



## ARTICLE

# Wu-5, a novel USP10 inhibitor, enhances crenolanib-induced FLT3-ITD-positive AML cell death via inhibiting FLT3 and AMPK pathways

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The kinase FLT3 internal tandem duplication (FLT3-ITD) is related to poor clinical outcomes of acute myeloid leukemia (AML). FLT3 inhibitors have provided novel strategies for the treatment of FLT3-ITD-positive AML. But they are limited by rapid development of acquired resistance and refractory in monotherapy. Recent evidence shows that inducing the degradation of FLT3-mutated protein is an attractive strategy for the treatment of FLT3-ITD-positive AML, especially those with FLT3 inhibitor resistance. In this study we identified Wu-5 as a novel USP10 inhibitor inducing the degradation of FLT3-mutated protein. We showed that Wu-5 selectively inhibited the viability of FLT3 inhibitor-sensitive (MV4-11, Molm13) and -resistant (MV4-11R) FLT3-ITD-positive AML cells with IC<sub>50</sub> of 3.794, 5.056, and 8.386 μM, respectively. Wu-5 (1–10 μM) dose-dependently induced apoptosis of MV4-11, Molm13, and MV4-11R cells through the proteasome-mediated degradation of FLT3-ITD. We further demonstrated that Wu-5 directly interacted with and inactivated USP10, the deubiquitinase for FLT3-ITD in vitro (IC<sub>50</sub> value = 8.3 μM) and in FLT3-ITD-positive AML cells. Overexpression of USP10 abrogated Wu-5-induced FLT3-ITD degradation and cell death. Also, the combined treatment of Wu-5 and crenolanib produced synergistic cell death in FLT3-ITD-positive cells via the reduction of both FLT3 and AMPKα proteins. In support of this, AMPKα inhibitor compound C synergistically enhanced the anti-leukemia effect of crenolanib, while AMPKα activator metformin inhibited the anti-leukemia effect of crenolanib. In summary, we demonstrate that Wu-5, a novel USP10 inhibitor, can overcome FLT3 inhibitor resistance and synergistically enhance the anti-AML effect of crenolanib through targeting FLT3 and AMPKα pathway.

**Keywords:** AML; Wu-5; crenolanib; USP10; FLT3-ITD; AMPKα; Compound C; metformin

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## INTRODUCTION

Fms-related tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase expressed on hematopoietic cells. FLT3 plays a critical role in both normal and malignant hematopoiesis [1, 2]. Activating mutations of FLT3 can be found in ~30% of acute myeloid leukemia (AML) cases [3]. There are two major FLT3 activating mutations: the internal tandem duplication (ITD) in the juxtamembrane domain of the tyrosine kinase and point mutations in the tyrosine kinase domain, typically at the D835 residue. FLT3-ITD is the most common form of FLT3 mutation, with an incidence rate of 15%–35% of patients with AML [4, 5]. This mutation causes the constitutive activation of FLT3 and therefore activates multiple intracellular signaling molecules, such as PI3K/AKT and MAPK/ERK, which in turn promote the proliferation and inhibit the apoptosis of leukemia cells [6–8]. Several studies have demonstrated that patients with FLT3-ITD exhibit a high relapse rate, poor clinical prognosis, and lower overall survival [3, 9, 10]. FLT3-ITD is a valid

target for the treatment of FLT3-ITD-positive AML. The first generation of FLT3 inhibitors, such as midostaurin and sorafenib, are relatively nonspecific for FLT3 and usually inhibit a variety of kinases, such as KIT and PDGFR. Off-target inhibition may be associated with increased toxicity and modest clinical benefit. Second-generation inhibitors, such as crenolanib and quizartinib, are more selective for FLT3 than are first-generation inhibitors [11, 12]; however, they are limited by the rapid development of acquired resistance and cancer that is refractory to monotherapy [13–15]. Therefore, novel strategies are required to improve the efficacy of FLT3 inhibitors.

In addition to directly inhibiting the kinase activity of FLT3, the induction of FLT3-ITD degradation is emerging as a novel strategy to target FLT3-ITD. FLT3 can be degraded by the ubiquitin-proteasome or lysosome pathway. C-Cbl is an E3 ubiquitin ligase that ubiquitinates and downregulates FLT3 [16]. However, the heterozygous deletion of c-Cbl in some FLT3-mutated cells, such

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as Molm13 cells [17], results in the stabilization of FLT3-ITD [18, 19]. USP10 deubiquitinates and stabilizes FLT3-ITD but not wild-type FLT3. Inhibiting USP10 causes the degradation of mutant FLT3, thus overcoming drug resistance [20]. In addition, the USP9X inhibitor WP1130 has also been reported to induce the degradation of FLT3-ITD [21]. The combination of homoharringtonine and the heat shock protein 90 inhibitor IPI504 exerted antitumor effects by reducing total FLT3 protein levels via degradation [22]. Compared with traditional FLT3 inhibitors, reducing total FLT3 protein not only inhibits the activity of FLT3 but also eradicates its kinase-independent activity. Therefore, inducing the degradation of the FLT3-mutated protein is an attractive strategy for the treatment of FLT3-ITD-positive AML, especially for those showing FLT3 inhibitor resistance [23].

In this study, we demonstrate that Wu-5 selectively induces the apoptosis and overcomes FLT3 inhibitor resistance of FLT3-ITD-positive cell lines and primary FLT3-ITD-positive AML cells. We also demonstrate that Wu-5 is a novel USP10 inhibitor. The combination of Wu-5 with crenolanib has a synergistic effect on FLT3-ITD-positive cells because both the FLT3 and AMPK $\alpha$  pathways are inhibited.

## MATERIALS AND METHODS

### Compounds and antibodies

Wu-5 was identified from an in-house compound library. The following antibodies were used in this study: anti-Caspase-3 (Cell Signaling Technology, 9662); anti-PARP1 (Cell Signaling Technology, 9532); anti-FLT3 (Cell Signaling Technology, 3462S); anti-P-AKT (Cell Signaling Technology, 4060S); anti-P-ERK (Cell Signaling Technology, 4370S); anti-USP10 (Santa Cruz, sc-365828); anti-AMPK $\alpha$  (Cell Signaling Technology, 5832S); anti-USP5 (Cell Signaling Technology, 12577S); anti-c-myc (Cell Signaling Technology, 18583); anti- $\beta$ -catenin (Cell Signaling Technology, 8480S);  $\beta$ -Actin-HRP (conjugated) (Proteintech, HRP-60008); anti-rabbit IgG, HRP-linked (Cell Signaling Technology, 7074); and anti-mouse IgG, HRP-linked (Cell Signaling Technology, 7076).

