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Thymidine Kinase 1 Mediates the Synergistic Antitumor Activity of Ubenimex and Celecoxib via Regulation of Cell Cycle in Colorectal Cancer[S]

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ABSTRACT

Colorectal cancer (CRC) is a common clinical malignant tumor of the digestive system that seriously affects the health and life of patients. Because it is difficult to cure CRC, the strategy of drug combination is often used in clinical therapy. This study mainly revealed that ubenimex and/or celecoxib exerted anti–colon cancer effects in vitro and in vivo, and the efficacy was significantly enhanced when the two drugs were combined. The combination of the two drugs induced significantly stronger cell-cycle arrest than did the single drug, and also enhanced the antitumor efficacy of 5-fluorouracil and its derivatives. At the same time, the expression of thymidine kinase 1 (TK1) protein was decreased through regulating the level of TK1 mRNA treated with celecoxib and/or ubenimex, but the combination drugs exhibited much more reduction of TK1 mRNA and protein as compared with the single agent alone. TK1 may be the molecular target of the combination of two drugs to exert the anti–colorectal cancer effect. In summary, this research demonstrates that celecoxib combined with ubenimex inhibits the development of colorectal cancer in vitro and in vivo, making them a viable combination regimen.

SIGNIFICANCE STATEMENT

In this study, our data reveal the great potential of celecoxib combined with ubenimex in the treatment of colorectal cancer, providing new ideas for clinical antitumor drug regimens and theoretical reference for drug development.

Introduction

Colorectal cancer (CRC) accounts for 10% of all cancer types globally (Argilés et al., 2020). At present, the clinical treatment of colorectal cancer is mainly radical surgery, radiotherapy, and chemotherapy that can improve the symptoms of patients (Wu, 2018), but the 5-year survival rate is low. Drug combination is the main treatment for this kind of malignant tumor (Lu et al., 2017), so finding a new drug combination is still an effective strategy to treat colorectal cancer and prolong the lifespan of colorectal cancer patients. In our laboratory, the colorectal cancer cell line HCT116 was used for drug combination screening, and it was found that the combination of celecoxib and ubenimex exerted more potent growth inhibitory effect on the cells than did each single drug. Therefore, we will further study the antitumor effect and mechanism of the combination of the two compounds on colorectal cancer.

Celecoxib as a nonsteroidal anti-inflammatory drug was an approved drug for rheumatoid arthritis and osteoarthritis by the U.S. Food and Drug Administration (FDA) in 1998 (Isakson et al., 1998). In addition, it was approved as a chemopreventive drug by the FDA in 2004 to reduce the formation of polyps in familial adenomatous polyposis in clinic (Saxena et al., 2020). Celecoxib was conducted in comprehensive clinical trials for a therapeutic agent for various cancers including colorectal cancer (Arber et al., 2011; Saxena et al., 2020), either as a single drug or combined with other drugs. Although celecoxib is a potently selective cyclooxygenase 2 (COX-2) enzyme inhibitor, it seems to have antitumor effect in a COX-2–independent manner (Chen et al., 2007). Due to the lack of COX-2 expression in HCT116 cells (Semaan et al., 2016), the antitumor mechanism of celecoxib combined with ubenimex may not depend on COX-2 enzyme activity. Therefore, a special mechanism of action needs to be further explored.

Ubenimex, an approved drug for adjuvant therapy in cancer patients with good safety, exerts antitumor activities via regulation of the immune response and direct inhibitory activity against tumor cells through binding to aminopeptidase (CD13) on the surface of immune cells and tumor cells (Ni et al., 2021). It reduces tumor cell growth and causes cell apoptosis, prevents tumor cell invasion and metastasis, and inhibits angiogenesis in tumors by inhibiting CD13 activity (Ni et al., 2018). As an immunomodulatory agent, it is often used to combine with chemotherapeutic agents to significantly improve therapeutic efficacies in many types of cancers, such as myelodysplastic syndrome and myeloid leukemia (Ota and Uzuka, 1992). The treatment of tumors is generally based on

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ABBREVIATIONS: COX-2, cyclooxygenase 2; CRC, colorectal cancer; 5-FU, 5-fluorouracil; TK1, thymidine kinase 1.

combination drugs, so it is still of practical significance to study the combination of ubenimex and other antitumor drugs. Until now, there are few reports on antitumor effects and the molecular mechanism of celecoxib combined with ubenimex in colorectal cancer.

The study will mainly explore the antitumor efficacy of the combination of celecoxib and ubenimex on colorectal cancer in vitro and in vivo, the molecular targets and signaling pathways that play their roles, so as to provide theoretical references for clinical drug regimens and new drug developments.

Materials and methods

Cell Culture. Human HCT116, HT29, and murine C26 colorectal cancer cell lines were kept in our laboratory and qualified by Short Tandem Repeat (STR) analysis resting by professional institutions (Shanghai Biowing Biotechnology Co. LTD, Shanghai, China). Colon epithelial cell line CCD-18C0 with clear STR was purchased from Shanghai Biowing Biotechnology Co. LTD. The cells were maintained in RPMI-1640 Medium (Hyclone, Thermo Fisher Scientific, Logan, Utah) at 37°C in a humidified incubator containing 5% CO_2, supplemented with 10% FBS (GIBCO, Thermo Fisher Scientific, Logan, Utah, USA) and 1% penicillin/streptomycin (Solarbio, Beijing, China).

Cell Proliferation Assay. HCT116, HT29, and C26 cells were seeded in a 96-well plate at a density of 2.0×10^3 cells/well, and then treated with drugs for 72 hours. 10 μL of Cell Counting Kit-8 (CCK-8) reagent (Meilunbio, Dalian, China) was added to each well, and detected at the absorbance of 450 nm after 1.5 hours at 37°C in the dark. Celecoxib (169590-42-5) and ubenimex (58970-76-6) were purchased from Solarbio (Beijing, China). 5-FU (3238-40-2) was purchased from J&K Scientific (Beijing, China). 5-Fluorouridine (316-46-1) was purchased from MCE (Shanghai, China). The CI values of drug interaction were calculated by the software CompuSyn.

Transfection. In brief, cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well and incubated for 24 hours, and then 50 nM siRNA and its control siRNA were transfected for 48 hours with Lipofectamine 3000 (Thermo Fisher Scientific, Logan, Utah) following the manufacturer's protocol. The nonspecific control and TK1 siRNAs used in this study were synthesized and purchased from JTS Scientific (Wuhan, China). The forward and reverse sequences used were as follows:

1# TK-1 sequences: forward, 5′-GGCCGAUGUUCUCAGGAAATT-3′; reverse, 5′-UUUCCUGAGAACAUCGGCCTT.

2# TK-1 sequences: forward, 5′-CCUUCCAGAGGAAGCCAUUTT-3′; reverse, 5′-AAUGGCUUCCUCUGGAAGGTT.

Apoptosis Detection. HCT116 and HT29 cells were seeded in 6-well plates at a density of 1×10^5 cells/well for 24 hours and treated with drugs for 72 hours. The operation was followed as the manufacturer's protocol of the Kit (Annexin V-eGFP/PI Kit,Beijing Sizhengbo Biologic Technology Co., Ltd.). In brief, the cells were collected and the pellets were resuspended with 100 μL (1× binding buffer), and then the cells were stained with 5 μL Annexin V/Alexa Fluor 488 for 5 minutes in the dark; after that, 10 μL PI (20 μg/mL) and 400 μL PBS were added. Apoptosis was analyzed by flow cytometer (BD FACSCalibur, NJ).

Cell Cycle Analysis. HCT116, HT29, C26 cells were seeded in 6-well plates at a density of 1 × 105 cells/well for 24 hours and then harvested after treated with drugs for 24 hours. In addition, HCT116 cells were cultured at the same density in 6-well plates for 24 hours and then collected after transfection of siTK1s and control siRNAs for 48 hours. The cells were fixed with 95% ethanol for 24 hours at 4°C, and then incubated with 400 μL PI/RNase staining buffer at 37°C for 30 minutes. The cell cycle was analyzed by flow cytometer (BD FACSCalibur, Franklin Lakes, NJ).

