



A novel Camptothecin analogue inhibits colon cancer development and downregulates the expression of miR-155 *in vivo* and *in vitro*

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Background: FL118 is a newly discovered camptothecin analogue that exerts anti-tumor activity on a wide range of cancers; however, the molecular mechanism underlying FL118's antitumor activity is still far from being completely understood. MicroRNAs (miRNAs) are believed to play an important role in the progression of human malignancies, and increasing evidence shows that these small RNAs also mediate the tumor-suppressing activity of many natural and/or synthetic compounds. Our previous studies indicated that miR-155, which has been confirmed as an oncogenic miRNA in colorectal carcinoma was significantly downregulated after the treatment of FL118.

Methods: MTT assay, scratch wound assay, BrdU cell proliferation assay and flow cytometry were employed to detect HCT-116 cell viability, mobility, proliferation, apoptosis and cell cycle under the treatment of FL118, respectively. Xenograft models were established to observe the effect of FL118 on tumor growth *in vivo*. Also, qRT-PCR was performed to detect the level of miR-155 in colon cancer cells and tumor samples after FL118 administration.

Results: Our results showed that FL118 induced cell apoptosis, inhibited cell viability and mobility, suppressed cell proliferation and limited the growth of colon cancer. The levels of miR-155 were downregulated significantly ($P < 0.05$) by FL118 both *in vivo* and *in vitro*.

Conclusions: FL118 effectively inhibits colon cancer development and downregulates the expression of miR-155 both *in vivo* and *in vitro*. With the understanding that miR-155 is closely associated with the pathogenesis and development of colon cancer, its downregulation resulting from FL118 may indicate that miR-155 is likely to participate in, and even mediate the anticancer activity of FL118 on colon carcinoma, which should be noted, and urges further study.

Keywords: FL118; Camptothecin analogue; colon cancer; miR-155

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Introduction

Camptothecin and its analogues are natural compounds with broad antitumor spectrums in clinics, such as gastric cancer, colon cancer, ovarian cancer and leukemia, etc. Studies on their pharmacological activity have demonstrated that camptothecin and its analogues exert their cytotoxicity on cancer cells mainly by targeting topoisomerase I to interrupt DNA replication, transcription and repair (1). FL118 is a special camptothecin derivate, although it shares similar structure (*Figure 1*) with clinically applied camptothecin analogues, irinotecan and topotecan, FL118 appears to demonstrate more effective properties of its antitumor activity (2). Further studies have revealed that FL118 is also able to overcome drug resistant colon cancer cells with efflux pump P-gp and ABCG2 overexpression (3,4), while irinotecan and topotecan cannot. Strikingly, Top1 inhibitor-resistant HCT-116 sub-lines (HCT-116-SN6/G7/SN50/A2) (4) and p53 null/mutant cells (SW620, FaDu, PC-3, 2008, EK VX) (2) have also been confirmed to be sensitive to FL118. It was also found that large SW620 and FaDu tumors (1,500–2,000 mm³) can be effectively eradicated by FL118 (2), suggesting that FL118 is a promising candidate to be applied to p53-mutant colon carcinoma therapy. Moreover, FL118 also demonstrated excellent pharmacokinetics profiles (4) and therapeutic effects and both *in vitro* and *in vivo* results looked promising for further development (2–8). Animal experiments observed better antitumor effect and lower toxicity of FL118 than first-line therapeutic drugs, including irinotecan, topotecan, adriamycin, 5-FU, gemcitabine, docetaxel, oxaliplatin, cisplatin and cyclophosphamide (2). The next question we must ask ourselves is what makes FL118 extraordinary in comparison with other Camptothecin analogues, even with some first-line therapeutic drugs? Finding the answer to this question is the research area regarding FL118 that needs further investigation and unraveling.

After some debate amongst our team, we elected to focus on colon cancer, a kind of cancer with high morbidity and mortality around the world. Colon cancer is also the most investigated cancer (2–5,8–12) of FL118 on treatment effect and action mechanism study. On the foundation of the previous studies, in this paper we tried to systematically study the properties of FL118's anti-colon cancer activity and extend the studies to the action mechanism of FL118.

