The Deubiquitinating Enzyme USP1 is Auto-Ubiquitinated and Destabilized by ML323 in Colorectal Cancer Cells

Objective:
Our previous study indicated that USP1 inhibitor ML323 downregulated USP1 in colorectal cancer (CRC) cells, but the specific mechanism was still unknown.

Methods:
CRC cells were lysed for immunoblotting to detect protein expressions. Quantitative real-time PCR was performed to examine mRNA levels. Cycloheximide chase assays were carried out to evaluate the half-life of USP1. Co-immunoprecipitation was used to analyze the polyubiquitination of USP1.

Results:
USP1 protein stability was enhanced by the proteasome inhibitor MG132 in CRC cells. The wild-type USP1 was upregulated by MG132, but not its catalytic mutant. Additionally, the polyubiquitination of USP1 was enhanced by MG132 as well, which indicated USP1 was degraded through the ubiquitin-proteasome pathway. Meanwhile, we confirmed ML323 downregulated USP1 expression in CRC cells, and cycloheximide chase assay also revealed ML323 reduced USP1 protein stability. Further results showed ML323-induced USP1 downregulation and destabilization were abolished by MG132. Moreover, USP1 protein destabilization was not reversed by the caspase inhibitor Z-VAD, which further suggested ML323-induced USP1 downregulation was not dependent on the effects of cell death in CRC cells.

Conclusion:
Our results showed USP1 was auto-ubiquitinated, and ML323 destabilized USP1 through the ubiquitin-proteasome pathway in CRC cells, providing a theoretical basis for anti-CRC drugs' development targeting USP1.

Keywords: Colorectal cancer, USP1, ML323

Currently, colorectal cancer (CRC) is the fourth deadliest tumor in the world, with nearly 900,000 people dead from CRC every year, and the incidence rate of CRC is increasing with each passing year. Meanwhile, the overall incidence rate of CRC has declined in people over 50 years old, but has increased in people under 50 years old, which suggests that CRC is showing a younger trend. The clinical treatments of CRC include surgery, local ablation therapy, chemotherapy, targeted therapy and immunotherapy. Despite recent progress in CRC therapy, it remains challenging for patients with advanced and metastatic CRC, and patients still cannot be cured. Therefore, further elucidating the pathogenesis of CRC and discovering novel drug targets remain particularly urgent for CRC therapy.
Protein ubiquitination is involved in many cellular processes, and most polyubiquitinated proteins can be recognized and degraded by the 26S proteasome.[5] The process of ubiquitination is complex, and mediated by the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3.[6] The ubiquitin (Ub) is activated by E1 in an ATP-dependent manner, then the activated Ub is transferred to an E2, and finally Ub is transferred to a substrate with the help of an E3.[7] Ubiquitination is also reversible, and the peptidases named deubiquitinating enzymes (DUBs) can cleave Ub from substrates.[8] Under normal conditions, ubiquitination and deubiquitination are dynamically balanced in cells, and once broken, it can cause abnormalities in cellular function. The dysregulation of DUBs and once broken, it can cause abnormalities in cellular function and deubiquitination are dynamically balanced in cells, including tumorigenesis.[9] Therefore, the main purpose of this study is to further elucidate the degradation mechanism of USP1 and the downregulation mechanism of USP1 mediated by ML323.

Ubiquitin specific protease 1 (USP1) is a deubiquitinating enzyme whose expression is elevated in many tumors, and more and more evidences suggest that USP1 can serve as an effective anti-tumor drug target.[11] Surprisingly, USP1 was also screened out to be a functional DUB in CRC by our previous work.[12, 13] Several USP1 inhibitors have been discovered, and ML323 is a novel USP1 inhibitor that exerts anti-tumor activity in some tumors.[14] Our previous work also indicated that the USP1 inhibitor ML323 could sensitize CRC cells to DNA-targeting agents, but the expression levels of USP1 itself was downregulated by ML323, whose mechanism was unclear.[15] Therefore, the main purpose of this study is to further elucidate the degradation mechanism of USP1 and the downregulation mechanism of USP1 mediated by ML323.

Methods

Cell lines, Cell Culture and Chemicals
CRC cell lines, including HCT116, RKO, SW480 and SW620, were obtained from ATCC, Manassas, VA. All the cells were cultured in DMEM medium (HyClone) with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO2. MG132, ML323 and Z-VAD were purchased from Selleck, Shanghai, China. CHX was purchased from Sigma-Aldrich, USA.

Quantitative Real-Time PCR
The trizol reagent RNAiso Plus (Takara) was used to isolate the total RNA from CRC cells, and the PrimeScriptTM RT reagent Kit (Takara) was used to reverse-transcribe mRNA into cDNA according to the manufacturer’s instruction. Quantitative real-time PCR (qRT-PCR) analysis was carried out to detect the mRNA levels of genes with SYBR Green qPCR Master Mix (Clontech) according to the manufacturer’s instruction. The primer sequences used were as follows: USP1, forward, 5’-ATACTGAAGCTGAACGAAGTC-3’; reverse, 5’-GATCTTGGAAAGTCCACCAC-3’; GAPDH, forward, 5’-GTATCGTGGAGGACTCATGAC-3’; reverse, 5’-ATGC-CAGTGAGCTTCCGTTCAG-3’.