Western Blot. The cancer cells in 6-well plates were treated with drugs or siRNAs. The cells were collected and then dissolved in cell lysis buffer (50 mM Tris-HCl, PH7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) with proteinase inhibitors (100:1) and incubated on ice for 30 minutes. The supernatant of the cell lysate was collected after centrifugation at 13,000 rpm and 4 °C for 15 minutes. The protein concentrations were determined using the bicinchoninic acid method according to the manufacturer's protocol. The proteins in supernatants were separated on 12% or 8% SDS-PAGE gels according to the molecular weight of our target proteins, and subsequently transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat milk for 1 hour at room temperature, and then incubated overnight at 4 °C or 1 hour at room temperature with primary antibodies as indicated. The membranes were washed with 1 × TBST (100 mM Tris, 1500mM NaCl, 1‰Tween-20, pH 7.6) for 6 times × 10 min and further incubated with an appropriate second antibody for 1 hour at room temperature. Signals were visualized using the Chemiluminescence imager (Tanon, Shanghai, China) after further washing with 1 × TBST for 3 times × 10 min. The bands of the target proteins were obtained, and the gray intensity of each band was scanned with Photoshop software.

The antibodies and their suppliers were as the following: antibodies against TK1 (cat no: 15691-1-AP) and Cyclin A2 (cat no: 18202-1-AP) were purchased from Proteintech (Wuhan, Hubei, China). Antibodies targeting P21 (cat no: SC6246) were purchased from Santa Cruz Biotechnology (Dallas, Texas). Antibodies for PARP1 (cat no: 9542S) were purchased from Cell Signaling Technology (Boston, MA), and antibodies for β-Tubulin (cat no: SC6246) were purchased from Booruijin (Haidian District, Beijing, China). Primary antibodies were respectively prepared in bovine serum albumin in different dilution based on manufacturers' instructions and detected by using the appropriate antimouse and antirabbit conjugate, prepared in bovine serum albumin in 1:5000 dilution.

Quantitative Real-Time PCR. Total RNA was extracted according to the protocol of the Fastagen RNAfast200 rapid extraction kit (Feijie, Shanghai, China), and cDNA was prepared with ReverTra Ace qPCR RT Master Mix with gDNA Remover (FSQ-301, TOYOBO, Osaka City, Japan). Then 100 ng of cDNA was added to the SYBR green master mixture (14370, Roche, Basel, Switzerland), and PCR was performed in the LightCycler 96 Instrument Real-time PCR System. The cycle program was 95°C for 2 minutes, 95°C for10 seconds, 55°C for 10 seconds, 72°C for 15 seconds, and there was a total of 45 cycles, and a high-resolution melting curve analysis was performed. Each sample was conducted for three replicates, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference to normalize the Quantification Cycle (C_q) value.

The forward and reverse primers used were as follows:

TK1 of Homo sapiens -F 5′-GGGGCAGATCCAGGTGATTC
TK1 of Homo sapiens -R 3′ CCATGGTGTTCCGGTCATGT
TK1 of Mus -F 5′-GGGGCAGATTCAGGTGATTC
TK1 of Mus - R 3′ CCATGGTGTTCCGATCATGT
GAPDH of Homo sapiens -F 5′-ACAACTTTGGTATCGTGGAAGG
GAPDH of Homo sapiens -R 3′ GCCATCACGCCACAGTTTC
GAPDH of Mus -F 5′-AGGTCGGTGTGAACGGATTTG
GAPDH of Mus -R 3′ TGTAGACCATGTAGTTGAGGTCA

Transcriptomic Analysis. C26 cells were divided into control, celecoxib, ubenimex, and celecoxib + ubenimex groups in triplicate. After the cells were treated with 20 μM celecoxib and/or 0.5 mg/mL ubenimex for 12 hours, RNA was extracted from 12 samples with Trizol and sent to BGI (Shenzhen, China) for transcriptome determination. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology Consortium (GO C) enrichment analysis of differential genes were carried out to obtain possible molecular targets of drug action.

Animal Studies. Animal experiments were carried out in accordance with the requirements of animal ethics in our country. Female BALB/C mice and nude mice weighing 18–22g were purchased from Sibeifu Laboratory Animal Technology Co., Ltd. (Beijing, China). Each mouse was injected with 0.2 ml of C26 tumor solution at a concentration of 1:60. A total of 32 mice or 24 nude mice were randomly

assigned to four groups, i.e., control, celecoxib (250 mg/kg), ubenimex (20 mg/kg), or celecoxib + ubenimex. The mice were given the drug or saline six days a week for each oral dose. After 18 days, the animals were killed and the tumors were removed and weighed. The combination index (CI) values in animal experiments were calculated according to the formula: tumor inhibition rate of the combination group/(celecoxib inhibitory rate × ubenimex inhibitory rate).

Statistical Analysis. Statistical analysis of the data was conducted using GraphPad Prism 8.0 software (Inc., La Jolla, CA). The data were reported as means ± S.D. or S.E.M., and they were evaluated by one-way analysis of variance. Differences were considered significant at $P < 0.05$. Statistically significant P values were presented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The experiments were performed in triplicate and independently, and the data were presented as three repeats from one independent experiment.

Results

Celecoxib and Ubenimex Exerted a Synergistic Effect of Anti–Colorectal Cancer Activity In Vitro. To determine the in vitro inhibitory effects of celecoxib and ubenimex on the proliferation of HCT116, HT29, and C26 cells, we used the CCK-8 method to determine the viability of cells treated with the indicated concentrations of drugs for 72 hours (Fig. 1A). The results showed that each of the two drugs possessed a dose-dependent inhibitory effect on the growth of the above three lines of colorectal cancer cells. At the same time, the IC_{50} values of ubenimex on the three lines of cells were 1.24 mg/ml, 0.43 mg/ml, 1.41 mg/ml, and the ones of celecoxib were 31.44 μM; 21.24 μM, 13.61 μM, respectively. In the meantime, the CCK-8 method was also used to detect the cell viability of CCD-18C0, a normal colon epithelial cells, after celecoxib or ubenimex treatment and there existed no significant change for the cell death at the indicated concentrations (Supplemental Fig. 1). In addition, the cell proliferation was examined after the cells were treated with the combination of celecoxib and ubenimex, and it was found that there existed an obviously enhanced effect on the proliferation of the cancer cells (Fig. 1B). Furthermore, the software compuSyn was used to calculate the combination index of the drug combination experiment in vitro, and the following data were indicated in Fig. 1C and the CI values were less than 1. Therefore, a