MicroRNA (miRNA) is a kind of non-coding RNA with

a length of 22 nt (13). It serves as a post-transcriptional regulator in various physical and pathologic processes by binding to the 3' untranslated region (3' UTR) in the target mRNA and thus altering the expression of its target gene. Increasing studies have showed that miRNA is closely related to carcinogenesis (14). Additionally, more and more microarray analysis demonstrate that natural compounds and their derivatives partially exert their antitumor activity through altering miRNA expression profiles and their downstream targets (15), such as curcumin (16), resveratrol (17), genistein (18), docosahexaenoic acid (DHA) (19), aplysin (20), methyl jasmonate (21) and oleanolic acid (22). Our previous studies indicated that the expression of miR-155 was significantly decreased by FL118. MiR-155 is a typical multifunctional gene related to neoplasm, inflammation, cardiovascular diseases and viral infection (23). In colon cancer, miR-155 is an oncogene. Clinical researches have elaborated the correlation between aberrant overexpression of miR-155 and colon cancer oncogenesis, development, proliferation, invasion and metastasis (24). In this paper, we will verify the downregulation of miR-155 in colon cancer after FL118 administration *in vivo* and *in vitro* and discuss the meaning of this downregulation for FL118 to colon cancer.

Methods

Cell culture

Human colon cancer cell lines HCT-8 and HCT-116. HCT-8 was purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. HCT-116 was kindly donated from School of Translational Medicine, Qingdao University. HCT-116 cells were grown in DMEM/HIGH GLUCOSE (HyClone) medium supplemented with 10% fetal bovine serum (FBS), along with 1% penicillin and streptomycin (HyClone) in a humidified incubator at 37 °C with 5% CO₂/95% air. HCT-8 cells shared same culturing condition with HCT-116 cells, except that the culture medium is RPMI 1640 medium (HyClone). Besides, the cells were cultured in a 25 cm² cell culture flask (corning) and removed by Trypsin 0.25% (1X) Solution (HyClone).

Cell viability assay

Cells were seeded in 96-well plates (NEST). After cells adhered for 24 h, FL118 was added to each well at a

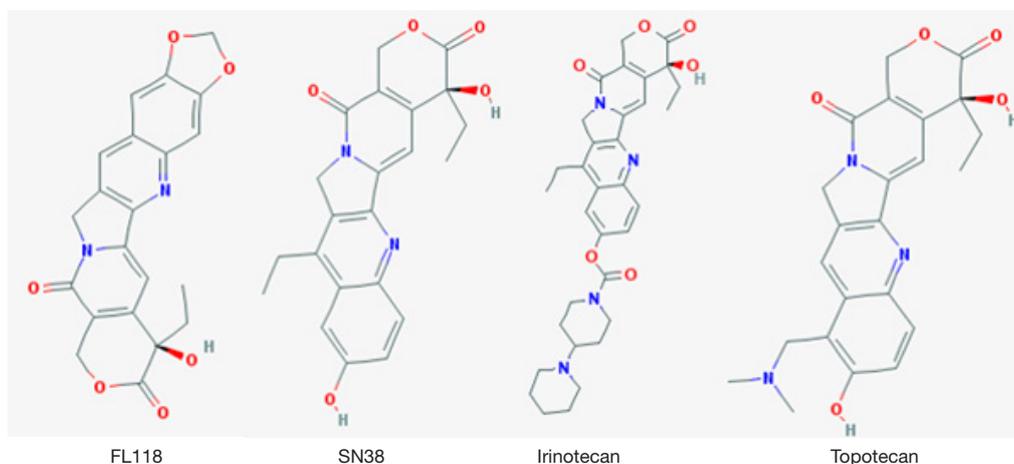


Figure 1 Structures of FL118, SN38 (the active metabolite of irinotecan), irinotecan and topotecan.

series of concentration: 0.01/0.1/1/10/100/1,000 nM. Cells treated with DMSO and SN38 (100 nM, the active metabolite of irinotecan) served as vehicle group and positive group. After 72 h, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was added to each well to indicate cell viability at the concentration of 0.5 mg/mL. Cells were further cultivated at 37 °C for 4 h. The OD value of each well was measured by microplate reader (Bio-Tek instruments) at 490 nm.

Scratch wound assay

Cells were seeded in 6-well plates (Costar) with 1×10^6 per well and adhered overnight. 10 μ L micropipette tip was used to scratch on the cell layer and then washed the cells surface twice by PBS. Culture medium with 1% serum was added to each well. Meanwhile, FL118 at 10 nM was added, too. The migration of cells was observed 24 and 48 h after incubated with FL118. Photos from three different sites of the wound in each well were collected.

