FL118 Induces p53-Dependent Senescence in Colorectal Cancer Cells by Promoting Degradation of MdmX

Xiang Ling1,2, Chao Xu1, Chuandong Fan1, Kai Zhong1, Fengzhi Li1, and Xinjiang Wang1

Abstract

Anticancer agent FL118 was recently identified in screening of small-molecule inhibitors of human survivin expression. Although FL118 is a camptothecin analogue, its antitumor potency is much superior to other FDA-approved camptothecin analogues (irinotecan and topotecan). The mechanism of action (MOA) underlying the antitumor effects of FL118 remains to be fully elucidated. Here, we report that FL118 activates tumor suppressor p53 as a novel MOA in p53 wild-type cancer cells. Our studies show that this MOA involves an induction of proteasomal degradation of MdmX, a critical negative regulator of p53, in a manner largely independent of ATM-dependent DNA damage signaling pathway but dependent on E3-competent Mdm2. FL118 inhibits p53 polyubiquitination and monoubiquitination by Mdm2–MdmX E3 complex in cells and in cell-free systems. In contrast, FL118 stimulates Mdm2-mediated MdmX ubiquitination. Combinonoprecipitation revealed that FL118 slightly decreases Mdm2–p53 interactions and moderately increases Mdm2–MdmX interactions, suggesting a change of targeting specificity of Mdm2–MdmX E3 complex from p53 to MdmX, resulting in accelerated MdmX degradation. As a result, p53 ubiquitination by Mdm2–MdmX E3 complex is reduced, which in turn activates p53 signaling. Activation of the p53 pathway by FL118 induces p53-dependent senescence in colorectal cancer cells. However, in the absence of p53 or in the presence of MdmX overexpression, FL118 promotes p53-independent apoptosis. These two distinct cellular consequences collectively contribute to the potent effects of FL118 to inhibit clonogenic potential of colon cancer cells. This study identifies a potential application of FL118 as an MdmX inhibitor for targeted therapies. Cancer Res; 74(24); 7487–97. ©2014 AACR.

Introduction

Evasion of apoptosis is one of hallmarks of human cancer (1). Damaged or unwanted cells are normally eliminated by apoptosis via extrinsic (2) and intrinsic apoptotic pathways (3). Deregulation of apoptosis in cancer occurs frequently by overexpression of inhibitor of apoptosis (IAP) and Bcl-2 family antiapoptotic proteins. The IAP family proteins, such as survivin, cIAP, and XIAP, possess an evolutionarily conserved domain of baculovirus IAP repeats. The Bcl-2 family antiapoptotic proteins, such as Mcl-1, Bcl-2, and Bcl-XL, possess four conserved Bcl-2 homology (BH) domains. Expression of these proteins is upregulated by chromosomal translocation, transcriptional, or posttranscriptional mechanisms (4–6). These proteins antagonize the proapoptotic activities of Bax/Bak and BH3-only proteins such as Bim, Puma, and Noxa (7). In an effort of screening for inhibitors of survivin expression, we identified FL118 as a potent inhibitor of survivin expression (8).

Structurally, FL118 is a camptothecin analogue with structural features of the FDA-approved camptothecin analogues irinotecan and topotecan, which are used for colon cancer treatment. However, the mechanism of action (MOA) for FL118 is quite different from that of irinotecan and topotecan. First, topotecan or SN-38 (active metabolite of irinotecan) are well-established topoisomerase 1 (TOP1) inhibitors, but FL118 weakly inhibits TOP1-mediated in vitro DNA nicking (8). Second, TOP1 mutation confers significant resistance to camptothecin, topotecan, and SN-38 but only mild resistance to FL118 (9, 10). Third, FL118 selectively inhibits expression of survivin, XIAP, cIAP2, and/or Mcl-1, but SN-38 and topotecan are 10 to 100 times less effective in the inhibition of these genes (X. Liu and F. Li, unpublished data; ref. 8). Nevertheless, FL118 exhibited superior antitumor effects in mouse models of human colon and head-and-neck cancer xenografts, when compared with topotecan and irinotecan (8). FL118 caused complete tumor regression of xenografted head-and-neck and colon tumors when administered either via intraperitoneal injection (8) or via an intravenous injection in different formulations (11). The superior antitumor effects of FL118 suggest that novel MOAs in addition to TOP1 inhibition underlie the potent antitumor effects of FL118. TP53 is the most frequently mutated tumor suppressor gene in human cancer (12). In colon cancer, KRAS, APC, and p53 mutations are the most frequent cancer driver mutations with p53 loss often occurs in the late stage of cancer progression (13, 14). Many chemotherapeutics including camptothecin activate the p53 pathway (15, 16). p53 activation leads to growth arrest,
senescence, or apoptosis (12), via induction of p53 target genes such as p21 for growth arrest (17) and/or senescence (18, 19), or Puma and Noxa for apoptosis (20–22), p53-dependent apoptosis and senescence prevent lymphomagenesis and determine lymphoma treatment outcomes in mouse models (23–27). However, studies with colon cancer cell lines indicate that p21 induction actually promotes colon cancer cells from p53-dependent apoptosis (28). How exactly p53 contributes to the therapeutic effects of colon cancer therapies is not fully addressed. Stress signaling activates p53 via disruption of p53/Mdm2 feedback loop (29–35). We recently reported that MdmX and Mdm2 form a polyubiquitination E3 ligase for p53 ubiquitin-dependent degradation (36) and MdmX stimulates Mdm2-mediated p53 multiple monoubiquitination (36–38). Therefore, Mdm2–MdmX complex is the key regulator of p53 protein stability and involved in p53 activation (39). In this report, we describe that FL118 induces Mdm2 degradation, leading to p53-dependent senescence in colon cancer cells. This novel MOA for FL118 identifies that MdmX is a FL118 target that contributes to FL118-induced inhibition of clonogenic growth of colon cancer cells.

