Original Article

Inhibition of ERK signaling potentiates the anti-tumor activity of FL118 on hepatocellular carcinoma cells

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Background: A novel camptothecin analogue, FL118, has been shown to exhibit superior anticancer activity over irinotecan on colon and lung cancer cells. It was reported that FL118 mainly performs its broad-spectrum anticancer activity through inducing cancer cell apoptosis. Accumulated evidence indicated that ERK pathway was constitutively activated in liver cancer, thereby promoting tumorigenesis, development, metastasis and recurrence. In this study, we aimed to investigate the anticancer potency of FL118 on liver cancer cell lines and the effect of FL118 on extracellular regulated protein kinase (ERK) pathway.

Methods: Viability of PLC and Huh7 cells was detected by MTT assay. Western blot assay was performed to assess phosphorylated and total ERK protein. And cell apoptosis was measured by Hochest-PI staining.

Results: Our results revealed that FL118 can inhibit PLC and Huh7 cell viability and markedly induce their apoptosis. Western blot results indicated that ERK pathway was activated in huh7 and PLC cells after administration of FL118. In addition, the pro-apoptotic and antiviability ability of FL118 can be potentiated by ERK-specific inhibitor U0126.

Conclusions: Our study confirmed good anticancer activity of FL118 on liver cancer, and targeting ERK pathway can significantly improve its anticancer potency.

Keywords: FL118; liver cancer; extracellular regulated protein kinase (ERK); apoptosis; cell viability

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Introduction

Hepatocellular carcinoma (HCC), one of the most widespread cancers with the characteristic of high incidence and mortality, is the third leading factor of cancer-related death in the world (1). Except for sorafenib, effective molecule-targeting medication is still lacked for advanced HCC. As an inhibitor of extracellular regulated protein kinase (ERK), sorafenib greatly improves the survival of liver cancer patients, and even, is the only choice for late liver cancer therapeutics (2,3). However, it is limited to improve 5-year patient survival due to the inevitable drug resistance (4).

FL118, also known as 10, 11-methylenedioxy-20(S)-camptothecin, 10, 11-MD-CPT, MDCPT (5), and 10, 11-mCPT (6), is a novel camptothecin derivative initially identified as a potent inhibitor of survivin, a member of IAP family. Recent studies have reported that FL118 is able to inhibit the expression of anti-apoptotic factors, such as X-linked inhibitor of apoptosis protein (XIAP) Baculoviral IAP repeat-containing protein 2 (cIAP2) and Induced myeloid leukemia cell differentiation protein 1 (Mcl-1), and induce the expression of pro-apoptotic

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proteins, such as Bcl-2-associated X protein (Bax) and bisindolylmaleimide inhibitor (Bim) in various cancer cell types (7). Interestingly, FL118 exerts its excellent anticancer activity regardless of p53 status. In cancer cells with wild type p53, FL118 induces cancer cell apoptosis by quickly activating p53 pathway, while in cells with null or mutant p53, FL118 can target survivin, XIAP, clAP2 and Mcl-1 to suppress cell growth (7). FL118 is observed to lack of Top 1 (Topoisomerase)—inhibiting activity compared with other camptothecin analogues, such as topotecan and irinotecan. However, experimental studies showed that FL118 significantly reduces cell proliferation and tumor growth in many types of cancer *in vitro* and *in vivo* (7,8). These dates suggested that FL118 may employ a different mechanism other than from Top 1 inhibition to exert its antitumor activity. Furthermore, it is worth noting that FL118 exhibits low toxicity due to targeting of FL118 to antiapoptotic proteins, such as survivin, XIAP, clAP2 and Mcl-1, which are expressed at a lower level in normal tissue compare with in cancerous cells (7).

ERK, a member of mitogen-activated protein kinases (MAPK) signaling pathway, plays an essential role in the pathogenesis of human cancer, under both pathological and physiological conditions. It has been found that ERK signaling can influence many functions of cancer cells, such as cellular proliferation, apoptosis, differentiation and survival (9). ERK pathway is closely associated with apoptosis by affecting the activity of apoptosis-related proteins, such as Bax, Bim and caspase 9 (1,10). Increasing evidence has indicated that FL118 plays an antitumor role by targeting apoptosis-related pathways. However, the relation between ERK and antitumor activity of FL118 still remains unclear so far.