BrdU labeling cell proliferation assay

Cells were seeded in 96-well plate at 2×10^5 cells/mL in 100 μ L/well culture media. Parallel wells with culture media only and cells plated without adding the BrdU Reagent should be prepared at the same time. Cells were treated with FL118 at 10 nM for 24 h. The 1 \times BrdU label was added into proliferating cells two and a half hours prior to the end of FL118 treatment. The subsequent steps were performed according to the protocol of BrdU Cell

Proliferation ELISA Kit (colorimetric, Abcom).

Cell cycle analysis

Cells at 1×10^5 /well were seeded in 6-well overnight and starved for 12 h for synchronization. Cells were collected after being treated with FL118 at 10 nM for 24 h and washed twice by precooling PBS. Next, 70% ethanol was added to immobilize cells overnight at 4 °C. The PI staining was performed according to the protocol of Cell Cycle and Apoptosis Analysis Kit (7sea biotechnology). At last, cells were detected one by one in flow cytometry at 488 nm. The portion of each cell cycle stage (G0, G1, S, G2/M) was analyzed by Motif analysis software.

Apoptosis assay

Annexin V-FITC/PI Apoptosis Detection Kit (7 sea biotechnology) was used to detect the apoptosis of cells. After being treated by FL118 at 10 nM for 36 h, cells were collected and resuspended in PBS. Following being washed by PBS twice, cells were suspended in loading buffer and incubated with Annexin V-FITC and PI for 15 and 5 minutes at room temperature away from light. Apoptosis detection was measured by flow cytometry (BD Accuri C6) within 30 minutes. Apoptotic and necrotic cells were analyzed by quadrant statistics.

Animal experiments

Female BALB/c nude mice (age 49 to 56 days, weight

15 to 22 g) were obtained from Beijing Vital River Laboratory Animal Technology Corporation (China) to establish xenograft models. HCT-116 cells were injected subcutaneously at 2×10^6 per mouse. Mice were divided into three groups: control group (injected vehicle only), lower dose group (treated with FL118 at 0.75 mg/kg), and higher dose group (treated with FL118 at 1.5 mg/kg).

FL118 formulation for intratumoral injection was prepared following the basic method reported recently (25): at first, 100 μ L dimethyl sulfoxide (DMSO) and 0.01 g hydroxypropyl- β -cyclodextrin were mixed completely to dilute 1mg FL118. Then this mixture was vortexed until no visible particle can be seen. Finally, 1.9 mL normal saline (NS) was added into the mixture above accompanied with fully vortexed. The control solution (vehicle) contains the same proportion of DMSO and hydroxypropyl- β -cyclodextrin in saline without FL118. Every procedure of solution preparation and treatment must be sterile.

The treatment schedule is one treatment per week for four weeks. During the treatment, the tumor volume and body weight were measured every other day. The tumor volume was calculated as $V = 0.5 \times (\text{length} \times \text{width}^2)$ and measured by caliper. And the weight of mice is measured by electronic balance. When the tumor volume is more than 1,500 mm^3 or the mouse is moribund, mice were sacrificed to collect the tumors that were then stored in liquid nitrogen or 4% paraformaldehyde. All animal experiments were performed according to IACUC-approved animal protocols.

RNA extraction

Total RNA was extracted by TRIZOL (ComWin Biotechnology Corporation) according its protocol. After cells were collected in DNase/RNase free 1.5 mL microcentrifuge tubes (ExGenTM) and 1 mL TRIZOL was added to fully lyse cells for 5 minutes at room temperature. Then, 200 μ L chloroform was added proportionally and kept quiescence for 2 minutes after being mixed. Later, tubes were centrifuged at 12,000 rpm to enrich total RNA in the supernatant. Following precipitation by isopropanol for 10 minutes at room temperature and centrifuged at 12,000 rpm, total RNA was accumulated at the bottom of the tube. Subsequently, the RNA precipitation was washed by 75% ethanol and centrifuged again. When the RNA precipitation dry out after ethanol discarded, DEPC water was added to dissolve RNA. RNA solution was stored at -70°C .

Reverse transcription and real-time PCR assay

TaqMan[®] Advanced miRNA Assay (Thermo Fisher Scientific, Cat. No. A25576) served as the primer of hsa-miR-155-5p. Since the TaqMan[®] Advanced miRNA Assay does not detect snRNAs or snoRNAs, neither of them could be used as endogenous control, a list of miRNAs working as good endogenous control offered by manufacture was given, has-miR-361-5p was one of them and was elected as the endogenous control. Reverse transcription and real-time PCR assay was performed by TaqMan[®] Advanced miRNA cDNA Synthesis Kit and the 2X Fast Advanced Master Mix according to the manufacture protocol. To limit the measurement errors, only spots' ratio with more 2-fold than the control were included.