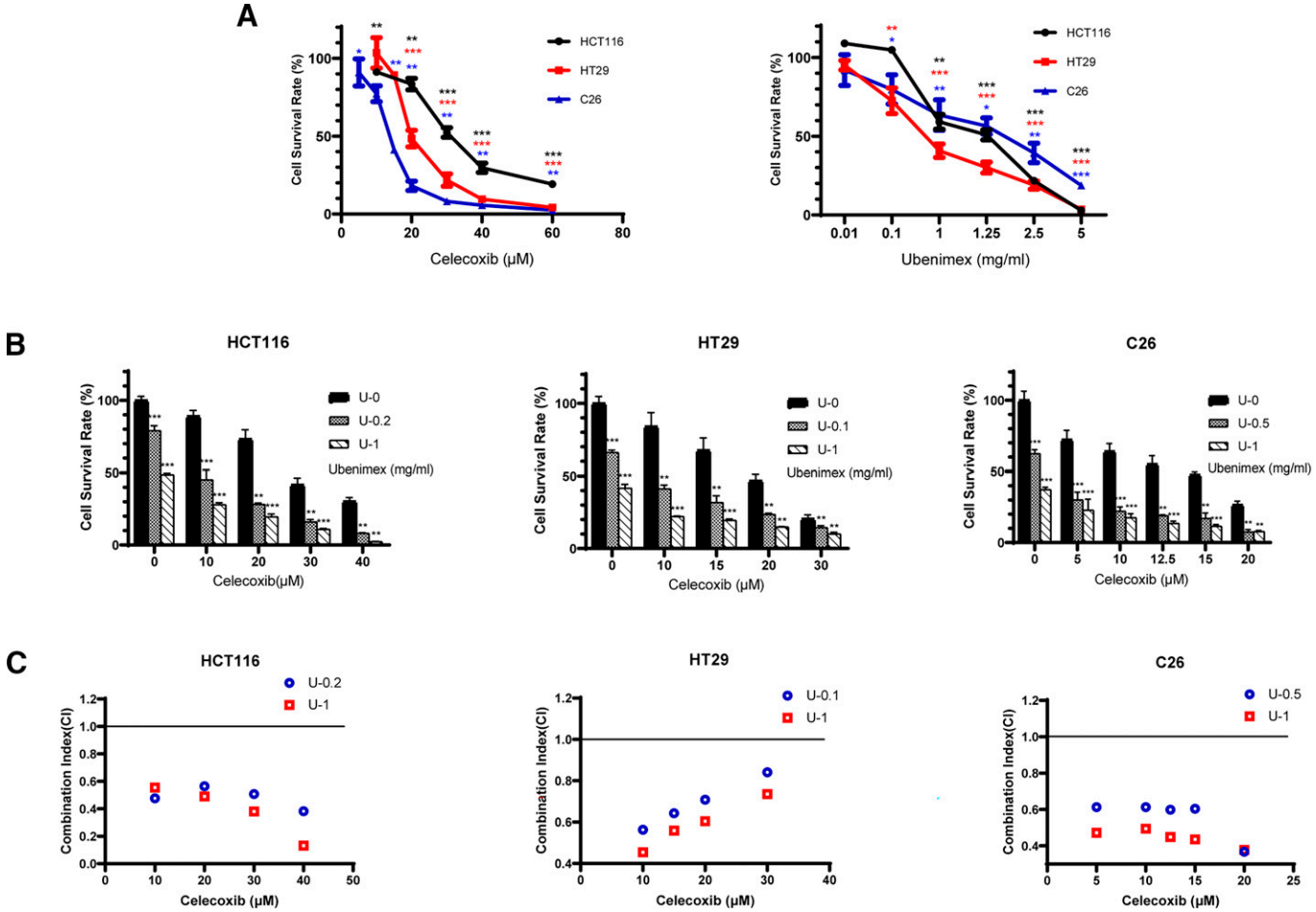

Fig. 1. Celecoxib and/or ubenimex inhibited the proliferation of colorectal cancer cells in vitro. (A) Celecoxib or ubenimex reduced the growth of cancer cells. HCT116, HT29, and C26 colorectal cancer cells were treated with celecoxib and ubenimex for 72 hours and then the cell survival was detected by CCK-8 assay. Results shown are mean ± S.D. of three independent experiments, each with three replicates. $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control (no agent treatment). IC50 values were determined by fitting a sigmoidal dose-response curve to the data using Graph Pad Prism. (B) The combination of the two drugs decreased cell survival. The three colorectal cell lines were treated with celecoxib combined with ubenimex for 72 hours and dittoed for three batches of repeated experimental results. $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with its own U-0 group. (C) The combination index was calculated using CompuSyn software and plotted with the celecoxib concentration gradient as the x-coordinate and the CI value as the y-coordinate. In the figure, U-0, U-0.1, U-0.2, U-0.5, and U-1 indicated the group of the cells treated with ubenimex at 0, 0.1, 0.2, 0.5, and 1 mg/ml, respectively.

synergistic effect on the proliferation of colorectal cancer cells was found when the two drugs were used together.

Celecoxib and Ubenimex Exerted a Synergistic Effect of Anti–Colorectal Cancer Activity In Vivo. From above the results, the combination exerted synergistic effects on the proliferation of colorectal cancer cells in vitro. So, we explored the anti–colorectal cancer activity of celecoxib and/or ubenimex in vivo. The animal experiment scheme was shown in Fig. 2A. In the BALB/C murine model, the ubenimex, celecoxib, and the combination group showed no significant change in body weight, but the tumor volume and weight in the drug treatment groups were smaller than that in the control group, and the combined group was the smallest among the groups. The inhibitory rate of the three groups was 25.12, 22.69, and 36.89%, respectively. Similar trend results were obtained in BALB/C nude mice and the inhibitory rate of tumor growth was 20.04, 44.74, and 59.77%, respectively. So, the results showed that celecoxib and ubenimex had anti–colorectal cancer function in mice. Based on the data, the CI values were calculated as 0.65 and 0.67 in the two animal models, respectively, indicating that the combination of the two drugs had a synergistic effect. Furthermore, the growth inhibitory effect of the combination group and the celecoxib group in the nude mouse model was stronger than that of the nude mouse model. Thus, we speculated that the difference in efficacy between mouse and nude mouse models might be related to the T cell–mediated immune response. (Fig. 2, B and C).

Celecoxib Cooperated with Ubenimex Affected Apoptosis in Colorectal Cancer Cells. To explore the mechanism by which the two drugs could inhibit the proliferation of colorectal cancer cells, we resuspend the cells of the control and the drug treated groups for 72 hours in binding buffer, and then carried out flow cytometry after Annexin V/Alexa Fluor 488 and PI staining. The results showed that the single drug alone could induce more cells into apoptosis in HCT116 and HT29 cell lines as compared with the control group, and the combination group resulted in the most apoptotic cells among the groups (Fig. 3A). For example, the rate of apoptosis of HCT116 cells was 4.44 ± 0.87%, 10.53 ± 2.55%, 7.90 ± 0.38%, and 39.98 ± 0.87% in the group of control, ubenimex, celecoxib, and ubenimex + celecoxib (C + U), respectively. There was a statistical significance ($P < 0.01$) between the combination group and the single agent alone group. However, in C26 cells, the number of apoptotic cells in the ubenimex group was close to that in the control group, and that in the celecoxib group was the highest, while in the combined group was less than that in the celecoxib group (Fig. 3A). To observe the effect of apoptosis induction by celecoxib and/or ubenimex, western blot analysis of cleaved PARP1 was performed in HCT116 and HT-29 cells, and it was found that cleaved PARP1 expression was significantly up-regulated by the single agent alone, with the highest expression level in the combination group (Fig. 3B). In C26 cells, there was no significant change of cleaved-PARP1 levels in the combination group as