Based on the previous studies, we speculated that ERK pathway may be involved in the anti-tumor effect of FL118. In this work, we tested the activation of ERK pathway in the treatment of FL118 in human liver cancer cells and explored the tumor suppressing effect of combined therapy of ERK inhibitor and FL118.

Methods

Cell culture

PLC and Huh7 cells were from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured in a humidified 37 ℃ condition with 5% CO_2. All cells lines were cultured with the same conditions.

Compounds

FL118 were obtained from Dr. Li research group, ERK inhibitor U0126 were purchased from Selleckchem (Shanghai, China). FL118 was dissolved in DMSO to make stock solution (1 mM) which was diluted into needed concentration when used. U0126 (10 μM) that selectively blocked ERK was added to cells with FL118 (10 and 100 nM) together.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The viability of PLC and Huh7 cells that treated with administration concentration of FL118 was determined by employing MTT assay. PLC (4×10^3/well) and Huh7 (5×10^3/well) cells were seeded into 96-wells plates incubated in 5% CO_2 at 37 ℃ for 12 h, and then treated with appropriate concentrations of FL118 or U0126 for 24, 48, and 72 h, respectively. Subsequently, the diluent MTT with 5 mg/mL was added into 96-well plates. The cells were culture for 4 h, then MTT was aspirated off and 200 μL DMSO was added to dissolved formazan crystals in each well. The plates were agitated for 30 s and the absorbance in each well was detected at 490 nm using Microplate Reader (Tecan, Shanghai, China).

Western blot analysis

The cells were washed twice with PBS, and then lysed on ice radio immunoprecipitation assay (RIPA) lysis buffer with protease inhibitors phenylmethanesulfonyl fluoride (PMSF) and all-in-one (Solarbio, Beijing, China). The concentration of total proteins was determined by BCA Protein Quantification Kit (Solarbio, Beijing, China). The proteins sample was separated by 15% SDS-PAGE gels, transferred to 0.45 micro polyvinyl difluoride (PVDF) membranes used semi-dry electrophoretic transfer, and then 5% skimmed milk in TBST was used to block the nonspecific binding site of PVDF membranes for 1h at room temperature. The membranes were incubated with antibodies which diluted with 5% skim milk at 4 ℃ overnight. Subsequently, the membranes were washed 3 times with TBST and then incubated with appropriate secondary antibodies for 2 h at room temperature. eECL western blot Kit (CWBIO,

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Beijing, China) was used to detect the protein binds. The antibodies involved in this study are as follows: ERK (Cell Signaling Technology, Boston, MA, USA), p-ERK (Cell Signaling Technology, Boston, MA, USA), GAPDH (Abcam, Cambridge, MA, USA).

Cell apoptosis assay

PLC and Huh7 cells were seeded into 6-well plates, the cells were divided into different groups randomly. Subsequently, after treated with appropriate concentration of FL118 or U0126 for 24 h, cells were washed with PBS and then incubated with Hoechst and propidium iodide (PI). Four random four file of view demonstrating total cells (Hochest-stained nuclei) or death (PI-stained nuclei) cells were observed by microscope (Nikon, Beijing, China). In each plate the percentage (PI/Hoechst) of cell apoptosis was determined by averaging of four files.

Statistical analysis

All the experiments were conducted for at least three times except western blot assays. All date are shown as means ± SD in figures. The samples' difference was analyzed by one-way ANOVA, statistical significance was considered as: *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; and ns, no significant difference.

Results

FL118 inhibits the viability of PLC and Huh7 cells

Different concentrations (0, 1, 10, 50, 100 and 200 nM) of FL118 was administrated on PLC and Huh7 cells for 24, 48, and 72 h. Cell viability was detected by MTT assay. As shown in *Figure 1A*,*B*, FL118 dramatically decreased the viability of PLC and Huh7 cells in a dose-dependent manner.