Statistical analysis

All the experiments and measurements were similarly performed in triplicate ($n=3$). All statistical analyses were performed using student's *t*-test. $P < 0.05$ was considered to be statistically significant. Besides specific illustration, the results shown in the figures are representatives.

Results

FL118 suppressed the viability, mobility and proliferation of HCT-116 cells

In addition to the well-investigated HCT-8 cells, HCT-116 cells are also sensitive to FL118.

MTT assay was performed on HCT-116 (p53 wide type) cells after FL118 treatment at a series of doses for 72 h (Figure 2), evident inhibitions were observed from 1 nM to 1,000 nM ($P < 0.01$). At 0.01/0.1 nM, FL118 failed to effectively inhibit the viability of HCT-116. However, under the treatment of FL118 at 0.01 nM for 72 h, the viability of SW620 (p53 mutant) was significantly inhibited ($P < 0.001$) (2), indicating that FL118 may exert more powerful effect on p53 mutant colon cancer cells. Intriguingly, the inhibitory effect of FL118 at 10 nM was comparable with SN38 at 100 nM.

Scratch wound assay was performed in HCT116 cells treated with FL118 (10 nM) for 24/48 h (Figure 3). Results clearly showed an inhibition of FL118 on the mobility of HCT-116 cells. The scratches in control group were gradually narrowed and almost confluent at 48 h, but in FL118-treated group, its scratches were still wider and clearer than control group.

BrdU cell proliferation ELISA Kit was used to study the inhibition of FL118 on the proliferation of HCT-116 cells for 24 h. The result revealed that FL118 significantly inhibited ($P < 0.01$) the proliferation of HCT-116 cells (Figure 4), and under same circumstances, the proliferation

of HCT-8 cells were also significantly suppressed ($P < 0.00001$) by FL118 (4). Consistently, FL118 induced S phase cell cycle arrest in HCT-116 cells at 24 h (Figure 5).

FL118 promotes apoptosis of HCT-116 cells

In our pre-experiments, under the treatment of FL118 (10 nM), obvious apoptosis occurred not at 12 or 24 h, but at 36 h in HCT-116 cells. Then, we performed parallel experiments to detect the apoptosis rate of FL118 at 10 nM for 36 h and successfully observed significant apoptosis in HCT-116 cells (Figure 6).

Taken together, we can figure out how the colon cancer cells go to death step by step under the treatment of FL118. At the same dose (10 nM) of FL118, HCT-116 cells suffered cycle arrest at S phase after treated for 24 h and their proliferation was also inhibited. Subsequently, obvious cell apoptosis was induced after FL118 treated for 36 h. Meanwhile, the weakened mobility of HCT-116 cells were observed since FL118 treated for 24 h and lasted for another 24 h. Eventually, after FL118 treated for 72 h, the viability of HCT-116 cells was suppressed on the whole.

FL118 limits the growth of HCT-116 tumor

HCT-116 xenograft models were established to test

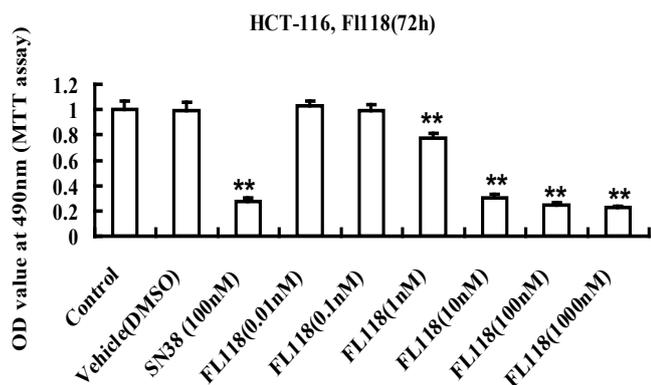


Figure 2 FL118 inhibits the viability of HCT-116 cells. MTT assay was performed to measure the cell viability after HCT-116 cells being treated by a series of FL118 doses. SN38 (100 nM) set as positive control and was added to a group of cells. The OD value of each well was detected by microplate reader at the wave length of 490nm. **, $P < 0.01$. Error bars are the SEM. MTT assay was performed in triplicate (n=3).