### Cell culture

The HL60 (WT-FLT3), MV4-11 (FLT3-ITD), Molm13 (FLT3-ITD), and MV4-11-R (FLT3-ITD with crenolanib resistance) cell lines were cultured in Iscove's modified Dulbecco's medium containing 10% FBS. U937 (WT-FLT3) and FLT3-ITD transfected Ba/F3 cells were cultured in RPMI-1640 containing 10% FBS. Parent Ba/F3 cells were cultured in 90% RPMI-1640 medium containing 10% FBS and 10 ng/mL IL-3. All the culture media contained 100 U/mL penicillin and 100 g/mL streptomycin. The cells were cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub> and 95% air.

### Transfection

USP10 plasmids were purchased from Addgene (#22543). The FLT3-ITD plasmid was kindly provided by Professor Rui-bao Ren from Ruijin Hospital, Shanghai. PLKO-shUSP10-1 and PLKO-shUSP10-2 plasmids were obtained from the DNA library of Shanghai Jiao Tong University School of Medicine. The retroviral vector PBABE-Puro and packaging vectors Gag-Pol and VSVG and lentiviral packaging plasmids PXPAX2 and PMD2G were developed in our laboratory. The USP10 genes were cloned into a PBABE-Puro vector. USP10, FLT3-ITD, or PBABE-Puro was cotransfected with Gag-Pol and VSVG into HEK293T cells. PLKO-shUSP10-1, PLKO-shUSP10-2, or PLKO-Puro was cotransfected with GPXPAX2 and PMD2G into HEK293T cells. The viral supernatants were collected 36 h after transfection, and used with 5  $\mu$ g/mL polybrene to infect MV4-11 cells. After 48 h, stably transfected cells were selected with puromycin. The transfection efficiency was confirmed by Western blotting.

Establishment of the crenolanib-resistant MV4-11 cell line  
MV4-11 cells were seeded onto 12-well plates (10<sup>6</sup> cells/well) and treated with crenolanib at different concentrations (3–20 nM) for

48 h. The medium containing the drug was then removed, and then, the cells were incubated in drug-free medium for another 72 h. The cells resistant to high concentrations of crenolanib were collected and plated on a new 12-well plate and treated with higher concentrations of crenolanib for another 48 h. These steps were repeated until the cells were resistant to 100 nM crenolanib.

### Mononuclear cell separation

Normal human bone marrow samples were obtained from Shanghai General Hospital. FLT3-ITD-positive and FLT3-ITD-negative AML bone marrow samples were obtained from Shanghai Tongren Hospital. Bone marrow mononuclear cells were isolated as described previously [24].

### Western blotting

MV4-11, Molm13, and MV4-11-R cells were treated with Wu-5 for 24 h, and then, the cells were harvested and washed with ice-cold PBS. The cells were lysed using 2 $\times$  SDS (1 M Tris-HCl, pH 6.8; 0.08 mM DTT; 10% sodium deoxycholate; and 50% glycerol). Cell lysates were separated on 8%–12% SDS-PAGE gels, transferred to nitrocellulose (NC) membranes and probed with primary antibodies.

### Cellular thermal shift assay (CETSA)

To evaluate the dose effect of Wu-5 on the thermal stability of USP10, cell lysates were incubated with different concentrations of Wu-5 (0–200  $\mu$ M) at room temperature for 40 min, heated at 52 °C (Veriti Thermal Cycler, Applied Biosystems/Life Technologies) for 3 min, and cooled for 3 min at 25 °C. Finally, the lysates were centrifuged at 20,000  $\times$  *g* for 20 min at 4 °C. To determine the temperature effect on Wu-5 mediation of USP10 thermal stability, the cellular supernatants were divided into two aliquots. One aliquot was treated with DMSO, and the other aliquot was treated with Wu-5 (100  $\mu$ M). After 40 min of incubation at room temperature, the respective lysates were divided into separate aliquots (30  $\mu$ L) and heated individually at different temperatures for 3 min. The supernatants were transferred to a new microtube, lysed with 2 $\times$  SDS at 99 °C for 10 min and analyzed by Western blotting.

### Deubiquitinating enzyme (DUB) activity assay

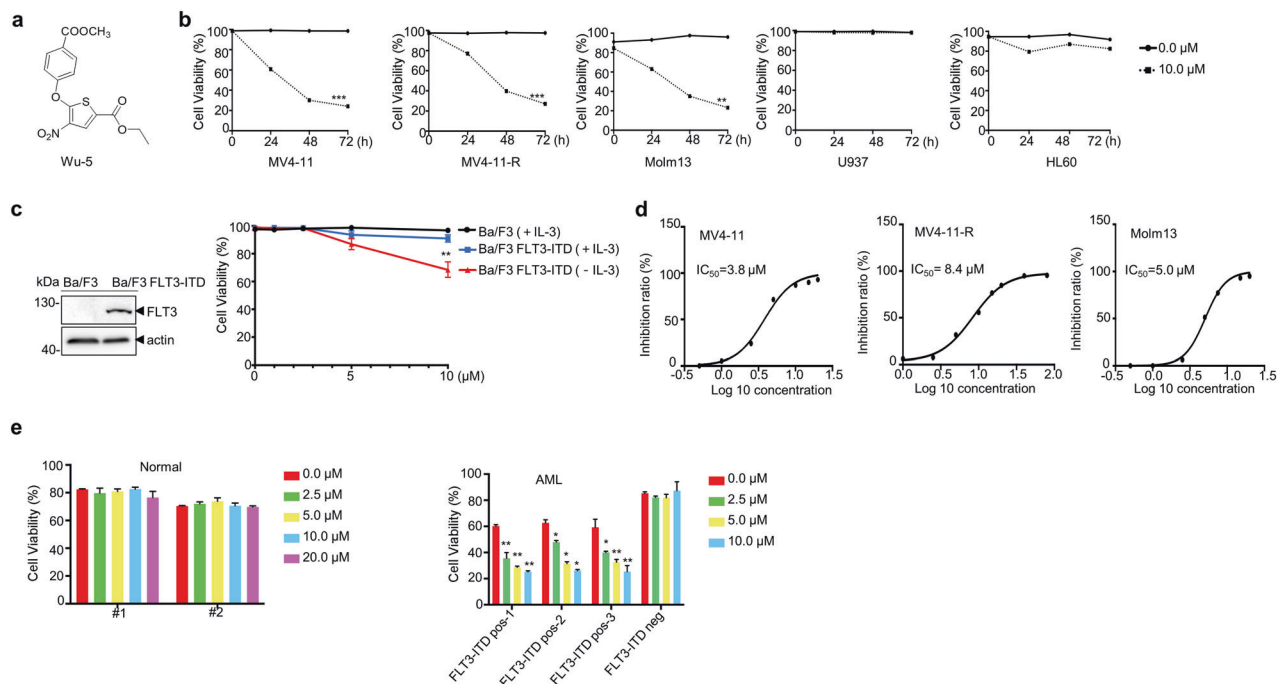
Recombinant human His-USP10 protein was purchased from Boston Biochem (01870119A). The GST-UbA52 and USP5 proteins were purified in our laboratory, and DUB activity was measured as described previously [25].