FL118 induces apoptosis of PLC and Huh7 cells

To observe the effect of FL118 on apoptosis of human liver cancer cells, PLC and Huh7 cells were treated with different concentrations (10 and 100 nM) of FL118 for 24 h. Apoptosis was assessed by Hochest-PI staining. As shown in *Figure 1C*,*D*, exposure to both 10 and 100 nM FL118 led to a significant apoptosis in cancer cells. Few apoptotic cells were observed in control groups. However, some apoptotic cells were detected under FL118 treatment and apoptotic events were found to be increased along with the elevated concentrations.

FL118 induces ERK activation in cancer cells in a time-dependent manner

Subsequently, to explore the potential anticancer activity of FL118 on liver cancer cells, we investigated if ERK pathway was activated after treatment with 100 nM FL118. Western blotting was used to detect the expression of total and phosphorylated ERK proteins at 0, 1, 2, 3, 6, 12 h. The results showed treatment of 100 nM FL118 decrease and increase the level of total and phosphorylated ERK proteins in a time-dependent manner, respectively (*Figure 2A*), indicating that FL118 can inhibit the ERK pathway, and that PLC and Huh7 cells may resist against FL118's anticancer potency via activating ERK pathway. Subsequently, we detected the expression of total and phosphorylated ERK after treatment with FL118 or/and U1026, an ERK-specific inhibitor, for 6 h. As indicated in *Figure 2B*, there is no significant changes in the level of total ERK protein between FL118 alone and combined treatment of FL118 and 10 μM U0126. However, the combined administration of U0126 and FL118 led to a significant suppression of ERK phosphorylation, compared with FL118 alone. These results indicated that FL118 treatment induce the activation of ERK pathways in liver cancer cells.

The capability of FL118 to inhibit cell viability and induce apoptosis is potentiated by suppression of ERK activation in cancer cells

To further determine the relationship between FL118 and ERK pathway, we detected the cell viability and apoptosis in PLC cells after the treatment with U0126 and FL118. *Figure 3A*,*B* showed that combined treatment of U0126 and FL118 significantly inhibited the growth of both PLC cells, compared to control and FL118 groups. Moreover, *Figure 3C*,*D* demonstrated that the apoptotic ratio was obviously higher in PLC and Huh7 cells after combined treatment with FL118 and U0126 for 24 h than that with only FL118 stimulation. The above results indicated that the combined therapy using both ERK inhibitor and FL118 might be more effective than FL118 alone for inhibiting proliferation and inducing apoptosis in PLC and Huh7 cells.

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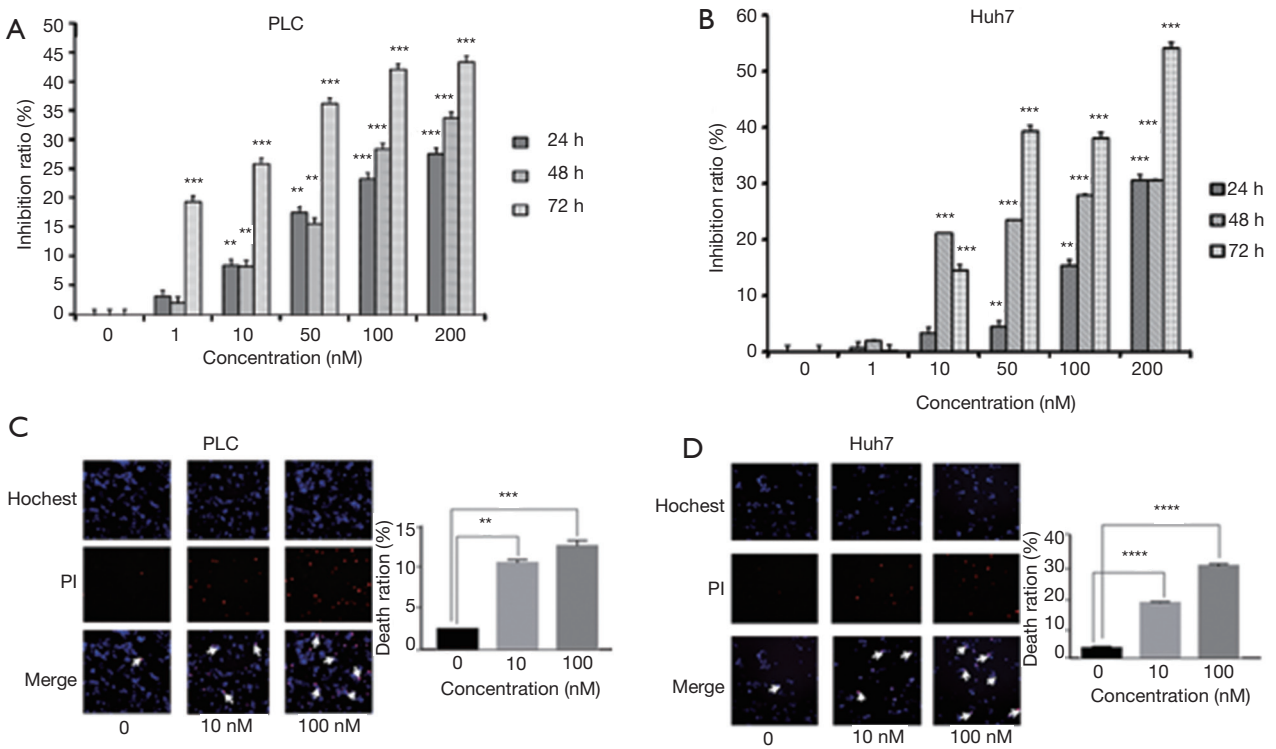