Materials and Methods

Cell culture, chemicals, and treatment

HCT116, HCT116-p53−/−, HCT116-p21−/− cells were originally provided to Dr. Terry Beerman by Prof. B. Vogelstein (Johns Hopkins University, Baltimore, MD). These cells were received in 2004 and cultured in McCoy’s 5A containing 10% FBS in an atmosphere of 5% CO2. The p53/mdm2/mdmx triple knockout (TKO) mouse embryonic fibroblasts (MEF; ref. 40) were obtained from Gigi Lozano, MD Anderson Cancer Center, Houston, TX, in January, 2013. The genetic status of these cell lines was confirmed by Western blot analysis and the last testing was June, 2013. HCT-8 was used in our recent studies (8) and originally purchased from the ATCC. TKO and HCT-8 lines were maintained in DMEM supplemented with 10% FCS (Atlanta Biologicals, Inc.) and antibiotics. Transfection was carried out with Lipofectamine 2000 (Invitrogen). Proteasome inhibitor MG-132 (Selleckchem; catalog no. S2619), a mixture of 7.1 and 13.1); anti-ubiquitin from BD (catalog number 550944), anti-HdmX (human MdmX) from Bethyl Laboratories Inc. (catalog number A300-287A) or Proteintech (catalog number 17914-1-AP); anti-p21 from Santa Cruz Biotechnology (sc-397, C-19); and anti-PUMA was from Enzo (ADI-905-237-100), respectively.

In vivo p53 ubiquitination

In vivo p53 ubiquitination assay was performed with HCT-8 cells. Briefly, whole cell lysates were denatured by 1% SDS followed by boiling for 5 minutes. After diluted 10 times with a buffer containing 20 mmol/L Tris, pH 7.5, 0.5% NP40, and 120 mmol/L NaCl followed by centrifugation at 22,000 × g for 10 minutes, ubiquitinated proteins were pulled down with an anti-ubiquitin antibody coupled with Western blotting for p53. Alternatively, HCT116 and HCT116-p53−/− cells transfected with a His-ubiquitin plasmid were treated with or without 25 nmol/L MG132 in the presence or absence of 10 nmol/L FL118 for 8 hours followed by His-tag pulldown as described previously (41) followed by Western blotting for p53.

In vitro ubiquitination

In vitro assays for p53 ubiquitination by Mdm2–MdmX were performed as described previously (36). Briefly, reactions were carried out at 30°C for 1 hour in a volume of 20 μL containing 40 mmol/L Tris/HCl (pH 7.5), 2 mol/L DTT, 5 mmol/L MgCl2, 10 μmol/L of ubiquitin, 40 mmol/L E1, 350 mmol/L UbCH5c, 5 mmol/L ATP, 100 mmol/L p53, 200 mmol/L Mdm2, 200 mmol/L MdmX, and different concentrations of FL118 or vehicle solvent DMSO, followed by Western blotting of p53 with DO-1. Similar procedure was performed for MdmX ubiquitination except for direct Western blotting with FLAG antibody or immunoprecipitation with an ubiquitin antibody followed by Western blotting for FLAG-HdmX.