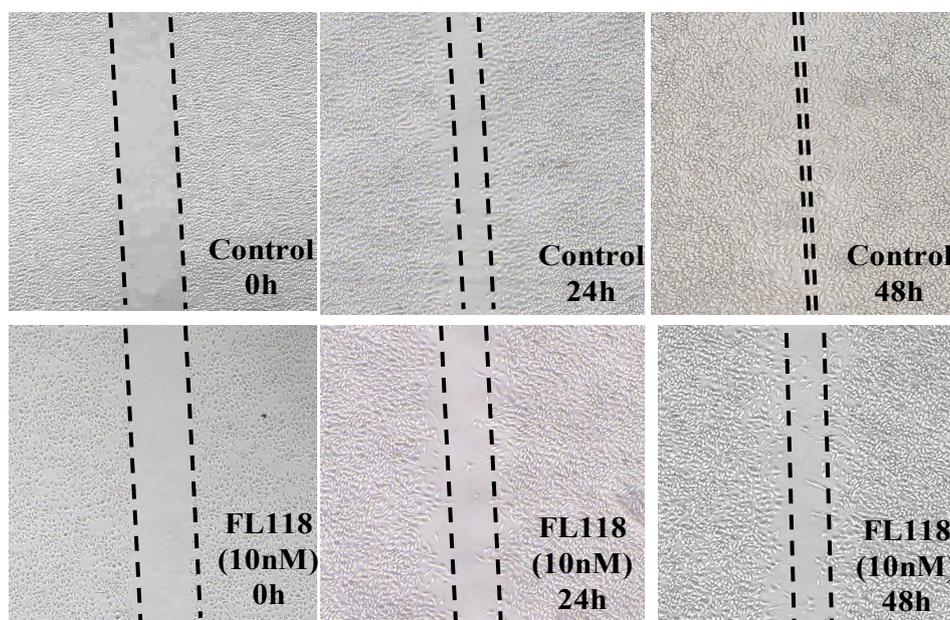


Figure 3 FL118 inhibits the mobility of HCT-116 cells. Scratch wound assay was performed to observe the mobility of HCT-116 cells after FL118 administration. Pictures were collected and the ledges of cell clusters were marked by dotted lines.

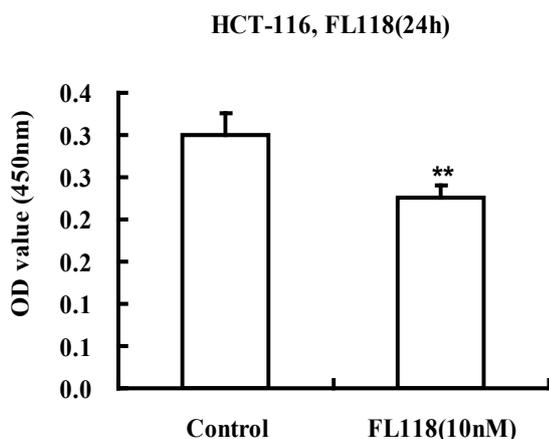


Figure 4 FL118 inhibits the proliferation of HCT-116 cells. BrdU assay was performed to detect the proliferation of HCT-116 cells after FL118 administration. BrdU reagent was added into each well two and a half hours prior to the end of FL118 treatment. The OD value of each well was detected by microplate reader at the wave length of 450 nm. **, $P < 0.01$. Error bars are the SEM. BrdU proliferation assay was performed in triplicate ($n=3$).

the anti-colon cancer activity of FL118 *in vivo*. FL118 solution was injected into HCT-116 tumor once a week for 4 weeks. The weight of mice and the volume of tumors were measured every other day (Figure 7). According to the results, the growth of tumor was effectively limited by FL118.

FL118 downregulates the expression of miR-155 in HCT-8 cells

Microarray analysis showed that miR-155 was significantly downregulated in the HCT-8 cells after treated by FL118 for 24 h. Subsequently, qRT-PCR was employed to confirm this result, the expression of miR-155 were detected in HCT-8 cells after being treated by FL118 for 24 and 48 h at 1/10/100 nM (Figure 8A).