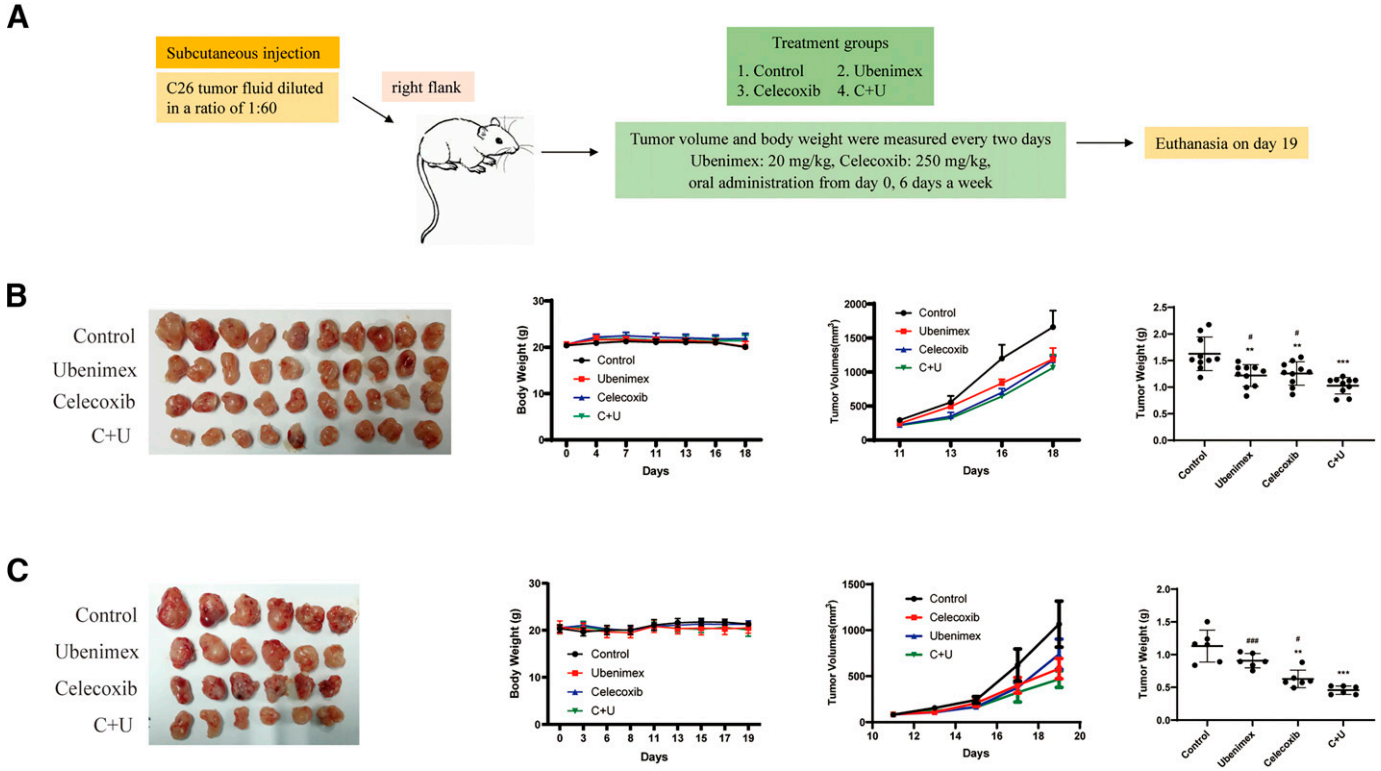

Fig. 2. Celecoxib and/or ubenimex suppressed the growth of colorectal cancer in vivo. (A) Pattern diagram was shown for establishing tumor model of heterotopic transplantation of C26 tumor fluid in BalB/C mice and nude mice, grouping, and drug administration. (B) Celecoxib and/or ubenimex reduced the growth of C26 tumors in BalB/C mice. From left to right: tumor images obtained after 18 days of treatment in a BalB/C murine model; body weight curve of mice; tumor volume curve; mean ± S.E.M. of tumor weight. (C) Celecoxib and/or ubenimex restrained the growth of C26 tumors in nude mice. From left to right: tumor images obtained after 18 days of treatment in a nude mice model; body weight curve of mice; tumor volume curve; mean ± S.E.M. of tumor weight. All data were processed using Graph Pad Prism. In the figure, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control; $^{\#}P < 0.5$, $^{\#\#\#}P < 0.001$ compared with the group of C + U. C + U represented the combined group of celecoxib (250 mg/kg) and ubenimex (20 mg/kg).

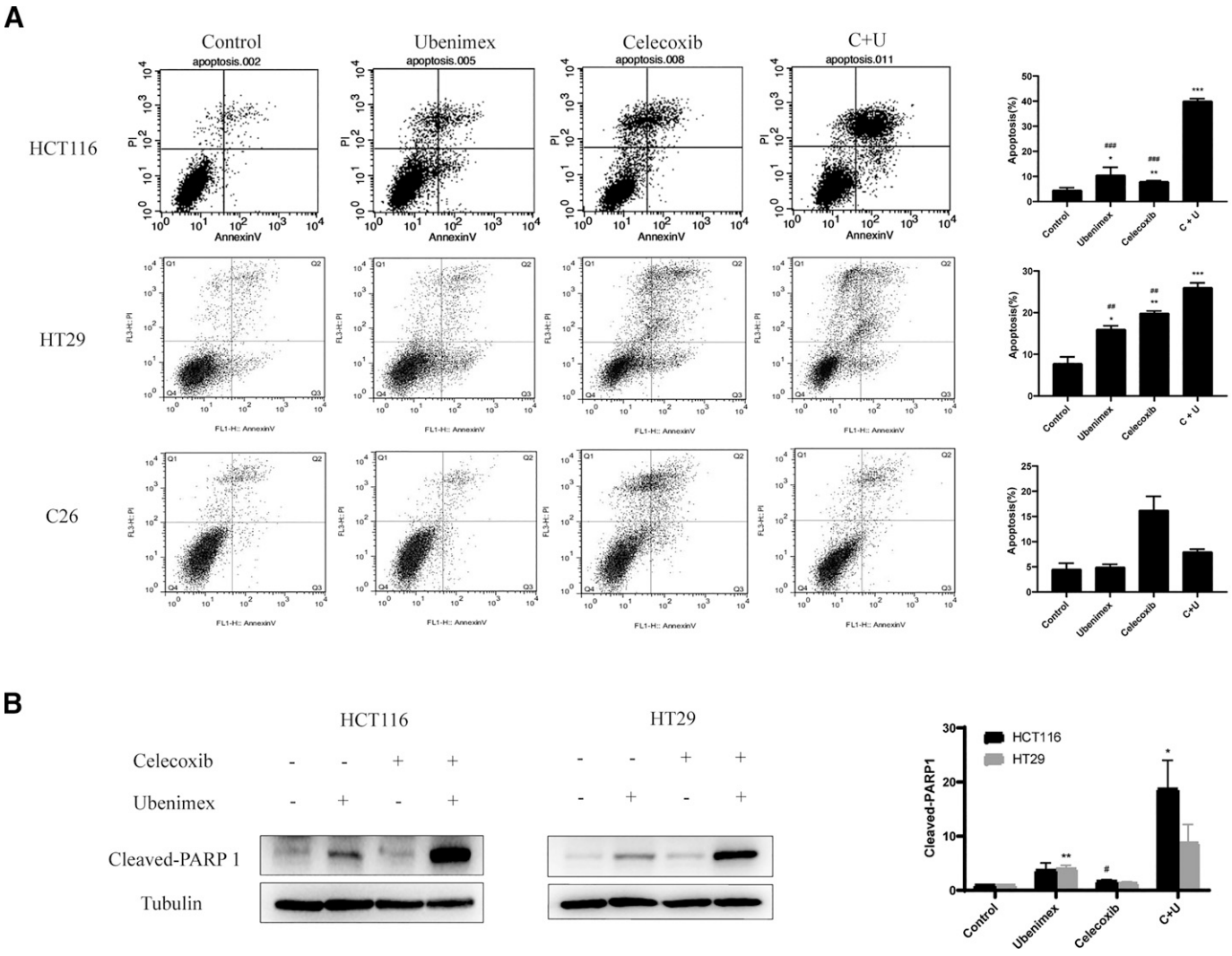

Fig. 3. Effect on the apoptosis of colorectal cancer cells induced by celecoxib and/or ubenimex. (A) Left: flow cytometric images of HCT116, HT29, and C26 were treated with celecoxib (40 μM) and ubenimex (0.5 mg/ml) for 72 hours. C + U represented celecoxib plus ubenimex. Right: the proportion of the number of apoptotic cells in all cells as the indicated groups. $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the group of C + U. (B) The expression of cleaved-PARP-1 as in the indicated groups were examined with western blot on the left. HCT116 and HT29 were treated with celecoxib (40 μM) and ubenimex (0.5 mg/ml) for 24 hours. Protein expression levels were quantified by using a standard curve and normalized to the housekeeping gene tubulin on the right. C + U represented celecoxib plus ubenimex. $^{*}P < 0.5$, $^{**}P < 0.01$ compared with control; $^{\#}P < 0.5$ compared with the group of C + U.

compared with the single agent alone (the data were not shown). Through the above experiment it was speculated that celecoxib and/or ubenimex inhibited the proliferation of HCT116 and HT29 cell lines by promoting cell apoptosis, but the same results were not achieved in C26 cells. Therefore, inducing apoptosis might not be the common mechanism by which the combination of the two drugs was used against colorectal cancer cells.

Celecoxib and/or Ubenimex Induced Cell Cycle Arrest in Colorectal Cancer Cells. From the above results, apoptosis was not the common mechanism of the combination for killing the colorectal cancer cells, so cell cycle related tests were performed with flow cytometry. The results showed that the number of synthesis phase (S phase) cells in the celecoxib, ubenimex, and the drug combination groups was reduced as compared with the control group in all three cell lines, and the effect of the drug combination group was the most obvious among all the groups (Fig. 4A). For example, the rate of S phase of C26 cells was 35.05 ± 4.02%, 31.09 ± 3.18%, 28.61 ± 0.62%, and 24.73 ± 2.58% in the group of control, ubenimex, celecoxib, and ubenimex + celecoxib (C + U), respectively. There was a statistical significance ($P < 0.05$) just between the combination group and the control group.

To further observe the change of cell cycle, cell cycle-related proteins cyclin A2 and P21 were detected with western blot, and it was found that celecoxib or ubenimex induced the down-regulation of the expression of cyclin A2 protein, up-regulation of the expression of P21 protein, and the combination group caused the most obvious change for cyclin A2 and P21 among all the groups in HCT116, HT29, and C26 cells (Fig. 4B). Based on the data, celecoxib cooperated ubenimex exerted antitumor activity through cell cycle arrest in colorectal cancer cells.