Immunoprecipitation

HCT8 cells were treated with or without 10 nmol/L FL118 in the presence of 10 μmol/L MG132 for 8 hours followed by lysis in immunoprecipitation buffer [20 mmol/L Tris-HCl, pH 7.5, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, 2 mmol/L EDTA, PMSF (50 μg/mL), and aprotinin (1 μg/mL)]. Total protein (500 μg) per sample was precleaned with 40 μL A-G beads (Santa Cruz Biotechnology) before immunoprecipitation with 2 μg control IgG (Santa Cruz Biotechnology) or MDM2 (ab-3) antibody (Calbiochem) at 4°C overnight. After incubation with 40 μL A-G beads in a cold room for 3 hours followed by 5 times washes with PBS containing 0.2% NP-40, proteins bound to A-G beads were released in 2 × SDS-PAGE sample buffer by boiling for 5 minutes and used for Western blotting with DO-1 for p53, 8C6 (Millipore) for MdmX, and ab-3 for Mdm2 (Calbiochem).

Clonogenic assay

HCT8 cells were infected with lentiviral particles expressing control shRNA or shRNA for p53 knockdown (pLKO.1 from Addgene) followed by selection with puromycin for one week at 5 μg/mL. The cells were plated at 200 cells/well in 6-well
plates for no-treatment group or 1,000 cells/well for FL118 treatment groups to have sufficient numbers of colonies in drug-treated groups for accurate colony counting. Cells were treated with 0.15 to 20 nmol/L FL118 for 3 days followed by two washes with PBS and replenished with drug-free complete medium. After two weeks of culture in an incubator at 37°C, 5% CO2, cells are fixed and stained with crystal violet solution. Colonies were defined as ≥50 cells/colony.

**Quantitative real-time PCR**

Total RNA was extracted from cells using TRI REAGENT RT (Molecular Research Center, Inc.). Total RNA (2 µg per sample) was converted to cDNA using anchored oligo (dT) primers (RevertAid First Strand cDNA Synthesis Kit; Thermo Scientific) following the manufacturer’s instructions. Individual reverse transcription (RT) reactions of 20 µL were then diluted to 200 µL with sterile H2O. Ten microliter of diluted RT reaction was used for real-time qPCR using the iTaq SYBR Green Supermix with ROX (Bio-Rad). The sequences of primers used in real-time qPCR reactions were as follows: 5'-GGGCCCTTGACCTAAAAATGGTTGCA-3' (HdmX forward) and 5'-TTATGCTATAAAA-ACTTAA-3' (HdmX reverse); 5'-CCTGTCACTGTCTTGAGGAAG-3' (PUMA forward) and 5'-TCACAGTCGCTCTCTAAACC-3' (PUMA reverse); 5'-GTTAGAAATCTGTCTACTGCT-3' (p21 forward) and 5'-GCGACGCTACTTCCATC-3' (p21 reverse); 5'-ATGCCGCGCTACCTTACATC-3' (GAPDH forward) and 5'-TCACAGTCGCTCTCTAAACC-3' (GAPDH reverse). GAPDH was used as an internal control using the Applied Biosystems (Catalog no. QIA117).

**Senescence-associated β-gal assay**

HCT8 cells were either treated with 10 nmol/L FL118 for 72 hours or left untreated, followed by additional 7-day culture. Then the cells were fixed and stained for senescence-associated β-galactosidase (SA-β-gal) activity with a commercial kit performed according to manufacturer’s instructions (Cambiochem; catalog no. QA117).

**Data analysis and quantification of protein levels**

Data plotting in relevant figures (Figs. 1A and B, 2D, 4, 5D, and 6C) was made using Microsoft Excel. The values of each bar are presented as mean ± SD from at least three independent assays. Statistical significance among the mean values was analyzed using a Student t test. The significance (P value) was set at the nominal level of 0.05 or less. The asterisk in Fig. 1A and B represents the P value is less than 0.05. Proteins band intensities were quantified using IMAGEJ software. The relative protein levels were normalized against loading controls. In case of the communoprecipitation, immunoprecipitation efficiency and inputs were both taken into consideration to determine the relative communoprecipitation efficiency.

