein (P-gp/MDR1) are both major efflux pump proteins. Although for many drugs, inhibition of ABCG2 and/or P-gp is an effective way to increase drug efficacy [26-30], FL118 can bypass ABCG2 and P-gp-mediated resistance and thus has an inherent advantages over many other drugs.

The molecular mechanisms of drug resistance are complicated. One of mechanisms is the enhancements in DNA damages repairing. Nucleotide excision repair (NER) system is an important way to repair DNA damage. In human tumor cells, DNA damages caused by ultraviolet rays and chemotherapy drugs are mainly repaired by NER pathway [31], and ERCC1 plays a key role during DNA damage recognition in the NER pathway [32, 33]. Inhibition of ERCC1 expression can reduce the ability of tumor cell to repair DNA damage, causing tumor cells to be sensitive to chemotherapeutic agents, such as cisplatin. In vitro transfection experiments showed that, down-regulated expression of ERCC1 can improve the chemosensitivity of cisplatin-resistant human ovarian cancer cells. meaning that the function of cellular DNA mismatch repair pathway is lost by inhibiting expression of ERCC1 [34]. Consistent with the previous conclusion that acquired cisplatin resistance of tumor cells have an overexpression of ERCC1 [35, 36], A549/DDP has an obvious expression of ERCC1 protein. Moreover as mentioned above, P-gp/MDR1 has a strong outer pumping action to platinum, thus reducing the concentration of the platinum drugs inside tumor cells and then rendering tumor cells with platinum-resistance. In our study we found the similar results that FL118 may inhibit A549/DDP cells even if the tumor cells have a higher expression of P-gp and ERCC1 (Figures 1B and 2). It suggested that FL118 may enhance drug-sensitivity of the NSCLC cells through decreasing the expression of resistant-associated proteins.

Nevertheless, there was an interesting discovery detected by real-time PCR (Figure 1C) displayed that, contrary to the protein expression of the ERCC1 and P-gp, the mRNA expression in A549/DDP cells obviously increased after the treatment of FL118. These data suggest that inhibition of ERCC1 and P-gp protein expression by FL118 likely occurs during transcriptional or post-transcriptional level, and there may be other potential protein targets interacted with FL118.

Another important theory which is closely related to chemoresistance is epithelial-mesenchymal transition (EMT). Many studies have shown that EMT plays an important role in the carcinogenicity, metastasis and poor prognosis in many types of tumor [37-40], and EMT is involved in drug resistance in NSCLC [41, 42]. During EMT, epithelial markers such as the expressions of E-cadherin decrease, while mesenchymal markers such as vimentin were overexpressed [43]. Our study showed that FL118 weakened the mesenchymal characteristics of A549/DDP (Figure 6A, 6B) including morphologic change and the reversal of EMT phenotypic markers, suggesting that FL118 may inhibit cisplatin resistance tumor cells by regulating EMT. We also observed the ability of migration and invasion in NSCLC cells after the treatment of FL118 (Figure 5). Its migration and invasion abilities was associated with EMT. Furthermore, FL118 may have more potential protein targets to exert inhibitory effect on movement ability.

In conclusions, the present study demonstrated that FL118 not only effectively inhibits the growth and motility of NSCLC cells with or without cisplatin resistance, but also significantly enhances drug sensitivity through decreasing resistance-associated proteins, ERCC1 and P-gp. Except that FL118 selectively inhibits survivin, Mcl-1, XIAP, and c-IAP2 and bypasses ABCG2 and P-gp-associated drug resistance, the effect of FL118 on EMT of tumor cells is also likely to play a key role for drug resistance.
and tumor relapse. Taken together, our and others’ studies showed that FL118 application is a novel therapeutic approach for cisplatin-resistant NSCLC.

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Disclosure of conflict of interest

None.

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