### DUB-labeling assay

HA-Ub-V5 was purchased from Boston Biochem (28333619 A). MV4-11 cells were treated with Wu-5 (20  $\mu$ M) for 4 h and washed twice with ice-cold PBS. The cell pellets were resuspended in 50  $\mu$ L of cold cell lysis buffer and lysed by sonication. The lysate was centrifuged at 20,000  $\times$  *g* for 15 min at 4 °C. Then, the protein concentration in the supernatant was determined by the BCA method. Fifty micrograms of the lysate protein was incubated with 2.5  $\mu$ M HA-Ub-V5 in a total volume of 50  $\mu$ L of DUB assay buffer at 37 °C for 1 h, boiled in reducing sample buffer, and resolved by SDS-PAGE. After the protein was transferred to an NC membrane, immunoblot analysis was performed with anti-HA (clone HA-7, Sigma-Aldrich), anti-USP10, or anti-USP5 antibodies.

### Apoptosis assays

MV4-11 cells were seeded onto 12-well plates (10<sup>6</sup> cells/well) and treated with Wu-5 for 24 or 48 h or in combination with crenolanib for 48 h. Then, 5  $\times$  10<sup>5</sup> cells were harvested and washed twice with cold PBS. The cells were washed with 1 $\times$  binding buffer and stained with 10  $\mu$ g/mL Annexin V-FITC for 15 min and 5  $\mu$ g/mL propidium iodide (PI) for 5 min at room temperature. The cells were washed with cold PBS for flow



**Fig. 1** Wu-5 selectively induces the death of FLT3-ITD-positive AML cells. **a** Chemical structure of Wu-5. **b** Viability of the U937, HL60, MV4-11, Molm13, and MV4-11-R cells treated with 10 μM Wu-5 for 24, 48, and 72 h, as determined by trypan blue exclusion assay. **c** Viability of the Ba/F3 FLT3-ITD-positive cells (supplemented with or without 10 ng/mL IL-3) treated with different concentrations of Wu-5. **d** Inhibition ratios of MV4-11, Molm13, and MV4-11-R cells treated with different concentrations of Wu-5 for 24 h, as measured by CCK-8 assay. The results are presented as the means ± S.D. ( $n = 3$ ). **e** Viability of mononuclear cells isolated from normal bone marrow ( $n = 2$ ), FLT3-ITD-positive AML patient blood ( $n = 3$ ), and FLT3-ITD-negative AML patient blood samples ( $n = 1$ ) treated with different concentrations of Wu-5 for 24 h was determined by trypan blue exclusion assay. \* $P < 0.05$ , \*\* $P < 0.01$  (Student's  $t$  test).

cytometric analysis. LYSIS II software (BD Biosciences) was used to analyze the flow cytometry data.

#### Quantitative real-time polymerase chain reaction

MV4-11 and Molm13 cells were treated with Wu-5 for 24 h, harvested and washed twice with PBS. Cellular mRNA was extracted with an EZ-press RNA purification kit (EZBioscience) and converted to cDNA with 4×EZscript Reverse Transcription Mix II (EZBioscience). Real-time PCR was carried out in a 96-well plate with TaqMan probes and a 7500 FAST real-time PCR system (Thermo Fisher). The ΔCT method was used to determine the expression levels of FLT3 relative to β-actin.

#### Cell viability assay

MV4-11, Molm13, and MV4-11-R cells were seeded onto 12-well plates ( $1 \times 10^6$  cells/well). The cells were treated with inhibitors for 24 h. DMSO was used as a vehicle control. Then, 10 μL of Cell Counting Kit-8 (CCK-8) reagent was added to each well, and the plates were incubated for 4–6 h (5% CO<sub>2</sub>, 37 °C). The absorbance was measured at 570 nm with a VICTOR3 1420 multilabel counter (PerkinElmer). Three wells were analyzed for each condition, and the wells containing only medium, CCK-8 agents or vehicle were used as controls. Inhibitory concentration 50% (IC<sub>50</sub>) values from the CCK-8 experiments were calculated with Graphpad PrismV7.04 software.

#### Synergy calculations

Synergy data were analyzed with online software (<https://synergyfinder.fimm.fi/synergy>). Combination indexes (CIs) were obtained. CI < 1 was considered to be synergistic, CI = 1 was considered to be additive, and CI > 1 was considered antagonistic.

#### Statistical analyses

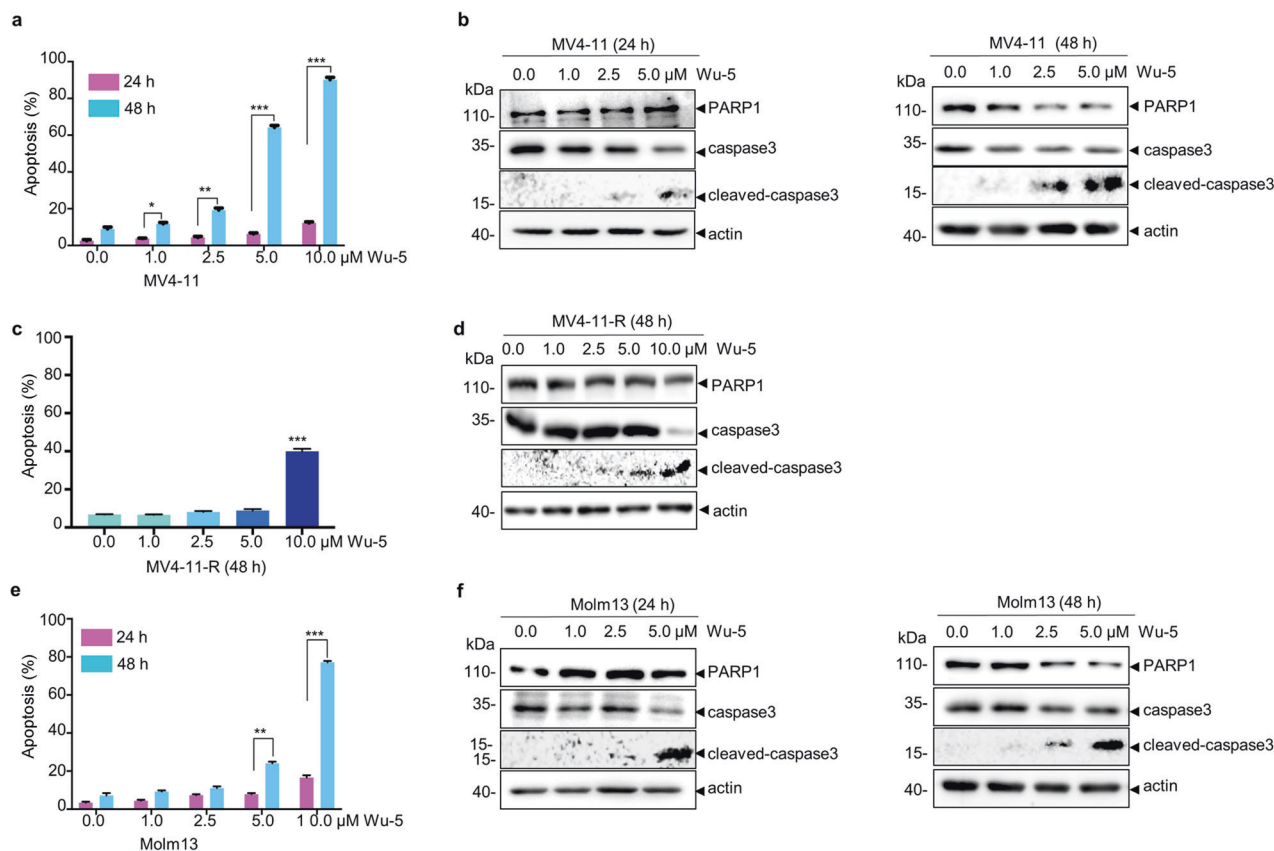
All experimental data are expressed as the means ± SD, and statistical analyses were performed with GraphPad PrismV7.04 software based