**Results**

**Anticancer drug FL118 activates the p53 signaling pathway**

To determine whether the p53 pathway is involved in the antitumor activity of FL118, we examined the effects of FL118 on cell growth and death with HCT116 and HCT116-p53−/− colorectal cancer cells in parallel. To our surprise, we found that FL118 induced 10% to 20% more growth inhibition across a wide range of concentrations (Fig. 1A) and approximately 2-fold more cell death (Fig. 1B) in HCT116-p53−/− cells than in wild-type (WT) p53−/− bearing parental HCT116 cells. Although this is consistent with our previous report that FL118...
antitumor effect is p53 independent (8), this observation suggests that p53 status indeed contributes to FL118 drug response. We then examined whether FL118 indeed activates the p53 pathway after FL118 treatment. As shown in Fig. 1C and D, p53 accumulation was induced by as low as 10 nmol/L FL118 treatment in WT p53–bearing HCT116 and HCT8 cells. Time course experiment with HCT8 cells indicated that FL118 induced evident p53 accumulation as short as 4 hours after treatment and reaching a peak after 24-hour treatment and sustained at least for 48 hours (Fig. 1D, left). Consistent with both p21 and PUMA being transcriptionally upregulated by p53 activation, p21 and PUMA were induced in these WT p53 cell
lines by FL118 but not in p53-null HCT116 (Fig. 1C and D). Interestingly, although p21 is induced in a FL118 concentration- and time-dependent manner, PUMA is induced by FL118 in a time-dependent manner but better induced by low concentration (10 nmol/L) than high concentration (100 nmol/L) of FL118.

To identify the mechanisms underlying p53 activation by FL118, we asked whether FL118 inhibits p53 ubiquitination since p53 is degraded by ubiquitin proteasomal pathway (36). As shown in Fig. 2A, FL118 treatment for 8 hours in the presence of proteasome inhibitor MG132 (to block proteasomal degradation of ubiquitinated proteins) significantly reduced polyubiquitination of endogenous p53 in HCT8 cells (Fig. 2A, left, pub-p53), accompanied with evident reduction in multimonoubiquitinated p53 (Fig. 2A, left, mub-p53). Equal immunoprecipitation efficiency was confirmed among the samples by reprobing polyubiquitin (Fig. 2A, right, low part, polyub), and equal amounts of p53 protein inputs after MG132 treatment were also confirmed (Fig. 2A, right, top part). To confirm whether p53 multimonoubiquitination is also significantly affected by FL118 treatment, a plasmid expressing His-tagged ubiquitin was transfected into HCT116 and HCT116-p53−/− cells followed by His-tag pulldown experiment under denaturing conditions in an in vivo p53 ubiquitination assay. Our results indicated that 10 nmol/L FL118 treatment for 8 hours significantly inhibited multimonoubiquitination of p53 detected either by direct p53 blotting (Fig. 2B, top) or by His-ubiquitin pulldown experiment (Fig. 2B, bottom). These results allow us to conclude that FL118 treatment inhibits p53 ubiquitination.

**FL118 downregulates MdmX, which involves MdmX protein degradation**

MdmX stimulates Mdm2-mediated p53 ubiquitination (38) and is required for p53 polyubiquitination and degradation in our previous report (36). Therefore, we asked whether FL118 inhibits Mdm2–MdmX activity by first testing the effects of FL118 on Mdm2 and MdmX protein levels in cells. As shown in Fig. 2C, left plot, FL118 treatment induced expression of human Mdm2 (Hdm2) protein but inhibited expression of human MdmX (HdmX) protein after treatment at 10 nmol/L and 100 nmol/L for 24 hours. Results from time course experiment indicated that HdmX downregulation by FL118 is a rapid event occurring within as short as 4 hours after FL118 treatment (Fig. 2C, right), a time point coincident with evident p53 accumulation induced by FL118 (Fig. 1D). Consistent with Mdm2 being a downstream target gene of p53, Mdm2 induction became evident at 4 hours and was nearly peaked at 8 hours after FL118 treatment (Fig. 2C, right), which follows similar kinetics of p53 accumulation (Fig. 1D). Therefore, loss of MdmX is responsible for FL118 to inactivate Mdm2–MdmX E3 activity. Our results from real-time qPCR indicated that FL118 treatment for 8 hours did not significantly alter the mRNA levels of HdmX (Fig. 2D). In contrast, the treatment significantly increased the p21, Hdm2, and PUMA mRNA levels (Fig. 2D), indicating that downregulation of HdmX protein levels by FL118 is a posttranscriptional event and FL118-induced p53 is transcriptionally active. Importantly, the presence of MG132 rescued HdmX downregulation by FL118 (Fig. 2E), indicating that FL118 promotes a proteasomal degradation of HdmX. To determine whether FL118 treatment affects Hdm2–p53 and Hdm2–HdmX physical interaction, we performed communoprecipitation experiments after treating cells with 10 nmol/L FL118 in the presence of MG132 to block p53 and MdmX degradation. After normalizing against inputs and immunoprecipitation efficiency, our results indicated that Hdm2–p53 interaction has a slight decrease to 90% of nontreated control, whereas the Hdm2–HdmX interaction was moderately increased to 145% by FL118 treatment compared with nontreated control (Fig. 2F).