Immunoblotting
Cells were collected by centrifugation, and lysed with the RIPA lysis (Beyotime, Beijing, China). Total protein was extracted and prepared for immunoblotting as described previously.[15] The anti-USP1 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-Myc tag and anti-Flag tag antibodies were bought from Medical & Biological Laboratories, Tokyo, Japan. The anti-GAPDH antibody was purchased from Proteintech Group, Wuhan, China. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgGs were purchased from Beyotime, Beijing, China.

Cycloheximide (CHX) Chase Assay
CHX chase assay was carried out as described previously. In brief, pre-treated cells were incubated with 100 μg/ml CHX (Sigma-Aldrich) for indicated times, and then collected cells were prepared for immunoblotting analysis.

Co-Immunoprecipitation
To evaluate the ubiquitination of USP1, co-immunoprecipitation (Co-IP) was performed as described previously. Briefly, cells were lysed by using the IP lysis (Beyotime, Beijing, China), and total protein were incubated with indicated primary antibody overnight. On the next day, the samples were subsequently incubated with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 2 hours. Then, the samples were washed, and the agarose beads were collected, boiled and subjected to immunoblotting analysis.

Plasmids and Gene Transfection
The wild-type (WT) and mutated USP1 (C90S) overexpression plasmids were constructed as described previously.[16] Myc-Ub plasmid was purchased from Addgene. These plasmids were transfected into HCT116 cells by using the Lipofectamine®3000 reagent (Invitrogen) according to the manufacturer’s instruction.

Statistical Analysis
Graphpad Prism was used to draw the pictures. One-way ANOVA was used to compare the differences among multiple groups, and a p value less than 0.05 was considered to be statistically significant.
Results and Discussion

USP1 Can be Degraded through the Proteasome Pathway

To evaluate whether USP1 could be degraded into 26S proteasome, the proteasome inhibitor MG132 was used to incubate with CRC cells. As shown in Figure 1a & 1b, MG132 could up-regulate the protein expression of USP1 in CRC cells in a dose-dependent manner. However, the mRNA levels of USP1 were not significantly changed by the treatment of MG132 (Fig. 1c). To further verify this phenomenon, CHX chase assay was conducted in the presence or absence of MG132. As shown in Figure 1d & 1e, the half-life of USP1 protein could be markedly prolonged by the treatment of MG132 in CRC cells, which suggested that USP1 could be degraded into 26S proteasome.

Wild-type USP1 Can be Polyubiquitinated But not its Catalytic Mutant

Since Cys90 was the catalytic active site of the deubiquitinating enzyme USP1, we then evaluated whether the mutation of Cys90 would affect the proteasome degradation of USP1. As shown in Figure 2a & 2b, MG132 could enhance the protein levels of the wild-type USP1, but not the catalytic mutant C90S, which revealed that the auto-ubiquitination of USP1 may be mediated by its catalytic site. Moreover, Co-IP was also performed to evaluate the ubiquitination of USP1. As shown in Figure 2c, the polyubiquitination of the wild-type USP1 could be enhanced by the treatment of MG132, but not the mutant, which further indicated that the auto-ubiquitinatuation of USP1 was dependent on its catalytic site and the accumulation of USP1 in CRC patients may be caused by the genetic mutations.

ML323 Downregulates USP1 Expression in Colorectal Cancer Cells

Given the above results, we further elaborated on the question in the previous article that why ML323, the USP1 inhibitor, downregulated the expression of USP1 in CRC cells. As shown in Figure 3a & 3b, we firstly collected more cell lines to verify the previous results and confirmed that ML323 downregulated the protein expression of USP1 in CRC cells, including HCT116, RKO, SW480 and SW620. At the same time, we found that ML323 downregulated USP1 in a dose-dependent and time-dependent manner (Fig. 3c & 3d). Furthermore, to confirm whether ML323 regulated the

![Figure 1](image1.png)  
**Figure 1.** USP1 can be degraded through the proteasome pathway. (a, b) HCT116 cells were incubated with increasing doses of MG132 for four hours, followed by immunoblotting analysis (a), and the optical density was also analyzed (b). (c) Above cells were also prepared for qRT-PCR analysis to detect the mRNA levels of USP1. GAPDH was used as an internal control. (d, e) CHX chase assay was carried out to evaluate whether MG132 regulated the stability of USP1 (d), and optical density was also analyzed (e). *p<0.05, **p<0.01.
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protein stability of USP1, CHX chase assay was conducted in the presence or absence of ML323 in CRC cells. As shown in Figure 4a & 4b, ML323 could markedly shorten the half-life of USP1 protein in CRC cells. CHX chase assay also revealed the same conclusion that MG132 could obviously abolish ML323-induced protein destabilization of USP1 in CRC cells (Fig. 5c & 5d). To further confirm that the downregulation of USP1 protein mediated by ML323 was not caused by cell apoptosis, Caspase inhibitor Z-VAD was added into the cells. As shown in Figure 5e, Z-VAD could not affect the effects of ML323 on USP1 reduction in CRC cells. These results indicated that ML323 reduced the expression of USP1 through the proteasome pathway.