on Student's  $t$  tests ( $P < 0.05$ ). All experiments were repeated three times.

## RESULTS

### Wu-5 selectively induces the death of FLT3-ITD-positive AML cells

To identify compounds with anti-AML effects, we treated various cell lines with compounds from an in-house compound library. Interestingly, we found that compound Wu-5 (Fig. 1a) significantly inhibited the growth of the FLT3-ITD-positive cells, namely, MV4-11 and MV4-11-R (crenolanib-resistant) cells (Fig. S2), and Molm13 cells but had no or little effect on the proliferation of the FLT3-ITD-negative U937 and HL60 cells, as evaluated by trypan blue exclusion test (Fig. 1b). Consistent with this observation, we found that, after the introduction of the FLT3-ITD mutation, the FLT3-ITD-positive Ba/F3 cells (IL-3 independent, in the absence of IL-3) were more sensitive to Wu-5 than were the parent Ba/F3 cells (in the presence of IL-3), indicating that Wu-5 can selectively impair the function of FLT3-ITD. In addition, the presence of IL-3 partially blocked the Wu-5-induced loss of FLT3-ITD-positive Ba/F3 cell viability. Moreover, Wu-5 reduced the viability of the MV4-11, MV4-11-R, and Molm13 cells in a dose-dependent manner with IC<sub>50</sub> values of 3.794, 8.386, and 5.056 μM, respectively (Fig. 1d). To verify the selective effect of Wu-5 on FLT3-ITD-positive AML, we treated normal human BM mononuclear cells, three primary AML cell lines with FLT3-ITD-positive expression and 1 FLT3-ITD-negative AML primary cell line with Wu-5. Wu-5 potentially reduced the viability of the primary AML cells with FLT3-ITD-positive expression in a dose-dependent manner but did not affect normal human BM mononuclear cells or the FLT3-ITD-negative primary AML cells (Fig. 1e). These results indicate that Wu-5 have a selective killing effect on FLT3-ITD-positive AML cells, including those cells showing crenolanib resistance.



**Fig. 2** Wu-5 induces the apoptosis of FLT3-ITD-positive AML cells. **a, c, e** Flow cytometric analysis of the apoptosis ratios of the MV4-11, MV4-11-R, and Molm13 cells treated with different concentrations of Wu-5. **b, d, f** Western blot analysis of the expression of PARP1, caspase3, and cleaved-caspase3 in the MV4-11, MV4-11-R, and Molm13 cells treated with different concentrations of Wu-5.  $\beta$ -actin was used as the loading control. The results are presented as the means  $\pm$  S.D. ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (Student's  $t$  test).

### Wu-5 induces apoptosis of FLT3-ITD-positive AML cells

We evaluated the effect of Wu-5 on the apoptosis of leukemia cells. MV4-11, MV4-11-R, and Molm13 cells were treated with Wu-5 at different concentrations for 24 or 48 h. The results showed that Wu-5 induced the apoptosis of MV4-11 and Molm13 in a concentration- and time-dependent manner (Fig. 2a, c, e; Fig. S3), as demonstrated by the Annexin V/PI double-staining assay. To further confirm the apoptosis-inducing effect of Wu-5 on AML cells, we used Western blotting to examine the expression of apoptosis-related markers. Wu-5 treatment decreased the levels of PARP1 and caspase3 and increased cleaved-caspase3 expression in the MV4-11 (Fig. 2b), MV4-11-R cells (Fig. 2d), and Molm13 cells (Fig. 2f). These results indicate that Wu-5 induce apoptosis of FLT3-ITD-positive AML cells.

### Wu-5 reduces FLT3-ITD levels

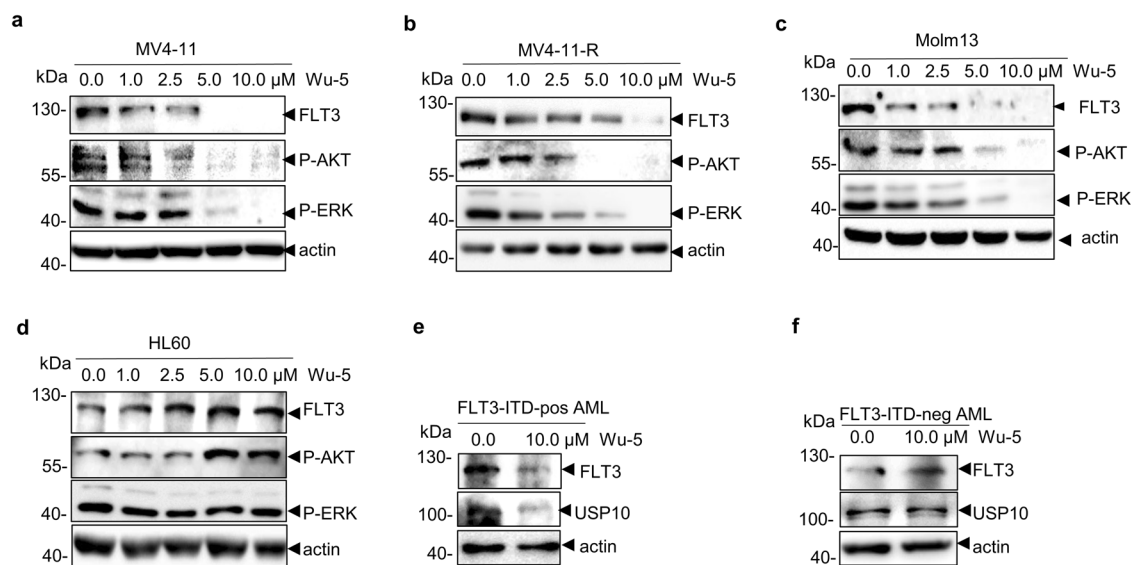
Based on the selective killing effect of Wu-5 on FLT3-ITD-positive AML cells, we assumed that it might affect the FLT3-ITD site and/or downstream signaling pathways. To prove our hypothesis, we treated MV4-11, MV4-11-R, Molm13, and HL60 cells with different concentrations of Wu-5 for 24 h and measured the levels of FLT3, P-AKT, and P-ERK by Western blot analysis. The results showed that Wu-5 decreased the level of FLT3 and simultaneously decreased the level of phosphorylation of AKT and ERK (P-AKT, P-ERK), the proteins downstream of FLT3 in the FLT3-ITD-positive cells (Fig. 3a–d). In addition, Wu-5 decreased the FLT3 protein in FLT3-ITD-positive but not in the FLT3-ITD-negative primary AML cells (Fig. 3e, f). These results indicate that Wu-5 inhibits the growth of crenolanib-sensitive and crenolanib-resistant FLT3-ITD-positive cells by decreasing the FLT3-ITD level and inactivating its downstream signaling pathways.