**FL118-induced MdmX protein degradation is independent of ATM, p53, and p21 status, but requires Hdm2**

To understand the role of several components of the p53 signaling pathway in accelerated HdmX protein degradation by FL118, we performed experiments using HCT116 cells with p53-null or p21-null status. Our results show that FL118-induced HdmX degradation was not affected by p53 or p21 status (Fig. 3A). Moreover, inhibition of ATM with KU55933 only slightly rescued the FL118-induced HdmX degradation or p53 accumulation (Fig. 3B, compare lane 2, 0.45-fold HdmX, with lane 3, 0.53-fold HdmX). In contrast, the ATM inhibitor KU55933 completely rescued HdmX downregulation by neocarzinostatin, a radiation-mimicking agent, indicating the ATM inhibitor worked (Fig. 3B, compare lane 4, 0.71-fold HdmX, with lane 5, 1.02-fold HdmX) and neocarzinostatin-induced HdmX downregulation is strictly ATM-dependent. These results indicate that FL118-induced HdmX degradation is largely independent of ATM-mediated DNA damage signaling pathway. However, a minor rescue of HdmX degradation by ATM inhibitor was observed and suggests that ATM makes a minor contribution to this process. HdmX degradation is mediated by Mdm2-dependent ubiquitination after DNA damage (42, 43). To understand whether FL118-induced HdmX degradation is also mediated by Hdm2, we performed experiments of siRNA knockdown of Hdm2 in HCT-8 cells. Our results indicated that knockdown of Hdm2 at least partially rescued FL118-induced HdmX degradation even though Hdm2 was not completely knocked down (Fig. 3C), suggesting that Hdm2 plays an important role in HdmX downregulation by FL118. To unambiguously address Hdm2 involvement, we used p53/mdm2/mdmx TKO MEFs, and found that Hdm2 is required for FL118-induced HdmX degradation in HdmX-cotransfected TKO cells, because the absence of Hdm2 totally abolished the effect of FL118 on HdmX degradation (Fig. 3D). To verify that Hdm2 is also required for FL118-induced HdmX degradation in colon cancer cells, we used HCT116 cells and explored the dominant negative effects of enzyme-dead Hdm2L468A mutant. Hdm2L468A is an E2-binding mutant but intact in RING–RING interaction with HdmX as demonstrated in our previous studies (36). Our results indicated that Hdm2L468A overexpression prevented HdmX degradation by FL118 treatment at multiple concentrations in HCT116 cells (Fig. 3E). These results allow us to conclude that FL118 induces Hdm2-dependent HdmX degradation.
FL118 inhibits p53 ubiquitination by Mdm2–MdmX but promotes Mdm2-mediated MdmX ubiquitination

To assess whether FL118 has direct effects on Mdm2–MdmX E3 complex and thus changes the biochemical property of the E3 complexes toward p53 ubiquitination, we performed in vitro p53 ubiquitination by Mdm2–MdmX using recombinant proteins in the presence or absence of FL118. This system allows us to exclude the effects of signaling events and other proteins on the Mdm2–MdmX E3 complex. We found that FL118 moderately inhibits p53 polyubiquitination by Mdm2–MdmX in vitro (Fig. 3F). In contrast, FL118 stimulated Hdm2-mediated HdmX ubiquitination in a concentration-dependent manner in both polyubiquitin pulldown experiments (Fig. 3G, left) and direct Western blotting of FLAG-HdmX (Fig. 3G, right). These results indicate that FL118 treatment switches substrate preference of the Mdm2–MdmX E3 complex from p53 to MdmX for ubiquitination that resulted in proteasomal degradation of MdmX and, consequently, leading to p53 accumulation.