Figure 1 FL118 effectively inhibits cell viability and induces apoptosis in PLC and Huh7 cells. (A,B) Cell viability assay was performed after stimulating with various concentrations (0, 1, 10, 50, 100 and 200 nM) FL118 for 24, 48 and 72 h in PLC and Huh7 cancer cells; (C,D) the fluorescent images of cancer cells stained with Hochest 33342 (blue) and PI (red) after FL118 treatment at 10 and 100 nM for 24 h (×200). Apoptosis ratio was measured in all groups. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

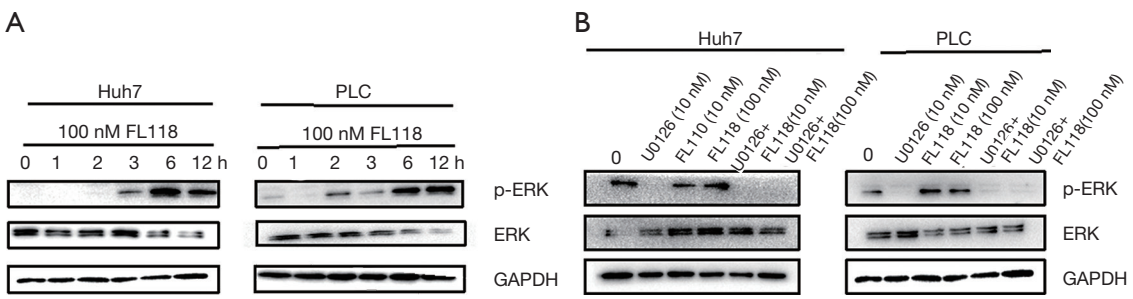

Figure 2 FL118 significantly induces ERK activation in cancer cells in a time-dependent manner. (A) Human liver cancer cells, PLC and Huh7 cells were treated with 100 nM FL118 at indicated timepoints (0, 1, 2, 3, 6 and 12 h), western blot analysis was used to detect the expression level of phosphorylated and total ERK proteins; (B) PLC and Huh7 cells was treated with U0126 (10 μM) or/and FL118 (10 and 100 nM). Six hours later, western blotting was performed to determine the expression level of phosphorylated and total ERK proteins.

Discussion

HCC is one of most common malignant tumors with the characteristics of high incidence, frequent relapse and poor prognosis (11). According to previous studies, liver cancer is the fifth and the seventh most common cancer in men and women, respectively (1). Currently, chemotherapy plays an important role in the treatment of patients who are not willing to or suitable for surgery (12). However, the occurrence of drug resistance limits the effect of chemotherapy (4).