TK1 Was Involved in the Antitumor Activity of Celecoxib and/or Ubenimex Against Colorectal Cancer. To explore the molecular mechanism of the combination of celecoxib and ubenimex, we tested the expression of CD13 (the molecular

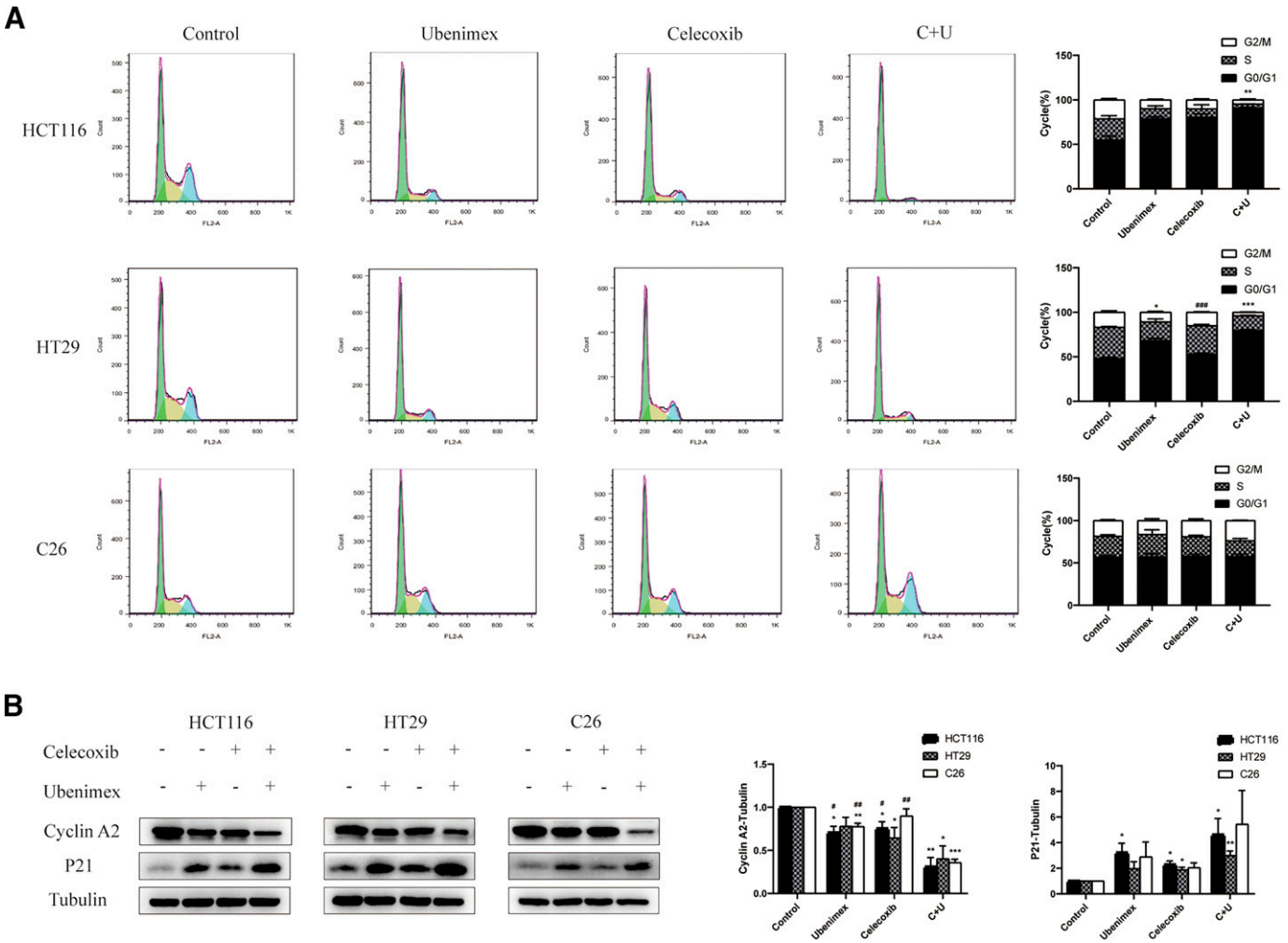

Fig. 4. Ubenimex cooperated with celecoxib enhanced cell cycle arrest of colorectal cancer cells. (A) Flow cytometric images of HCT116 was treated with celecoxib (20 μM) and/or ubenimex (0.5 mg/ml) for 24 hours; HT29 was treated with celecoxib (10 μM) and ubenimex (0.5 mg/ml) for 24 hours; C26 was treated with celecoxib (10 μM) and/or ubenimex (1.0 mg/ml) for 24 hours. The cell cycle distribution ratio compared with the control group. (B) The expression of cyclin A2 and P21 as in the indicated groups were examined with western blot on the left. HCT116 was treated with celecoxib (20 μM) and ubenimex (0.5 mg/ml) for 24 hours; HT29 was treated with celecoxib (10 μM) and ubenimex (0.5 mg/ml) for 24 hours; C26 was treated with celecoxib (10 μM) and ubenimex (1.0 mg/ml) for 24 hours. Cyclin A2, P21 protein expression levels were quantified by using a standard curve and normalized to the housekeeping gene tubulin on the right. Error bars represented S.E.M. In the figure, $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control; $^{\#}P < 0.5$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with C + U. C + U represented celecoxib plus ubenimex and the dose used in combination was the corresponding single drug dose for cells.

target of ubenimex for antitumor effects) and COX2 (the molecular target of celecoxib for antitumor effects) protein in three cell lines, and found that CD13 was highly expressed in HCT116 cells and was not obvious in HT29 and C26 cells, while COX2 was highly expressed in C26 cells and not significantly expressed in HCT116 and HT29 cells (Fig. 5A), so we believe that the molecular targets of the two-drug combination were not highly correlated with these proteins. Because of the COX-2- and CD13-independent mechanism of the antitumor effect of the combination, transcriptome analysis was performed on C26 cells to further explore the molecular target of celecoxib combined with ubenimex against colorectal cancer cells. The number of genes differed between celecoxib group, ubenimex group, drug combination group, and control group were 448, 968, and 2473, respectively (Fig. 5B). And the number of common differential genes between the three groups was 92. KEGG pathway and GO C enrichment analysis were carried out for these 92 differential genes (Fig. 5, C and D). After literature review, cytosol and pyrimidine metabolism related gene TK1 was selected as a target gene.

The results of transcriptome analysis showed that TK1 mRNA decreased after the treatment of the single drug alone compared with the control group, and the expression level of the combination group was the lowest among all the groups (Fig. 5E). Next, the expression of TK1 mRNA in three colorectal cancer cell lines was verified by qRT-PCR, and the results were consistent with the results of transcriptomics (Fig. 5F). Therefore, the next step was to explore the effects of the two drugs on the expression level of TK1 protein in the cell species. The results from western blot showed that both drugs reduced the expression of TK1 protein in the three lines of colorectal cancer cells, and the combination group exhibited the lowest expression level in the all groups (Fig. 5G). Based on the data, it was supposed that TK1 was involved in the molecular mechanism of the antitumor effect of celecoxib combined with ubenimex.