MdmX levels modulate FL118 sensitivity of HCT116 cells

Our results indicate that p53-null status makes colon cancer cells more sensitive to FL118 (Fig. 1A and B). Because MdmX negatively regulates the p53 pathway, we hypothesized that overexpression of HdmX would nullify the p53 function, thus sensitizing colon cancer cells to FL118. To test this hypothesis, we performed experiments with overexpression of HdmX in
increased FL118-induced cell death (4.2%, 8.4%, and 8.8% increase for 50 nmol/L, 100 nmol/L, and 250 nmol/L of FL118, respectively, with statistical significance of \( P < 0.03 \); Fig. 4C). These results suggest that HdmX levels can modify FL118-induced cell killing via its effect on the p53 pathway.

**FL118-induced p53 activation leads to senescence response in WT p53–bearing colon cancer cells**

Previous studies reported that HdmX overexpression inhibits oncogene-induced senescence program (44). We asked whether HdmX elimination by FL118 induces p53-dependent senescence. We tested this idea in HCT8 cells with or without knockdown of p53 in a clonogenic assay for the effects of p53 status on long-term cell proliferation. Knockdown efficiency for p53 by shp53-lentiviral infection (shp53) was nearly complete as shown by Western blot analysis as compared with cells infected with empty lentiviral vector (shC; Fig. 5D, inset). To our surprise, knockdown of p53 alone did not alter the plating efficiency or clonogenic potential in nontreated HCT8 cells (Fig. 5A, compare shC with shp53). In contrast, FL118 treatment at very low doses (0.15 and 0.3 nmol/L) significantly reduced the numbers of colony-forming cells (colony is defined as >50 cells/colony after a 3-day FL118 treatment followed by a 14-day culture in drug-free medium; Fig. 5B, only 0.3 nmol/L results are shown). The antionclonogenic effect of FL118 at very low doses (<0.3 nmol/L) is independent of p53 status, because there was no significant difference in the colony numbers between control (shC) and p53 knockdown (shp53) groups (Fig. 5B and D). In contrast and impressively, FL118 at doses of \( \geq 10 \) nmol/L (10, 20, and 100 nmol/L) resulted in eradication of colonies (>50 cells per colony) in both shC and shp53 groups; however, the shC group left a lot of small colonies (<50 cells per colony; Fig. 5C). In other words, two distinct phenotypes were observed in this experiment: shp53 HCT8 cells are nearly clear of viable cells, whereas some shC HCT8 cells remained alive as tiny colonies (5 to 20 cells/colony; Fig. 5C, only 10 nmol/L results are shown). Under microscope, cells in normal colonies in nontreated HCT8 cells are round, stacking on each other, and densely stained with thousands of very small cells (Fig. 5A, nontreatment, right plot). In contrast, cells in the tiny colonies after 10 nmol/L FL118 treatment have morphology of enlarged flat cell, adherent to the surface of plate without stacking on each other and only lightly stained (Fig. 5C, FL118, 10 nmol/L, right plot). These morphologies suggest that these cells gradually lost their clonogenic potential during replication, thus are unable to form normal colonies, a phenotype reminiscent of p53-induced senescence. As expected, results from SA-β-gal activity staining indicated that 10 nmol/L FL118 treatment induced approximately 92% of SA-β-gal positivity in the surviving cells (Fig. 6A, right). To confirm that FL118 preferentially induces apoptosis in the absence of p53, we compared apoptotic cleavage of PARP between HCT116 and HCT116-p53–null cells after 48-hour FL118 treatment. Our results indicated that 10 nmol/L FL118 induced significant PARP cleavage in HCT116-p53–null cells but not in HCT116 cells (Fig. 6B, left). PARP cleavage in HCT116 cells was only detected with a high concentration of FL118 at 100 nmol/L. To gain insight into differential apoptotic response among WT p53 colorectal
cancer cell lines, we compared HCT8 with HCT116 cells. Our results indicated that HCT8 cells are more sensitive than HCT116 cells in FL118-induced PARP cleavage (Fig. 6B, right, compare 10 nmol/L FL118 treatment). Because p21 protects p53-dependent apoptosis (28), we tested whether absence of p21 will give a phenotype similar to that of HCT116-p53–null cells. Our results indicated that indeed, loss of p21 sensitizes HCT116 to FL118 for induction of sub-G1 population (Fig. 6C, top) and apoptotic cleavage of PARP (Fig. 6C, bottom), similar to HCT116-p53–null cells (Fig. 6B). These results indicate that the long-term effect of FL118 treatment triggers p53/p21-dependent senescence program in nonapoptotic cells, but it triggers apoptosis independent of p53, because the cells with senescent morphology disappeared in p53-null-HCT8 cells (Fig. 5C).