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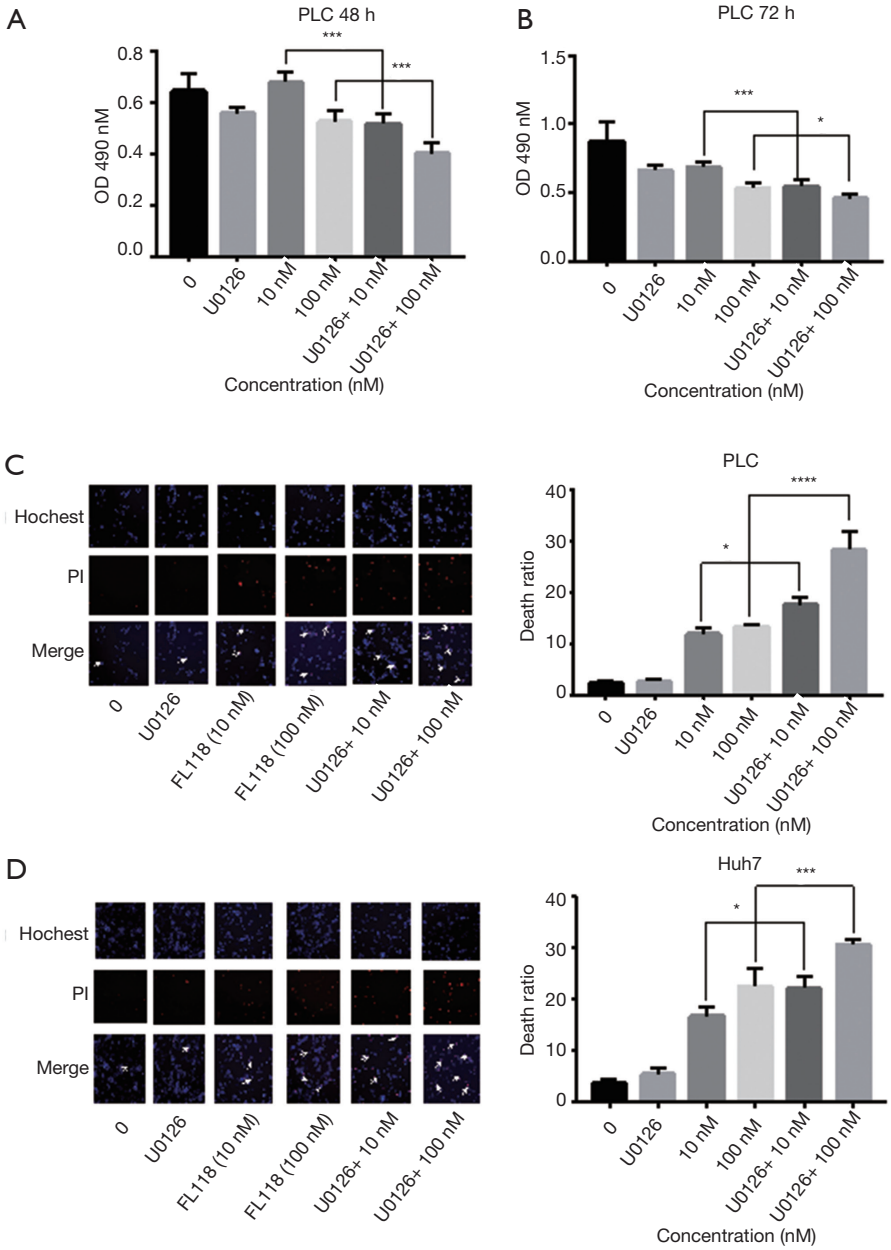

Figure 3 The capability of FL118 to inhibit cell viability and induce apoptosis was potentiated by suppression of ERK activation in cancer cells. (A,B) PLC cells were treated with various concentrations of FL118 with U0126 (10 μM) or/and FL118 (10 and 100 nM) for 48 and 72 h. MTT assay was conducted to measure cell viability; (C,D) Hochest 33342 and PI staining (×200) was done in PLC and Huh7 cells with U0126 (10 μM) or/and FL118 (10 and 100 nM). The apoptotic events were detected at 24 h. Apoptosis ratio of cancer cells was determined in all groups. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

Hinted by the resistance of cancer to drugs, it appears not to be an effective way to target a single molecular pathway for eliminating tumor cells. Therefore, it is believed to be a more effective strategy to target multiple cancer-associated signaling pathways. A newly-developed camptothecin analogue, FL118, has been observed to inhibit the growth of colon and head-neck cancer with a lower toxicity in mice models, compared with irinotecan or