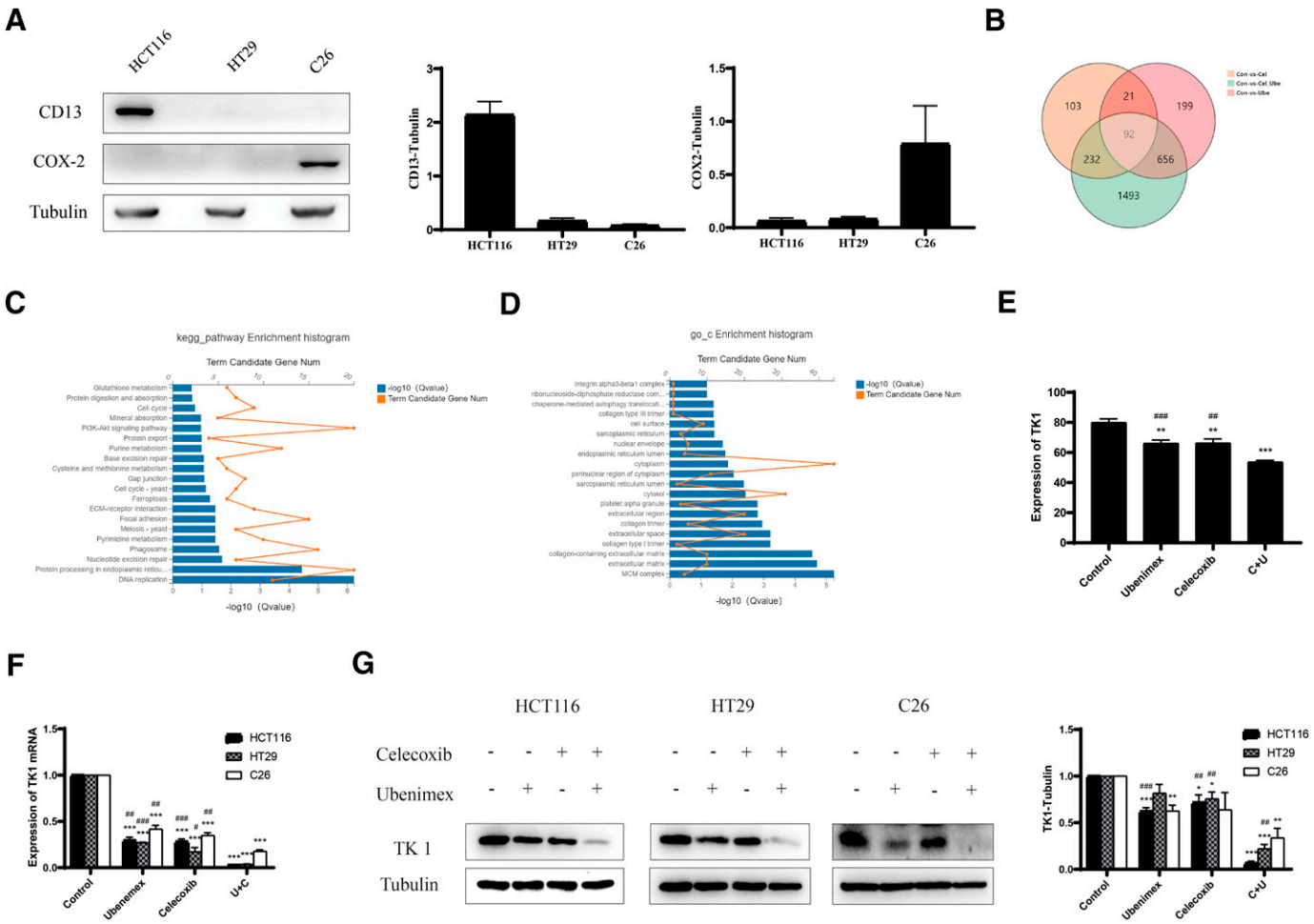

Fig. 5. Background expression of CD13 and COX2 in three colorectal cell lines. (A) Protein expression levels were quantified by using a standard curve and normalized to the housekeeping gene tubulin on the right. TK1 was involved in the mechanism of the two drugs against colorectal cancer. Differential gene Venn diagrams of transcriptomic analysis after C26 was treated with celecoxib (20 μM) and ubenimex (0.5 mg/ml) for 12 hours. The number of common differential genes was 92. (B–D) KEGG pathway and GO C enrichment analysis of common differential genes. (E) FPKM value of TK1 gene relative expression in ubenimex, celecoxib and C + U group of C26 cell line compared with the control group. (F) qRT-PCR was used to confirm that celecoxib and ubenimex could down-regulate the level of TK1mRNA in the three cell lines. HCT116 was treated with celecoxib (20 μM) and ubenimex (0.5 mg/ml) for 24 hours; HT29 was treated with celecoxib (10 μM) and ubenimex (0.5 mg/ml) for 24 hours; C26 was treated with celecoxib (10 μM) and ubenimex (1.0 mg/ml) for 24 hours. (G) The expression of TK1 as in the indicated groups were examined with western blot on the left. TK1 protein expression levels were quantified by using a standard curve and normalized to the housekeeping gene tubulin on the right. HCT116 was treated with celecoxib (20 μM) and ubenimex (0.5 mg/ml) for 24 hours; HT29 was treated with celecoxib (10 μM) and ubenimex (0.5 mg/ml) for 24 hours; C26 was treated with celecoxib (10 μM) and ubenimex (1.0 mg/ml) for 24 hours. Error bars represented S.E.M. Statistical significance was determined by *t* test ($^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control; $^{\#}P < 0.5$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with C + U) . C + U represented celecoxib plus ubenimex and the dose used in combination was the corresponding single drug dose for cells.

TK1 Modulated Cell Cycle in Colorectal Cancer Cells. To further verify TK1 functions in the anti–colorectal cancer effect, two siRNAs of TK1 were used to knock down the expression of TK1 protein in HCT116 cells. At first, siTK1s effectively decreased the level of TK1 protein (Fig. 6A). The results from TK1 function related experiments showed that siTK1s inhibited the proliferation of colorectal cancer cells as compared with the control group (Fig. 6B). At the same time, we also excluded the effect of siNC and Lipo3000 on the protein expression in cells (Supplemental Fig. 2). As the following, flow cytometry was used to examine the change of cell cycle, so it was found that the S-phase ratio of cells transfected with siTK1s was decreased and cell cycle arrest occurred (Fig. 6C). At last, the expression of cyclin A2 protein was decreased and P21 protein was increased in the transfected group through western blot assay (Fig. 6A). These data suggested that decreased TK1 protein expression may lead to inhibition of cell proliferation by inducing S-phase reduction.

Celecoxib Combined with Ubenimex Promoted the Antitumor Efficacy of 5-Fluorouracil And 5-Fluorouridine in Colorectal Cancer Cells. It is reported that 5-fluorouracil (5-FU) increases TK1 protein at mRNA level in HT29 cells (Lee et al., 2010) so that 5-FU and 5-fluorouridine were applied to further prove the involvement of TK1 in molecular mechanism of antitumor activity by the combined two drugs. As shown in Fig. 7A, 5-FU or 5-fluorouridine inhibited the proliferation of colorectal cancer cells as indicated cell lines in a dose-dependent manner. We also obtained the CI value of 5-FU and 5-fluorouridine in combination with celecoxib and ubenimex, and found that the most of CI values, after the combination of the three drugs, could be less than 1 (Fig. 7B), indicating that the combination of three drugs possessed a synergistic effect, and the combination of celecoxib and

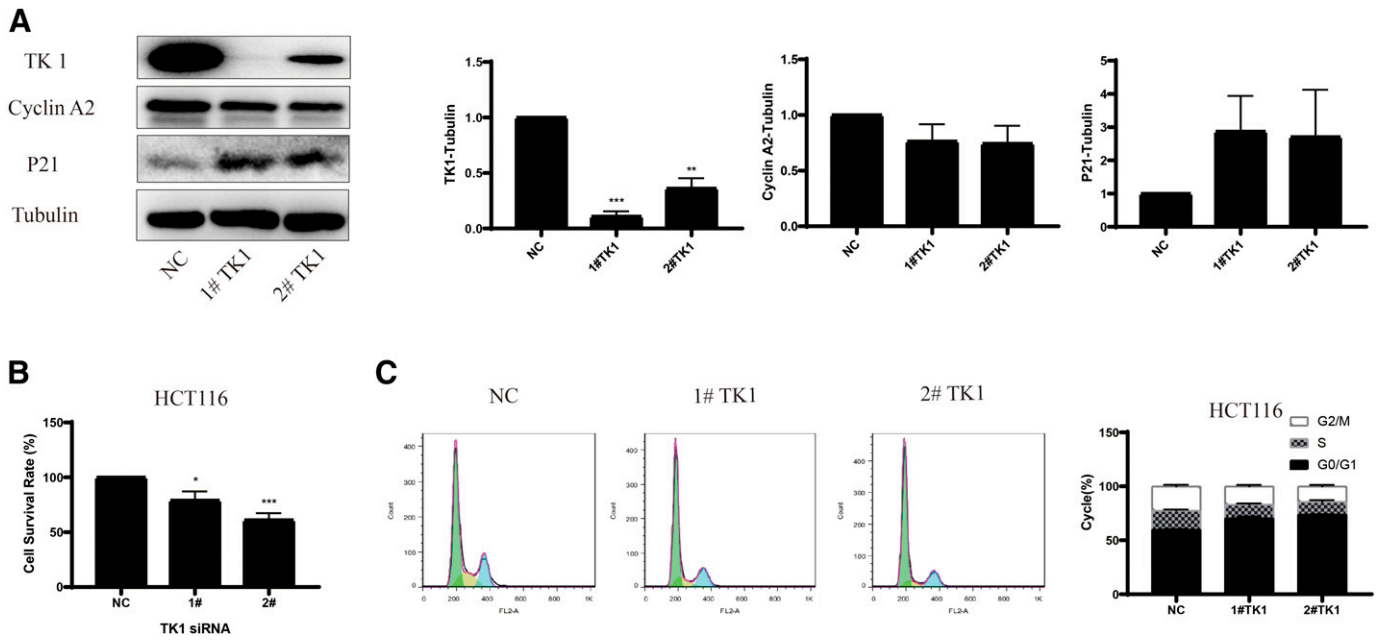

Fig. 6. Reduced expression of TK1 protein led to cell cycle arrest and inhibited the cell survival in colorectal cancer cells. (A) Western blot was applied to examine the expression of TK1, cyclin A2, P21 in HCT116 cells after TK1 siRNA transfection on the left. The level of TK1, cyclin A2, and P21 proteins was quantified by using a standard curve and normalized to the housekeeping gene tubulin. (B) CCK-8 assay was used to show the average cell survival rates of HCT116 treated with TK1 siRNAs. (C) Flow cytometric images of HCT116 cell line treated with TK1 siRNAs and the cell cycle distribution ratio compared with the control group. In the figure, error bars represented S.E.M. Statistical significance was determined by *t* test (*$P < 0.5$, **$P < 0.01$, and ***$P < 0.001$ compared with NC; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with C + U). NC represented control (no agent treatment).