Obviously, the expressions of miR-155 were significantly downregulated by FL118 at 48 h (1/10/100 nM) and 24 h (1 nM). It seems that longer treatment time and lower dosage of FL118 could effectively inhibit the

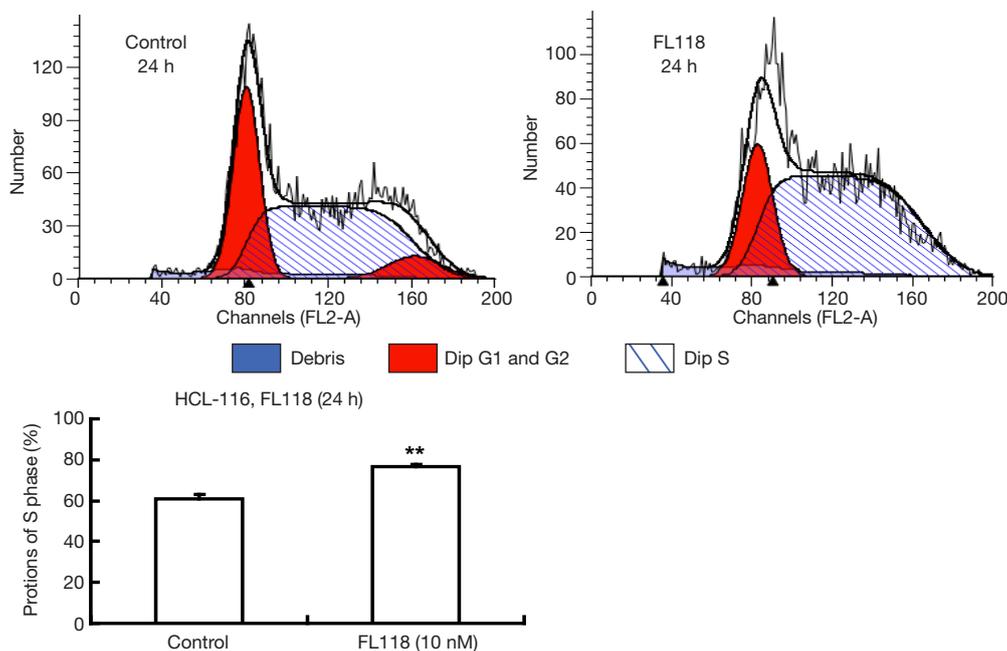


Figure 5 FL118 induced cell cycle arrest at S phase. HCT-116 cells were starved for 12 h for synchronization before FL118 treatment. After cells were collected, immobilized by 70% ethanol and dyed by PI, they were detected one by one in flow cytometry at 488 nm. The portion of each cell cycle stage (G0, G1, S, G2/M) was analyzed by Motif analysis software and the portions of S phase were shown in the bar graph. Colors corresponded to cell cycle stages were illustrated. Besides, the abnormal lines and colors (grey) can be explained by tears resulted from mechanical damage, however, their portion ($< 9\%$) do not influence the analysis of cell cycle. **, $P < 0.01$. Error bars are the SEM. Cell cycle analysis was performed in triplicate ($n=3$).