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its active metabolite, SN-38 (7,13,14). ABCG2 (15), a drug efflux pump (16), plays a pivotal role in cancer resistance to drugs, such as irinotecan, SN-38 (17), methotrexate (18) and a variety of tyrosine kinase inhibitors (19,20). Intriguingly as a poor substrate for ABCG2, FL118 displays better therapeutic efficacy in colon and lung cancer models than irinotecan through bypassing the ABCG2-induced drug resistance. Different from other camptothecin analogues, the antitumor ability of FL118 is irrelevant to the suppression of Top 1 enzyme activity (21). Thus, these data demonstrated that FL118 has a unique mechanism against cancer. In a previous study, FL118 exhibited strong anticancer activity, low toxicity and drug resistance in colon and head-neck cancer (8). We aimed to detect if FL118 has anticancer activity on human liver cancer cells and to create a potential rationale for its clinical application. Our results showed that FL118 reduced cell viability and induced apoptosis in dose- and time-dependent manners in PLC and Huh7 cells.

Accumulated evidence has demonstrated that dysregulated apoptosis is closely associated with initiation and progression of cancers (12). MAPK signaling cascade has been indicated to mediate cellular response to the intracellular and extracellular stimuli (22). Among the members of MAPKs, ERK is usually associated with cell apoptosis (23). Many studies have elucidated that ERK signaling is able to induce apoptosis in cancer cells (24). Experimental studies demonstrated that ERK pathway activation influences the expression of survivin, and thus to inhibit apoptosis in human cancer cells (25). Furthermore, other recently studies showed that the ERK pathway in conjugation with AKT regulated survivin activation on cancer cell lines (26). Based on the relationship between FL118 and survivin, ERK signaling might be an important pathway to FL118's anticancer effect. However, it is still yet to be determined if ERK pathway is affected by the treatment of FL118. Thus, we further detected the status of ERK pathway after treatment of FL118. Our results showed that the phosphorylation of ERK is enhanced after treatment of FL118 in PLC and Huh7 cells. Notably, the level of total ERK remained unchanged. Taken together, we found that FL118 induced the activation of ERK signaling pathway in liver cancer cells. In our work, it is the first time to demonstrate that FL118 is associated with ERK pathway in tumor cells. As reported previously, camptothecin can activate the ERK pathway through influence its upstream proteins (27). Some studies showed that camptothecin activates ERK by suppressing mitogen-activated protein kinase phosphatase-1 (MKP1) in various types of cancer cells (27). Based on the structural similarity between camptothecin derivatives, FL118 may also exert its effect on the activation of ERK pathway through MKP1, However, the mechanism by which FL118 activates the ERK pathway is still unfounded so far. And more, it should be noted that this finding is inconsistent with our initial speculation, because ERK activation is well believed to decrease apoptosis in cancer cells. These data suggested that FL118 may utilize a molecular signaling other than ERK pathway to exert its ability to trigger apoptosis. We will search for the exact molecular mechanism by which FL118 leads to the apoptosis in these malignant cells in further work.

Subsequently, it is interesting to us if the proapoptotic activity of FL118 can be further potentiated by the suppression of ERK pathway. Our results showed that suppression of ERK pathway can enhance the apoptosis and inhibit cell viability after treatment of FL118 in PLC and Huh7 cells, indicating that abrogation of ERK signaling is indeed an effective strategy to further enhance the anticancer activity of FL118. Previous studies have also reported that ERK signaling is activated during the administration of anticancer compounds for clinical therapeutics (28), which is consistent with our results. And this phenomenon is believed to probably induce the drug resistance of cancer cells to this antitumor molecule.

Intriguingly, some studies demonstrated that the activation of ERK signaling can be inhibited by some natural products, such as celastrol (29) and curcumin (30), which may also be used to enhance the anticancer effect of FL118. Although ERK activation is involved in the impairment of FL118's antitumor efficacy, it's still worthy of being further investigated if other possible mechanisms are associated with a weakened antitumor activity of FL118. It is an effective strategy to improve the clinical outcomes of FL118 by targeting these signaling pathways.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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