ubenimex enhanced the efficacy of 5-FU and 5-fluorouridine against colorectal cancer cells. Next, to further verify the molecular mechanism of the three drugs combination against colorectal cancer, we tested the expression of TK1 and found that 5-FU upregulated the expression of TK1, but the combination of celecoxib and ubenimex down-regulated the protein (Fig. 7C). That could be the reason why the combination of three drugs exerted synergistic effects on colorectal cancer.

Discussion

Drug combination is an important therapeutic strategy for antitumor treatment, which can significantly improve drug efficacy or reduce drug toxicity (Rawal et al., 2019). Our study focused on the anti–colorectal cancer efficacy of celecoxib in combination with ubenimex in vitro and in vivo, as well as its possible molecular targets and signaling pathways. This is the first study of celecoxib combined with ubenimex against colorectal cancer and their molecular mechanisms. Furthermore, celecoxib and ubenimex are both clinically used drugs with lower toxicity than chemotherapeutic drugs, so studying their combined antitumor effects and mechanisms can provide references for clinical use.

Celecoxib is reported to exert antitumor activities in various types of cancers through COX-2-dependent or independent mechanisms (Egashira et al., 2017). Ubenimex is widely applied as an adjunct drug for cancer therapy, especially modulation of anticancer drugs' activity (Wang et al., 2018), so there are still many unknown combinations of ubenimex and different antitumor drugs, which need further research and clarification. In our experimental system, in vitro antitumor activities of celecoxib and/or ubenimex were evaluated with CCK-8 assay in colorectal cancer cells. Our results showed that celecoxib or ubenimex inhibited the proliferation of colorectal cancer cells and there existed a synergistic effect of antitumor activities between celecoxib and ubenimex. Based on this, we investigated the in vivo antitumor effect of celecoxib and/or ubenimex in transplanted C26 tumor model for BALB/c mice and nude mice, and then it was found that celecoxib or ubenimex alone reduced the growth of C26 tumor in the two types of mice while the combined two drugs showed more potent decrement of tumor volumes and weights without accompanying changes in murine body weight. From this, it is supposed that a better therapeutic efficacy may be obtained if the dosages of the drugs is adjusted. Therefore, ubenimex enhances the antitumor activities of celecoxib in vitro and in vivo.

Apoptosis, a kind of programmed cell death, causes the disciplined and efficient exclusion of damaged cells without inflammatory responses and is finely modulated at gene level (Xu et al., 2019). Apoptosis is one of the main mechanisms by which antitumor drugs inhibit cell proliferation (Pistritto et al., 2016). We first explored the apoptosis of colorectal cancer cells, and found that celecoxib cooperated with ubenimex induced the percentage of apoptosis significantly more than the single drug alone did in HCT116 and HT29 cells, but no similar results were obtained in C26 cells. PARP1 is a protease with polyadenosine diphosphate ribosyl (PAR) catalytic activity in eukaryotic cells. It is activated when DNA is damaged and broken and participates in DNA repair and cell apoptosis. PARP1 can accelerate cell instability when enzyme activity is lost, and its splicing is considered to be an important indicator of apoptosis (Alemasova et al., 2019; Diamantopoulos et al., 2014). Therefore, the detection of apoptosis related protein cleaved-PARP1 also verified the above results. In other

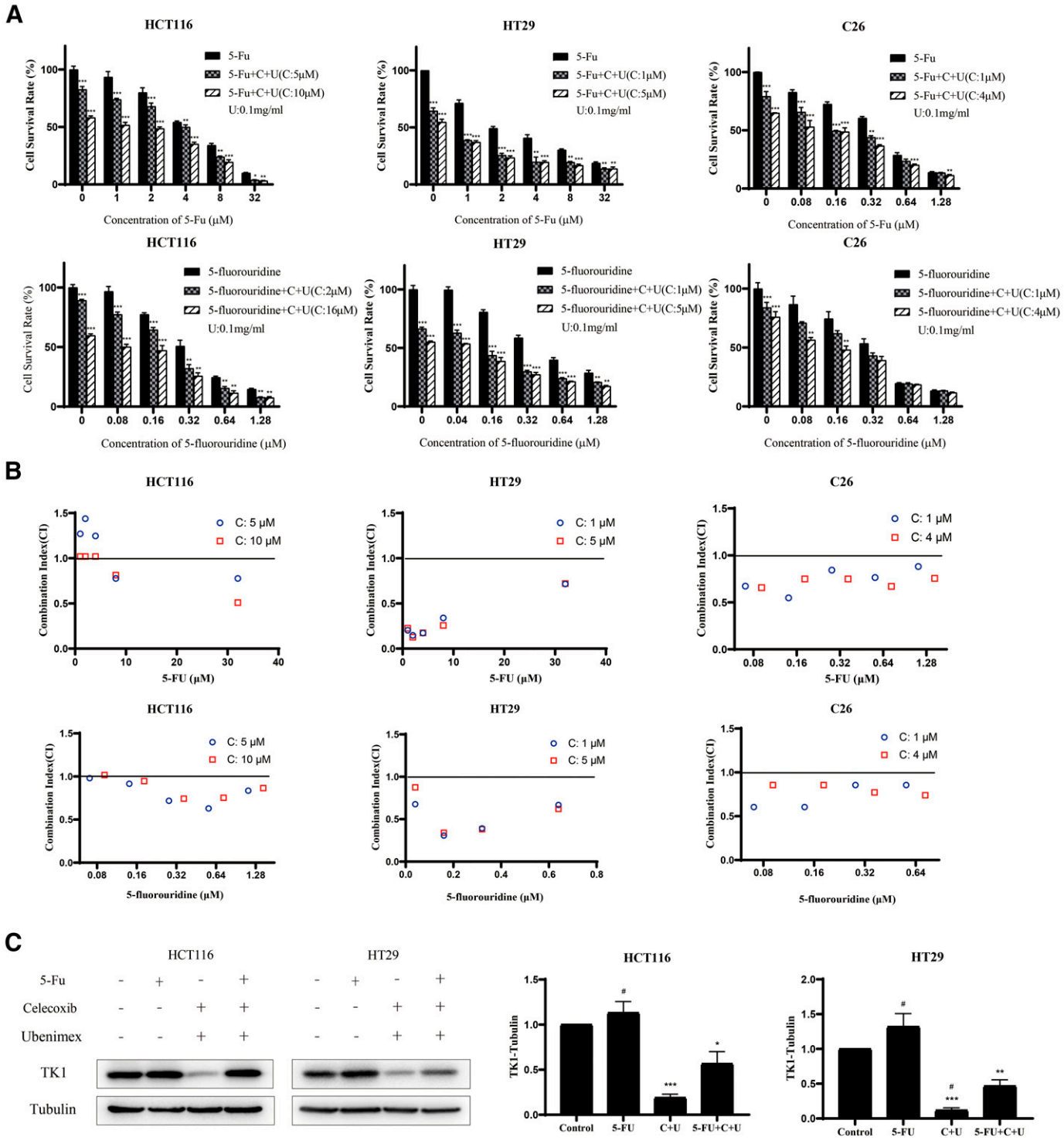

Fig. 7. The combination of ubenimex and celecoxib promoted the anti–colorectal cancer effect of 5-fluorouracil (5-FU) and 5-fluorouridine. (A) CCK-8 assay was used to determine the average cell survival rates of colorectal cancer cells. HCT116, HT29, and C26 cell lines were treated with the indicated drugs for 72 hours. $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the corresponding group of 5-FU or 5-fluorouridine. (B) CI value of colorectal cancer cells treated with the combination of three drugs. CI values were calculated by CompuSyn software. C, celecoxib; U, ubenimex; C + U, celecoxib plus ubenimex. Effect of the combination of three drugs on TK1 expression. (C) The expression of TK1 as in the indicated groups were examined with western blot on the left. HCT116 was treated with celecoxib (10 μM), ubenimex (0.5 mg/ml) and 5-fluorouracil (8 μM) for 24 hours. HT29 was treated with celecoxib (10 μM), ubenimex (0.5 mg/ml), and 5-fluorouracil (2 μM) for 24 hours. TK1 protein expression levels were quantified by using a standard curve and normalized to the housekeeping gene tubulin on the right. Error bars represented S.E.M. Statistical significance was determined by t test ($^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control; $^{\#}P < 0.5$ compared with 5-FU + C + U). 5-FU + C + U was presented as 5-FU plus celecoxib and ubenimex.