Figure 2. Wild-type USP1 can be polyubiquitinated but not its catalytic mutant. (a, b) The wild-type USP1 (Flag-USP1-WT) (a) and catalytic mutant of USP1 (Flag-USP1-C90S) (b) were respectively transfected into HCT116 cells. Twenty-four hours later, cells were incubated with 20 μM MG132 or DMSO for four hours, and then lysed for immunoblotting analysis. (c) Co-immunoprecipitation was carried out, and immunoblotting was performed to detect the poly-ubiquitination of wild type USP1 (USP1-WT) and mutated USP1 (USP1-C90S). IP, immunoprecipitation. IB, immunoblotting.

Figure 3. ML323 downregulates USP1 expression in colorectal cancer cells. (a) The chemical structure of ML323. (b) Four colorectal cancer cell lines were respectively incubated with 200 nM ML323 for 8 hours, followed by immunoblotting against USP1 and GAPDH. (c) HCT116 cells were incubated with indicated increasing concentrations of ML323 for 8 hours, and then cells were prepared for immunoblotting. (d) HCT116 cells were incubated with 200 nM ML323 for 0, 4, 8 or 16 hours, followed by immunoblotting analysis.

Figure 4. ML323 destabilizes USP1 in colorectal cancer cells. (a, b) CHX chase assay was carried out to evaluate whether ML323 regulated the stability of USP1 (a), and optical density was measured (b).

ML323 Downregulates USP1 Expression Through the Proteasome Pathway

Subsequently, the proteasome inhibitor MG132 was added into the ML323-treated CRC cells, and we found that ML323-induced USP1 downregulation could be significantly reversed by the treatment of MG132 (Fig. 5a & 5b), which suggested that ML323 induced USP1 protein to enter 26S proteasome for degradation. Meanwhile, CHX chase assay also revealed the same conclusion that MG132 could obviously abolish ML323-induced protein destabilization of USP1 in CRC cells (Fig. 5c & 5d). To further confirm that the downregulation of USP1 protein mediated by ML323 was not caused by cell apoptosis, Caspase inhibitor Z-VAD was added into the cells. As shown in Figure 5e, Z-VAD could not affect the effects of ML323 on USP1 reduction in CRC cells. These results indicated that ML323 reduced the expression of USP1 through the proteasome pathway.

To identify functional DUBs in CRC cells, a high-content screening based on the lentivirus-derived shRNAs targeting DUBs was carried out in our previous study, and several functional DUBs were screened out, including USP1.[13] Our further investigations showed that USP1 was elevated and predicted as a negative index for CRC patients.[12] And inhibiting USP1 by its inhibitor ML323 significantly sensitized CRC cells to the DNA-targeting agents.[12] These results further confirmed that USP1 was functional in CRC, and could be a potential target for anti-CRC drug discovery. Gradually, some USP1 inhibitors have been discovered, but they are still in early development.[17, 18] Therefore, further research on the molecular mechanism of the target and the characteristics of existing inhibitors will promote the successful clinical application of USP1 inhibitors in the future.

There are two main ways of protein degradation, one is the ubiquitin-proteasome pathway, and the other is the lysosomal pathway. Most of the proteins are degraded through the ubiquitin-proteasome pathway.[19] Although USP1 is a DUB that removes the Ubs from the substrates, itself is also a protein, which will definitely be degraded in the cellular processes.[11] In this study, we firstly found that USP1 could be polyubiquitinated and degraded into the 26S proteasome. Interestingly, the catalytic mutant of USP1 was also used for investigations, and we found that MG132 could not upregulate and enhance the polyubiquitination of the mutant, which suggested that the autoubiquitination of USP1 may be dependent on its catalytic site.
Proteolysis Targeting Chimeras (PROTACs) have been used to degrade disease-relevant targeted protein, which has been a novel strategy for the drug discovery. In general, proteins of interest are degraded into 26S proteasome by PROTACs through hijacking E3 ligases. In our current study, we noticed that ML323 could markedly downregulate the expression of USP1 in CRC cells, and our current study indicated that even low-dose ML323 could still downregulate the expression of USP1 in CRC cells, which attracted our attention. Therefore, several investigations were conducted and we found that ML323-induced USP1 downregulation was markedly inhibited by the proteasome inhibitor MG132. And further CHX chase assay showed that the half life of USP1 was significantly shortened by ML323 treatment, which could be also reversed by the treatment of the proteasome inhibitor MG132. We then propose a hypothesis that ML323 may act as a natural ‘PROTAC’ to promote USP1 degradation? This hypothesis requires further experiments to be proved, and we will demonstrate it in our future work.

In conclusion, this study elucidated the degradation mechanism of USP1, and clarified that ML323 downregulated the protein level of USP1 by inducing it go into the proteasome, which provides a theoretical reference for the further development of ML323 as an anticancer drug in clinic.

Disclosures
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References