### Wu-5 induces proteasome degradation of FLT3-ITD

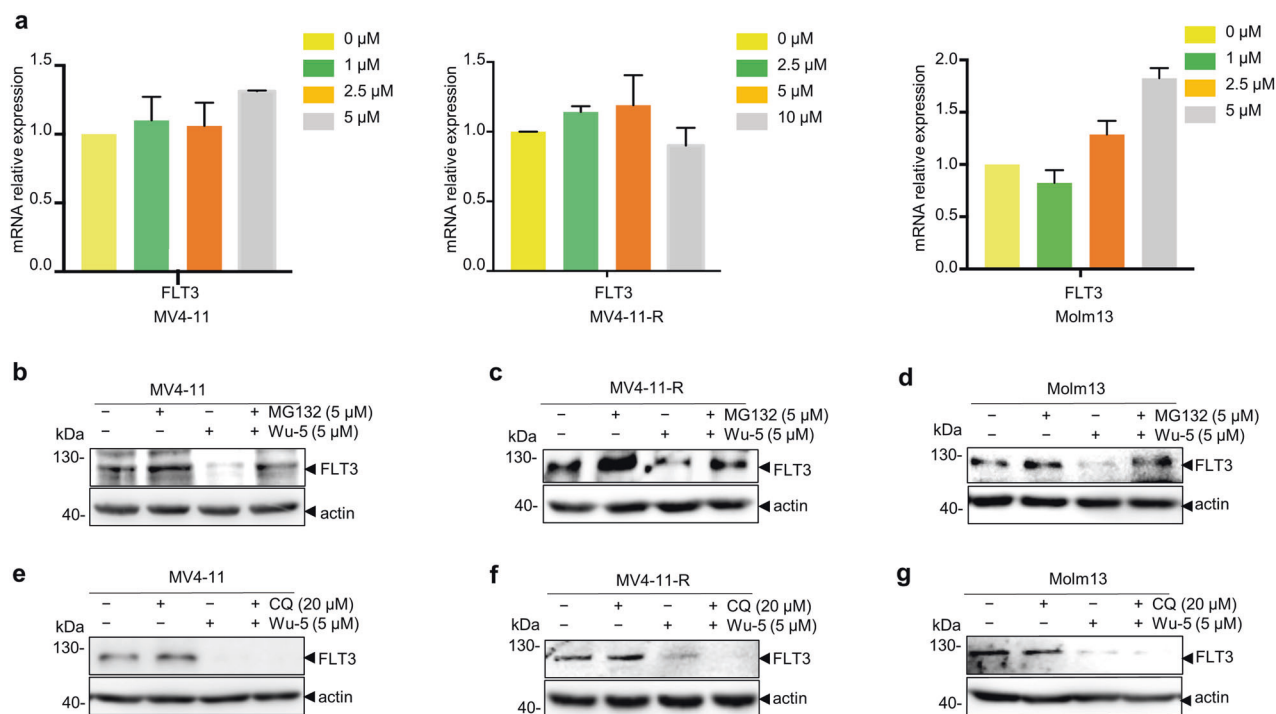
To investigate the underlying mechanism by which Wu-5 induced the downregulation of FLT3-ITD, we first examined its mRNA level by qPCR analysis. The results showed that Wu-5 did not reduce the mRNA level of FLT3-ITD (Fig. 4a). We then sought to determine whether the Wu-5-induced low expression of FLT3-ITD was caused by degradation. Interestingly, the proteasome inhibitor MG132 (5  $\mu$ M) (Fig. 4b–d) but not the lysosome inhibitor CQ (20  $\mu$ M) (Fig. 4e–g) partially rescued Wu-5-induced FLT3-ITD from degradation. These results suggest that Wu-5-induced FLT3-ITD degradation is mediated by the proteasome pathway.

### Wu-5 inhibits USP10 activity in vitro and interacts with USP10 in cells

The ubiquitination of FLT3-ITD is regulated by its ubiquitin E3-ligase c-Cbl and deubiquitinase USP10. However, c-Cbl is mutated in Molm13, which significantly prolongs the half-life of the FLT3-ITD in AML cells. Therefore, we hypothesized that Wu-5 might affect USP10. Using an in vitro gel-based assay, we found that Wu-5 inhibited the USP10-mediated cleavage of GST-Uba52 (Fig. 5a). The  $IC_{50}$  of Wu-5 on USP10 activity was 8.3  $\mu$ M (Fig. 5b). However, Wu-5 (50  $\mu$ M) did not affect the activity of USP5 (Fig. S4). Next, we performed CETSA to assess the binding of a drug to target proteins in cells and tissue samples [26, 27]. The results showed that, compared to the DMSO group, the incubation with Wu-5 reduced the thermal stability of USP10 in the cell lysates as the temperature increased, which was observed in both the MV4-11 cells (Fig. 5c, d) and Molm13 cells (Fig. 5e, f). Consistent with these results, Wu-5 also reduced the thermal stability of USP10 in a



**Fig. 3** Wu-5 reduces the protein level of FLT3-ITD. **a–d** Expression of FLT3, P-AKT, and P-ERK in the MV4-11, Molm13, MV4-11-R, and HL60 cells treated with different concentrations of Wu-5 for 24 h. **e, f** Expression of FLT3 and USP10 in the FLT3-ITD-positive and FLT3-ITD-negative primary AML cells treated with 10  $\mu$ M Wu-5.  $\beta$ -actin was used as the loading control.