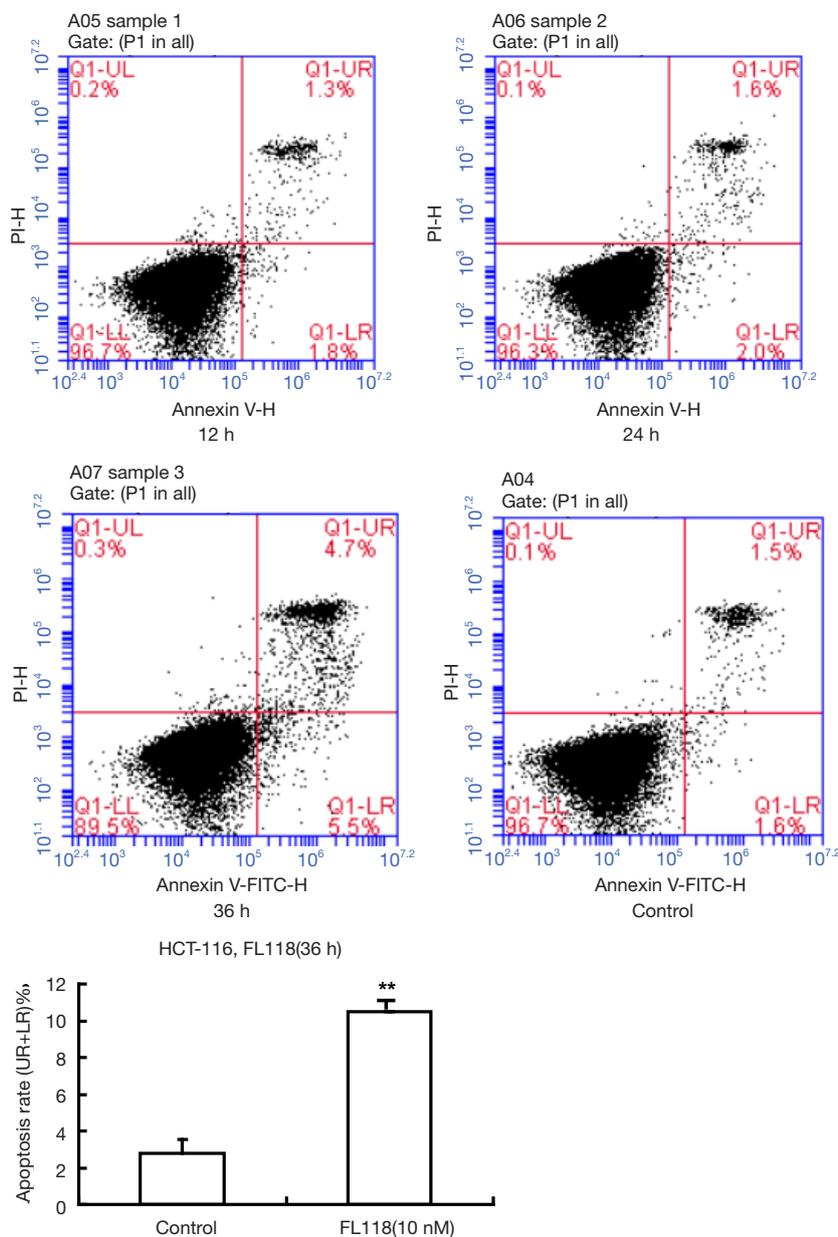


Figure 6 FL118 promotes apoptosis of HCT-116 cells. After treated by FL118 at 10 nM for 36 h, HCT-116 cells were collected, washed by PBS and dyed by Annexin V-FITC and PI. Apoptotic and necrotic cells were analyzed by quadrant statistics. In the picture, black particles in the UR, LR, UL and LL parts represent cells of late apoptosis and death, early apoptosis, necrosis and living cells, respectively. **, P<0.01. Error bars are the SEM. Apoptosis assay was performed in triplicate (n=3).

expression of miR-155.

FL118 downregulates the expression of miR-155 in HCT-116 cells both in vivo and in vitro

The level of miR-155 in HCT-116 cells is higher (P<0.01)

in comparison with HCT-8 cells (Figure 8B). Under the same treatment condition (Figure 8C), the expressions of miR-155 were significantly downregulated by FL118 at 48 h (1/10/100 nM) and 24 h (1/10 nM) indicating that colon cancer cells with higher levels of miR-155 may be more sensitive to FL118.

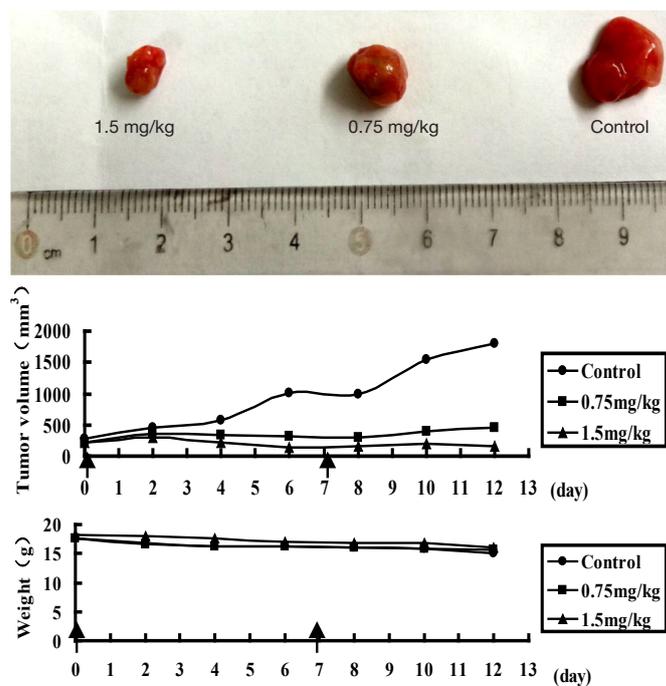


Figure 7 Tumor samples, mice weight and tumor volume change during FL118 administration. FL118 was treated through intratumoral injection and the days of FL118 administration are annotated by bold arrow symbols. Totally, FL118 treatment was lasted for 12 days. The institutional and national guide for the care and use of laboratory animals was followed.