Discussion

FL118 structurally is an analogue of irinotecan (pro-drug of SN-38) and topotecan, two FDA-approved camptothecin analogues for cancer treatment in clinical practice. Evidence suggests that FL118 may have novel MOAs distinct from other camptothecin analogues. Camptothecin was reported to activate p53 with serine 20 phosphorylation, indicating ATM activation in colon cancer cells, including RKO cells (16, 45, 46). Interestingly, camptothecin treatment does not induce p53-dependent transactivation of Mdm2 gene (16), which is opposite to the effect of FL118-induced p53-dependent Mdm2 expression (Figs. 2C and 3A, C, and E). These observations suggest that FL118 activates p53 via alternative mechanisms different from that used by other camptothecin analogues. We demonstrated here that FL118-induced p53 activation is
largely ATM independent (Fig. 3B). This is distinct from irinotecan and topotecan, which induce ATM activation (47), and ATM is involved in repair of topotecan-induced double-strand breaks (48). FL118, rather, induces proteasomal degradation of HdmX as an underlying mechanism for p53 activation. These findings have two important implications. First, this further supports the notion that MdmX depletion is a critical mechanism to inactivate oncogenic Mdm2–MdmX E3 complex and accordingly activates p53 signaling in cells (39), because MdmX stimulates Mdm2-mediated p53 monoubiquitination and is required for p53 polyubiquitination (36, 38). Second, FL118 can be used as an MdmX-depleting agent in MdmX-targeting therapies for a subgroup of patients with cancer (i.e., a type of personalized medicine) such as in chronic lymphocytic leukemia (49) and melanomas (50), in which MdmX overexpression confers treatment resistance. Our studies provided evidence that FL118 inhibits growth of MdmX-overexpressing cells better than non–MdmX-overexpressing cells (Fig. 4), probably because it nullifies p53-dependent senescence and the cells switch to apoptotic response to FL118. Currently, how FL118 changes the biochemical properties of Mdm2–MdmX E3 complex remains an open question, even though it can be partially explained by FL118 slightly decreasing Hdm2–p53 interaction and moderately increasing Hdm2–HdmX interaction (Fig. 2F). It remains to be determined whether FL118 directly binds to Mdm2 or MdmX or to E2 enzymes that alter the ubiquitination reaction, or FL118 binds to other Mdm2/MdmX complex–associated proteins for its effects in cells.

Another question is how FL118-induced activation of p53 signaling pathway contributes to the antitumor effects of FL118. Our studies in this report from multiple cancer cell lines of different p53 status demonstrated that FL118 induces p53-independent apoptosis and p53/p21-dependent senescence, both contribute to the inhibition of clonogenic growth of colon cancer cells by FL118 (Figs. 5 and 6). Importantly, without WT p53, FL118 more effectively induces cancer cell apoptosis. Given the fact that many types of later-stage cancers.

Figure 6. FL118 induces p53/p21-dependent senescence and p53/p21-independent apoptosis. A, FL118 effects on SA-β-gal positivity, HCT8 cells without treatment or treated with 10 nmol/L FL118 for 3 days followed by another 10-day culture in drug-free medium were stained for SA-β-gal activity. SA-β-gal positivity in nontreatment control and 10 nmol/L FL118-treated HCT8 cells are shown in the images and in percentages (under the images). B, Western blot analysis of the effects of FL118 in induction of apoptotic cleavage of PARP (cPARP) in the indicated cells after 48-hour FL118 treatment. C, effects of p21 on FL118-induced apoptosis. HCT116 and HCT116-p21−/− cells were treated with and without 10 and 100 nmol/L FL118 for 72 hours. Sub-G1 populations were analyzed by flow cytometry (top), and Western blot analysis of apoptotic cleavage of PARP in these cells are shown. D, a proposed model of antitumor mechanisms for FL118 in colon cancer cells (see text).
frequently lose functional p53, the double antitumor mechanisms of FL118 (p53-independent apoptosis and p53/p21-dependent senescence) support potential application of FL118 for both early- and later-stage cancers. In short, this newly identified MOA of FL118 constitutes a novel component of the FL118 antitumor mechanisms. Of note, regarding p53-dependent p21 induction in WT p53 cells, the conclusions drawn from this study are inconsistent with our previous report (8). We would like to clarify this here. This inconsistency was due to the use of different p21 promoter sequences in our previous reporter assays: In our previous report, we used p21 core promoter-driven (234 bp long from −208 to +26) luciferase reporter construct, which does not contain p53 binding sites (distal site: −2313 to −2212 as one p53 binding site and proximal site: −1452 to −1310 as the other site; ref. 9), whereas in this study, we used qPCR to directly measure the p21 transcripts. Because of the use of the p53-binding sites negative p21 core promoter as one of the four negative controls plus a poor p21 Western blot, the p53-dependent p21 induction by FL118 was overlooked in the previous report, in which the inhibitory effects of FL118 on antipoptotic gene promoters were a focus.