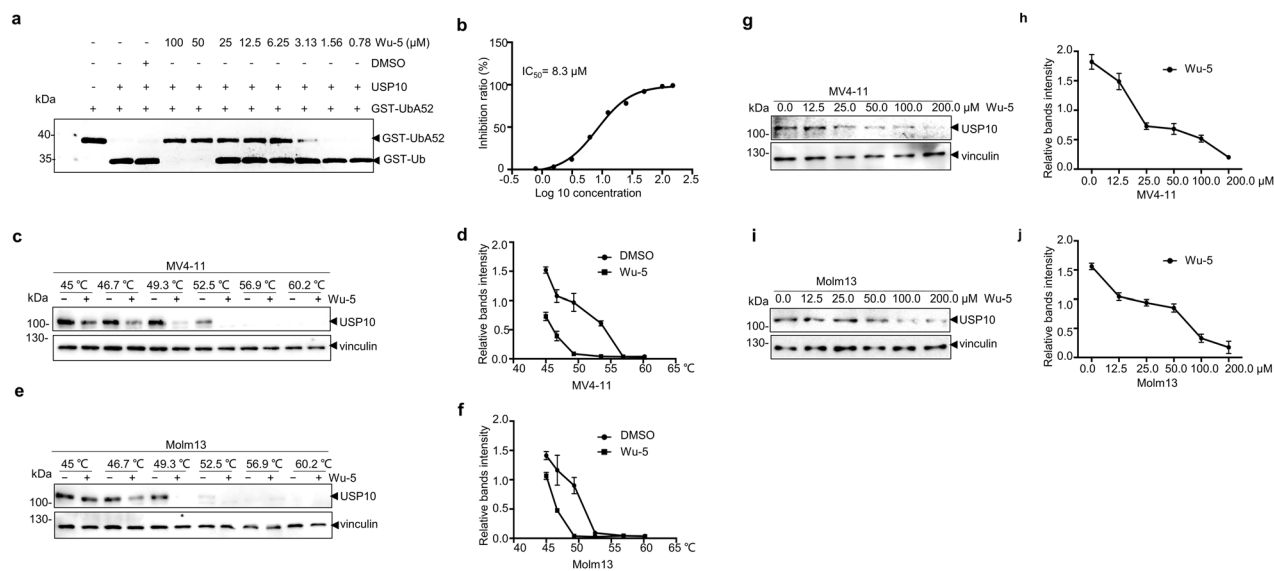
**Fig. 4** Wu-5 induces the proteasome degradation of FLT3-ITD. **a** Relative mRNA levels of FLT3 relative to that of  $\beta$ -actin in the MV4-11, Molm13, and MV4-11-R cells treated with different concentrations of Wu-5 for 24 h. **b–d** Expression of FLT3 in the MV4-11, MV4-11-R, and Molm13 cells treated with 5  $\mu$ M MG132 and 5  $\mu$ M Wu-5. **e–g** Expression of FLT3 in the MV4-11, MV4-11-R, and Molm13 cells treated with 20  $\mu$ M CQ and 5  $\mu$ M Wu-5.

concentration-dependent manner (Fig. 5g–j). These results suggest that Wu-5 directly interacts with USP10 in vitro and in cells.

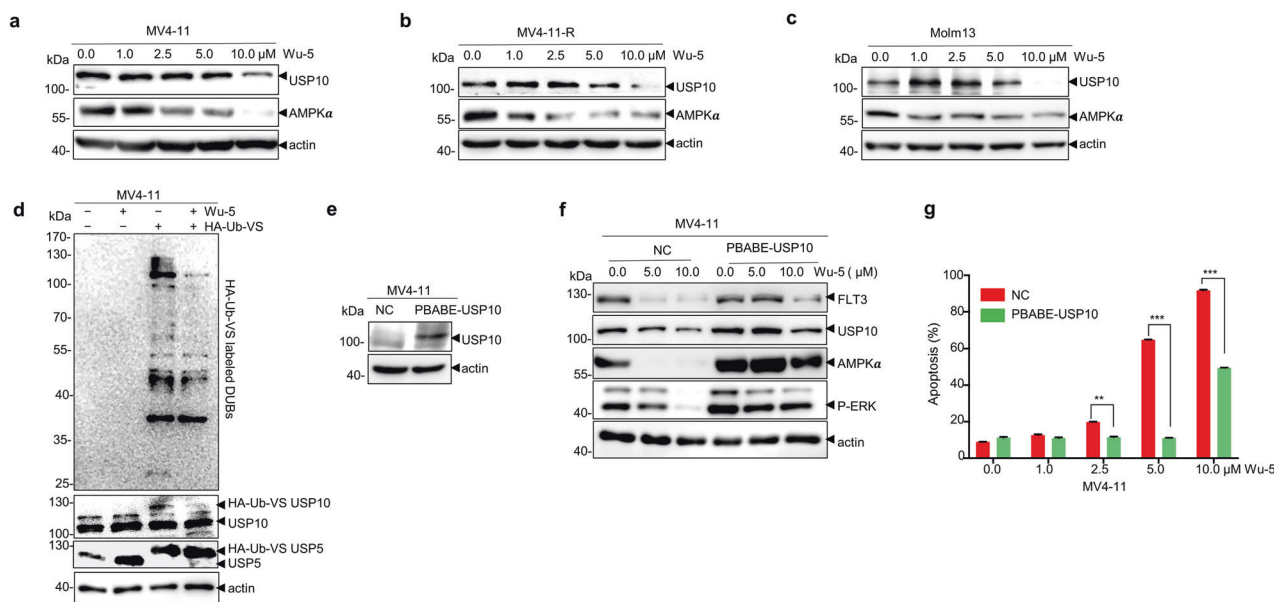
Inactivation of USP10 contributes to Wu-5-induced apoptosis  
We next evaluated the effect of Wu-5 on USP10 in FLT3-ITD-positive cell lines. The results show that the Wu-5 treatment significantly downregulated the protein level of AMPK $\alpha$ , a downstream substrate of USP10 [28]. A slight decrease in USP10 protein was also observed (Fig. 6a–c). These results suggest that

Wu-5 inhibit the activity of USP10 in cells. To evaluate the selectivity of Wu-5 on DUBs in cells, a DUB-labeling assay was performed. The results showed that Wu-5 inhibited the binding of HA-Ub-V5 to USP10 but not to USP5 (Fig. 6d).

To investigate the role of USP10 in Wu-5-induced death of FLT3-ITD-positive cells, USP10 was overexpressed in the MV4-11 cells (Fig. 6e). As expected, overexpression of USP10 abrogated the Wu-5-induced downregulation of the FLT3-ITD-positive protein and cell death (Fig. 6f, g and Fig. S5).



**Fig. 5** Wu-5 inhibits USP10 activity *in vitro* and interacts with USP10 in cells. **a** Coomassie blue staining of GST-UbaA52 and GST-Ub in the GST-UbaA52 samples incubated with different concentrations of Wu-5 and USP10 at 37 °C for 30 min, **b** statistical results. **c, e** CETSA on USP10 in the MV4-11 and Molm13 cells treated with different concentrations of Wu-5 and DMSO at different temperatures, **d, f** statistical results. **g, i** CETSA on USP10 in the MV4-11 and Molm13 cells treated with different concentrations of Wu-5, **h, j** statistical results.