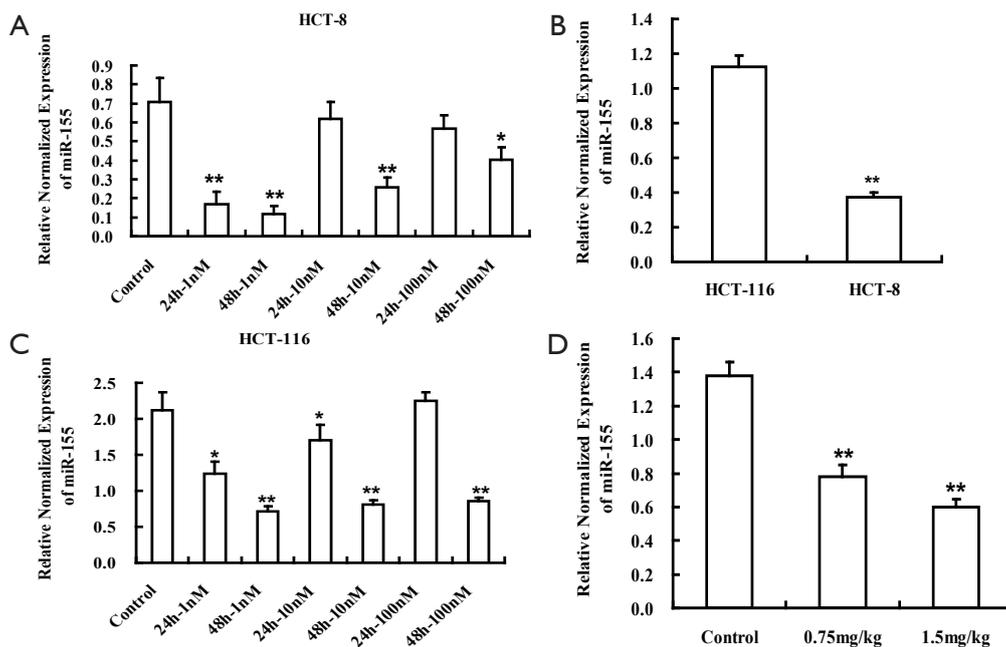


Figure 8 Relative normalized expression of miR-155 in cells and tumor samples after treated with FL118. (A-C) The expression of miR-155 is higher in HCT-116 cells than it in HCT-8 cells, but their levels were both decreased after being treated by FL118; (D) the downregulation of miR-155 in FL118-treated tumor samples compared to control group is shown. *, $P < 0.05$; **, $P < 0.01$. Error bars are the SEM. Each experiment was performed in triplicate ($n = 3$).

In addition, results from animal experiments also demonstrated that the expressions of miR-155 were significantly downregulated ($P < 0.01$) in FL118-treated groups compared to the control group (Figure 8D).

Conclusions

Previous studies have indicated that FL118 exhibits more effective ways to inhibit HCT-116 cancer cell growth and induce apoptosis when HCT-116 colon cancer cells lost the functional p53 (8). In these studies, we used HCT-116 cancer cells with an intact wild type p53. Therefore, we may get even better *in vitro* and *in vivo* results if we use the HCT-116 colon cancer cells without a wild type p53. Nevertheless, our work has clearly determined the multiple inhibition of FL118 on HCT-116 cells *in vitro* and *in vivo*. Furthermore, both *in vitro* and *in vivo* experiments have detected the significant downregulation of miR-155 after FL118 treated.

Surprisingly, it has been reported that miR-155 is closely associated with colon cancer pathogenesis (26), tumor growth (27), cell proliferation (28-30), chemoresistance (30), cell cycle arrest (31), migration and invasion (28,32-33), which resembled the anti-colon cancer activities of FL118. Taken together, it is reasonable to propose that this critical oncogenic miRNA, miR-155, is likely to participate in, and even mediate the anticancer activity of FL118 on colon carcinoma.

In short, our work highlighted a new insight to elucidate the action mechanism of an innovative anticancer Camptothecin analogue, FL118.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2017.06.20>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived. All animal experiments were performed according to IACUC-approved animal protocols.

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