words, celecoxib plus ubenimex caused more cleaved-PARP1 protein in HCT116 and HT29 cells as compared with the single drug alone. However, there was no obvious change for cleaved-PARP1 in C26 cells treated celecoxib and/or ubenimex. These findings showed that apoptosis was not the common mechanism of the inhibitory effect on the colorectal cancer cells and

then the common mechanism of the combination therapy need to be further studied.

Cell cycle dysfuction plays an important role of unscheduled cell proliferation and causes the malignant phenotype. Targeting cell cycle possesses the therapeutic potential for cancer patients and many anticancer drugs inhibit tumor growth by induction of cell cycle arrest, such as 5-FU (Kim et al., 2020). Next, the effects of celecoxib and/or ubenimex on the cell cycle were investigated. The results from flow cytometry showed that celecoxib or ubenimex could reduce the percentage of cells in S phase of three types of colorectal cancer cell lines, but celecoxib plus ubenimex decreased the rate of S-phase cells much more than the single drug alone did. The G1, S, G2, and M phases control programmed cell proliferation. Cell cycle checkpoints (G1, G2, and intermediate (M) or spindle checkpoints) and the order activation of cyclin-dependent kinases are needed during cell cycle transition and ensure that the DNA replicates correctly so that the cell is ready to divide (Chen et al., 2019). Cyclin A2 initiates and controls DNA replication, which is essential for DNA synthesis (S phase) (Wolgemuth, 2011). At the same time, the presence of cyclin A2 is restricted by p21, especially after DNA damage (Silva Cascales et al., 2021). In our research, celecoxib or ubenimex reduced the level of cyclin A2 protein and increased the expression of P21 in the three colorectal cancer cell lines. In addition, celecoxib plus ubenimex had a more significant effect on reducing cyclin A2 and increasing P21 as compared with the single drug alone. Therefore, ubenimex potentiated the antitumor activity of celecoxib through regulating the cell cycle in colorectal cancer. Subsequently, the molecular target of the combined drugs should be explored in our experimental system.

There was an enhanced effect between celecoxib and ubenimex, which was found by combination drugs screening in COX-2-deficient HCT116 cells. Our experiment also proved that there was no clear expression of COX-2 in HCT116 and HT29 cells. At the same time, there was no clear expression of CD13 in HT29 and C26 cells. Therefore, the molecular mechanism of celecoxib plus ubenimex was independent of COX-2 and CD13. To investigate the molecular mechanism, the transcriptome analysis was performed on C26 cells and it was found that TK1 might be involved in the mechanism. TK1 is one of the key enzymes in DNA synthesis and is involved in cell proliferation.TK1 converts DT to DTMP by transferring phosphate from ATP, and further phosphorylates DTTP, whose activity is regulated by the cell cycle (Bitter et al., 2020). TK1 activity in cell cycle is highest at late S/early G2 and decreases significantly to nondetectable levels by the end of M phase. In malignant tumor cells, TK1 activity remains elevated during the G2 and M phases (Singh et al., 2020). Studies have shown that serum levels of TK1 are significantly elevated in patients with colorectal cancer compared with healthy controls (Velazquez et al., 2020) and Weagel and colleagues have also found that TK1 expression are increased in colorectal adenocarcinoma tissues by analyzing RNA-sequencing data (The Cancer Genome Atlas) and it is only expressed on the cell membrane of malignant cells in extreme proliferation tissues (Weagel et al., 2018). In the following experiments, we found that the combination of the two drugs could reduce TK1 mRNA level and its protein expression much more potently than the single agent alone did. Moreover, siTK1 diminished the rate of S-phase cells, reduced cyclin A2 protein level, and raised the expression of P21. According to the induction of TK1 expression at mRNA level by 5-FU (Lee et al., 2010), celecoxib plus ubenimex promoted the inhibitory effect of proliferation by 5-FU in colorectal cancer cells. TK1 is involved in the signaling pathway in which 5-FU plays an antitumor role (Kidd et al., 2005), but 5-FU can increase the expression of TK1 protein, while celecoxib and/or ubenimex reduced the expression of TK1 protein. Thus, the decreased expression of TK1 can play an anti–colorectal cancer role through cell cycle regulation. This result was consistent with the research one (Zhu et al., 2018). To the best of our knowledge, it is the first report that the involvement of TK1 in the antitumor effect in colorectal cancer cells through cell-cycle modulation by celecoxib and/or ubenimex.

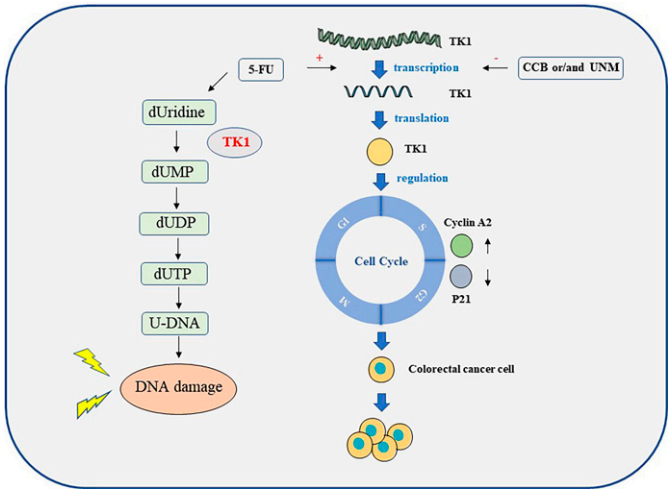

Fig. 8. The summary diagram of this paper and the TK1 part of 5-FU signaling pathway related to literature (Lee et al., 2010).

To further study the mechanism of the combination of the two drugs causing colorectal cancer cell cycle arrest, we further analyzed the results of the transcriptome and found that there might be a relationship of cell cycle-related proteins MYBL2, RRM2 with TK1 (Supplemental Fig. 3A). The results of transcriptomic analysis showed that MYBL2 and RRM2 mRNA decreased after the treatment of the single drug alone compared with the control group, and the expression level of the combination group was the lowest among all the groups (Supplemental Fig. 3B). Qian Liu (Liu et al., 2021) and colleagues showed that MYBL2 can directly bind to the promoter region of RRM2 in colorectal cancer cells to activate the transcription of RRM2 and affect the cell cycle. Thus, TK1 may influence the cell cycle through MYBL2/RRM2 axis. Based on this, we will further study other mechanisms involved in the signaling pathway of the combination in the future.

In summary, ubenimex enhances the antitumor activity of celecoxib in vitro and in vivo, and TK1 is involved in the mechanism through cell cycle modulation independent of COX-2 and CD13 in colorectal cancer. Celecoxib and/or ubenimex decreases the expression of TK1 protein at mRNA level (Fig. 8). In this study, our data reveal the great potential of celecoxib combined with ubenimex in the treatment of colorectal cancer, providing new ideas for clinical antitumor drug regimens and theoretical reference for drug development.

Authorship Contributions

Participated in research design: Chen.

Conducted experiments: A. Wang, Shang, Ni, W. Wang.

Performed data analysis: A. Wang, Ni, W. Wang, Chen.

Wrote or contributed to the writing of the manuscript: A. Wang, C. Wang, Li, Chen.

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