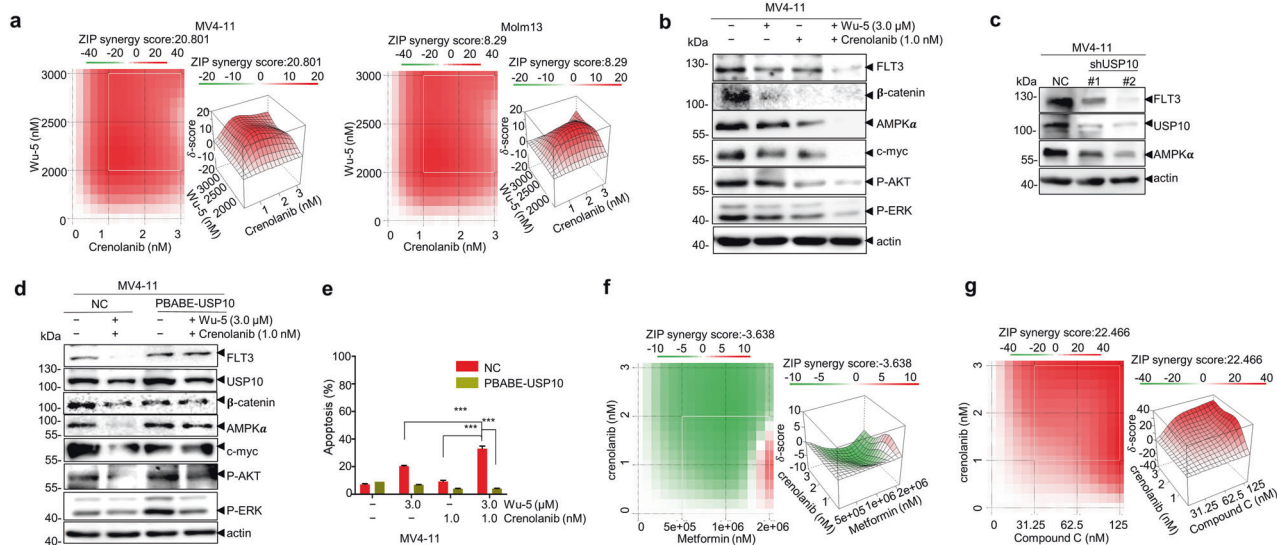


**Fig. 6** Inactivation of USP10 contributes to Wu-5-induced apoptosis. **a–c** Expression of USP10 and AMPK $\alpha$  in the MV4-11, MV4-11-R, and Molm13 cells treated with different concentrations of Wu-5 for 24 h. **d** HA-Ub-V5 labeling assays of USP10 and USP5 in the MV4-11 cells treated with/without Wu-5. **e** Expression of USP10 in the MV4-11 cells transfected with PBABE-USP10. **f** Expression of FLT3, USP10, AMPK $\alpha$ , and P-ERK in the control or USP10-overexpressing MV4-11 cells treated with different concentrations of Wu-5. **g** Flow cytometric analysis used to the apoptosis ratios of the control group and USP10-overexpressing MV4-11 cells treated with different concentrations of Wu-5. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Wu-5 synergistically enhances the crenolanib-induced death of FLT3-ITD-positive AML cells via the dual inhibition of AMPK $\alpha$  and FLT3-ITD.

Crenolanib is a novel FLT3-ITD inhibitor used for the treatment of AML and shows significant inhibition of the growth of FLT3-ITD cells. Its  $IC_{50}$  values are 5.94 nM for the MV4-11 cells and 3.54 nM for the Molm13 cells (Fig. S6a, b). To test whether Wu-5 can synergize with FLT3 inhibitors to suppress the proliferation of AML cells, we treated MV4-11 and Molm13 cells with Wu-5 in the presence or absence of crenolanib. The results showed that cotreatment of Wu-5 with crenolanib synergistically inhibit the proliferation of the MV4-11 and

Molm13 cells (Fig. 7a). To investigate the mechanism by which Wu-5 and crenolanib synergistically induced apoptosis, we examined the protein levels of FLT3-ITD, USP10 and their downstream substrates. Compared with the effect of the single treatment, cotreatment with Wu-5 and crenolanib reduced the protein levels of FLT3, P-AKT, P-ERK, AMPK $\alpha$ ,  $\beta$ -catenin, and c-myc (Fig. 7b). With an effect similar to that of the Wu-5 treatment, knocking down USP10 reduced the protein levels of FLT3-ITD and AMPK $\alpha$  (Fig. 7c). These observations suggest that Wu-5 and crenolanib synergistically inhibit the USP10 and FLT3 signaling pathways. To confirm the important role of USP10 inhibition in the combination effects, USP10-overexpressing



**Fig. 7** Wu-5 synergistically enhances crenolanib-induced death of the FLT3-ITD-positive AML cells via the dual inhibition of AMPK and FLT3-ITD. **a** The combination index (CI) of Wu-5 and crenolanib in the FLT3-ITD-expressing MV4-11 and Molm13 cells ( $n = 2$ ). Red indicates synergy, and green indicates antagonism. **b** Expression of FLT3,  $\beta$ -catenin, AMPK $\alpha$ , c-myc, P-AKT, and P-ERK in the MV4-11 cells treated with Wu-5 and/or crenolanib. **c** Expression of FLT3, USP10, and AMPK $\alpha$  in the shUSP10 MV4-11 cells. **d**, **e** Expression of FLT3, USP10,  $\beta$ -catenin, AMPK $\alpha$ , c-myc, P-AKT, and P-ERK in the control and PBABE-USP10 MV4-11 cells treated with Wu-5 and/or crenolanib, and apoptosis ratios were determined based on the flow cytometric analysis. **f** CI of metformin and crenolanib in MV4-11 cells. **g** CI of compound C and crenolanib in the MV4-11 cells. The results are presented as the means  $\pm$  SD ( $n = 3$ ). \*\*\* $P < 0.001$ .

MV4-11 cells were treated with Wu-5 plus crenolanib. Compared with the control group, overexpression of USP10 abrogated Wu-5 plus crenolanib-induced loss of FLT3 and AMPK $\alpha$  (Fig. 7d) and apoptosis of the MV4-11 cells (Fig. 7e, Fig. S7). Next, to investigate the therapeutic potential of the combined inhibitory effect on AMPK $\alpha$  and FLT3-ITD, we treated MV4-11 cells by inhibiting FLT3 in the presence or absence of an AMPK $\alpha$  activator and inhibitor. As expected, the AMPK $\alpha$  activator metformin inhibited (Fig. 7f, Fig. S6c), while the AMPK $\alpha$  inhibitor compound C synergistically enhanced the anti-leukemic cell effect of crenolanib (Fig. 7g, Fig. S6d). These results show that targeting USP10 synergistically enhance FLT3 inhibitor-induced apoptosis.