Another point we would like to mention here is that although we observed massive senescence in surviving WT p53 HCT8 cells after FL118 treatment (Fig. 6A, right), it is noteworthy that the extent of FL118-induced senescence and apoptosis programs may vary among WT p53–bearing colorectal cancer cell lines as shown by the difference of FL118-induced PARP cleavage between HCT8 and HCT116 cells (Fig. 6B, 10 nmol/L FL118). Nevertheless, our results suggest that both senescence and apoptosis contribute to elimination of clonogenic potentials of cancer cells (Fig. 5). As summarized in our proposed model (Fig. 6D), FL118 treatment of WT p53–bearing cancer cells induces p53-independent apoptosis by downregulating antipoptotic genes such as survivin, XIAP, cIAP2, and Mcl-1, and p53-dependent apoptosis via PUMA induction. However, the surviving cells also undergo p53/p21-dependent senescence, which inhibits the apoptotic pathway. This is consistent with a role of p53-dependent senescence in tumor suppression and treatment outcomes (23, 26). In contrast, FL118 treatment of p53-null colon cancer cells exclusively induces p53-independent apoptosis due to lack of choice for p53-dependent senescence. The collective effects of FL118 in induction of both apoptosis and senescence inhibit clonogenic potentials of both WT p53 and p53-null colon cancer cells. Moreover, when WT p53 function is inhibited by alternative mechanisms such as HdmX overexpression, FL118 appears to kill these cells better (Fig. 4). Therefore, FL118 exerts a targeted and better cytotoxicity in cancer cells with either p53 deletion or with WT p53 plus HdmX overexpression. Given the fact that normal cells possess WT p53 function, FL118-induced p53 activation might be one of the possible explanations for the favorable toxicity profile of FL118: highly toxic to cancer cells but low toxicity to the normal organs of animals, i.e., p53 activation in normal cells by FL118 may result in temporary G1 arrest that protects cells from FL118 insult, whereas p53 activation by FL118 in cancer cells results in senescence and antipoptotic cell death.

On the basis of the current studies of FL118, apoptosis induction by FL118 is largely mediated by its inhibition of antipoptotic protein expression, including survivin, Mcl-1, XIAP, and cIAP2 (8). The mechanism of this inhibition occurs at least partially at transcriptional level for survivin promoter, and likely so for Mcl-1, XIAP, and cIAP2 promoters as well, because the 2-kb promoter region of these genes upstream of the transcriptional start site(s) shows similar transcription factor–binding patterns but highly different from the p21 core promoter and the dhfr (dihydrofolate reductase) promoter (9). Certainly, FL118 may also suppress the expression of antipoptotic proteins via posttranscriptional mechanisms, which is one of the research areas under investigation. Inhibition of antipoptotic proteins empowers FL118 to eliminate the antiapoptotic activities resulted from upregulation of these proteins, a common event in many types of cancer (5). As a result, this may partially explain the p53-independent apoptosis induction by FL118 in general (8).

In conclusion, our studies demonstrated that FL118 is capable of activating p53-dependent senescence program via a unique MOA in WT p53 cancer cells that bypassed apoptosis. Together with our previous studies, FL118 inhibits multiple oncofactors including antipoptotic proteins and MdmX for cancer cell elimination. These findings extend the uniqueness of FL118 as a superior anticancer drug and support its further development toward clinical application.

Disclosure of Potential Conflicts of Interest
X. Ling and X. Wang have ownership interest (including patents) in Canget BioTekpharma LLC. F. Li is CSO and a consultant/advisory board member for Canget BioTekpharma LLC. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: X. Ling, F. Li, X. Wang
Development of methodology: X. Ling, C. Xu, C. Fan, X. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Ling, C. Xu, C. Fan, K. Zhong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Ling, C. Xu, X. Wang
Writing, review, and/or revision of the manuscript: F. Li, X. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Fan, X. Wang
Study supervision: F. Li, X. Wang
Others (performed experiments): C. Fan

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FL118 Induces p53-Dependent Senescence in Colorectal Cancer Cells by Promoting Degradation of MdmX

Xiang Ling, Chao Xu, Chuandong Fan, et al.


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