## DISCUSSION

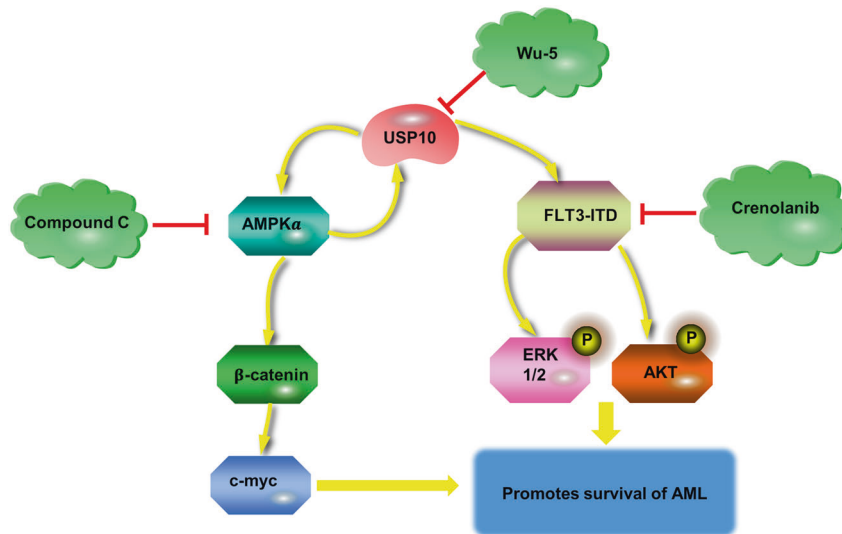
Several new FLT3 inhibitors have been developed to treat FLT3-mutated AML. However, as a monotherapy, each FLT3 inhibitor has been limited by its incomplete and transient clinical responses and acquired resistance [29]. In this study, we demonstrated that Wu-5, a novel USP10-targeting compound, can induce the degradation of FLT3-ITD and synergistically enhance crenolanib-induced apoptosis.

FLT3 mutations, such as FLT3-ITD and FLT3-D835, stimulate constitutive activation of the FLT3-expressing tyrosine kinase [30]. Correspondingly, most FLT3 inhibitors in clinical practice aim to inhibit FLT3 activity directly. However, frequent drug resistance to FLT3 inhibitors creates difficulty for the use of this treatment [31]. Rather than inhibiting the kinase activity of oncoproteins, a novel “degradation of oncoproteins” strategy represents a new possibility for anticancer therapy as it bypasses the drug resistance problem, especially those induced by various mutations. For example, quizartinib-based PROTAC has been shown to decrease the protein level of FLT3-ITD [23]. In the case of our study, the known E3 ligase for FLT3 is c-Cbl. However, mutations in c-Cbl have been found in AML patients, which makes the activation of c-Cbl inapplicable to AML patients. Our work demonstrates that complementing this E3-ligase-based strategy, the inhibition of DUBs promotes the degradation of FLT3-ITD in both crenolanib-sensitive and crenolanib-resistant cells. Specifically, we demonstrated that Wu-5,

a compound screened from our in-house compound library (Fig. S1), selectively inhibited the growth of FLT3-ITD-positive leukemia cells. Derivatives of Wu-5 have been reported to have potential therapeutic effects on human breast cancer [32, 33] and neuroprotective effects in Parkinson’s disease [34]; however, their direct targets remain unknown. Our results show that Wu-5 is a novel USP10 inhibitor, as evidenced by its inhibitory effect on purified USP10 but not USP5 in vitro and its ability to bind USP10 in cells. The selectivity of Wu-5 for USP10 was also confirmed by the DUB-labeling assay results.

Our results revealed USP10 as a novel target for the treatment of FLT3-ITD-positive leukemia cells. The role of USP10 in cancer is dependent on cell type. In liver and lung cancer, USP10 is a tumor suppressor, while in colon and prostate cancer and chronic myeloid leukemia, USP10 appears to be an oncogene [35–37]. However, the role of USP10 in leukemia has not yet been established. Our results support the use of USP10 as a novel target for the treatment of FLT3-ITD-positive leukemia cells from two respects: a, inhibiting USP10 by Wu-5 resulted in the degradation of FLT3-ITD, and b, overexpression of USP10 inhibited Wu-5 and crenolanib-induced cell death. Further analysis of USP10 in a large number of FLT3-mutated AML samples may address whether the expression level of USP10 has prognostic value for FLT3-mutated AML.

In our previous work, we demonstrated that the combination of arsenic trioxide and imatinib was effective in the treatment of chronic myeloid leukemia [38]. In this combination, imatinib inhibited the activity of BCR-ABL, and arsenic trioxide induced the degradation of BCR-ABL. Similar to this strategy, our present work and that of others demonstrated that the combination of an FLT3 inhibitor with an FLT3-degradation inducer was also effective [20]. Moreover, in addition to inhibiting the activation of FLT3 downstream substrates, this combination had a synergistic effect in reducing AMPK $\alpha$  levels. As has been reported previously that, under energy stress, USP10 removes ubiquitin from AMPK $\alpha$ , whereas AMPK $\alpha$  phosphorylates USP10 and activates USP10, forming a positive feedback loop [39]. Consistent with this finding, our results showed that Wu-5 inhibited USP10 and resulted in the reduction of AMPK $\alpha$ , which may further inhibited the activation of USP10. In addition, we present, for the first time, evidence that



**Fig. 8** Mode of action of Wu-5 and its combination effect with crenolanib in FLT3-ITD positive cells.

crenolanib also reduces the protein level of AMPK $\alpha$ . The combined effect of Wu-5 and crenolanib on AMPK $\alpha$  may contribute to the marked reduction in  $\beta$ -catenin and c-myc. Accordingly, we also demonstrated that the AMPK $\alpha$  inhibitor compound C and the activator metformin enhanced and inhibited the anti-leukemia activity of crenolanib, respectively. Taken together, our data lead us to propose that the highly synergistic effect of Wu-5 with crenolanib on FLT3-ITD-positive AML cells is attributed to their combined effect on FLT3-ITD and AMPK $\alpha$  (Fig. 8).

In summary, using Wu-5, identified as a novel USP10 inhibitor, we demonstrate that the combination of an FLT3 inhibitor and an FLT3-degradation inducer is a promising strategy to overcome FLT3 inhibitor resistance. Moreover, the combination of an AMPK $\alpha$  inhibitor with an FLT3 inhibitor deserves further investigation in the future.

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#### AUTHOR CONTRIBUTIONS

YLW and WL conceived and designed the study. MY and ZXF performed the experiments, collected data, conducted preliminary analysis of data and wrote the initial article. WWW, YZ, ZLB, ML, XHX, ZLZ, XMZ, YC, YYW, HL, HZX and YZW assisted in data analysis, manuscript writing and editing. All authors reviewed the manuscript.

#### ADDITIONAL INFORMATION

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**Competing interests:** The authors declare no competing interests.

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