**Original Paper**

# Efficacy of the MEK Inhibitor Cobimetinib and its Potential Application to Colorectal Cancer Cells

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## Key Words

Cobimetinib • Colorectal Cancer • Cancer therapy • Thymidylate synthetase

## Abstract

***Background/Aims:*** Mutations in the Ras/Raf/MEK/ERK pathway are detected in 50% of colorectal cancer cases and play a crucial role in cancer development and progression. Cobimetinib is a MEK inhibitor approved for the treatment of advanced melanoma and inhibits the cell viability of other types of cancer cells. ***Methods:*** HCT116 colorectal cancer cells were treated with cobimetinib, and MTT assay, colony formation assay, and flow cytometry were used to evaluate cell viability, cell cycle, and apoptosis, respectively. The expression of genes associated with the cell cycle and apoptosis were evaluated by quantitative real-time PCR and western blotting. To explore use of cobimetinib in colorectal cancer treatment and further understand its mechanisms, RNA-seq technology was used to identify differentially expressed genes (DEGs) between cobimetinib-treated and untreated HCT116 cells. Furthermore, we compared these DEGs with Gene Expression Omnibus data from colorectal cancer tissues and normal colonic epithelial tissues. ***Results:*** We found that cobimetinib not only inhibited cell proliferation but also induced G1 phase arrest and apoptosis in HCT116 colorectal cancer cells, suggesting that cobimetinib may useful in colorectal cancer therapy. After cobimetinib treatment, 3,495 DEGs were obtained, including 2,089 upregulated genes and 1,406 downregulated genes, and most of these DEGs were enriched in the cell cycle, DNA replication, and DNA damage repair pathways. Our results revealed that some genes with high expression in colorectal cancer tissues were downregulated by cobimetinib in HCT116 cells, including CCND1, E2F1, CDC25C, CCNE2, MYC, and PCNA. These genes have vital roles in DNA replication and the cell cycle. Furthermore, genes with low expression in colorectal cancer tissues were upregulated by cobimetinib, including PRKCA, PI3K, RTK, and PKC. Based on our results, the PKC and PI3K pathways were activated after cobimetinib treatment, and inhibition of these two pathways can increase the cytotoxicity of cobimetinib in HCT116 cells. Notably, cobimetinib appeared to enhance the efficacy of 5-fluorouracil (5-FU) by decreasing

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TYMS expression, high expression of which is responsible for 5-FU resistance in colorectal cancer. ***Conclusions:*** Our results suggest the potential use of cobimetinib in colorectal cancer therapy.

## Introduction

The Ras/Raf/MEK/ERK pathway regulates cell proliferation, survival, differentiation, and angiogenesis, and plays a critical role in tumorigenesis and development [1, 2]. Mutations in the composition of this pathway, especially the acquired mutation of K-Ras or B-Raf, can result in its hyperactivation, which is highly correlated with tumorigenesis [3]. The occurrence of colorectal cancer is accompanied by mutations in many genes, though K-Ras is the most common type of mutation [4]. As a downstream molecule of Ras, Raf mutations also play an important role in the pathogenesis of colorectal cancer [5]. Together, the Ras/Raf/MEK/ERK pathway is an important target for the discovery of anticancer drugs to treat colorectal cancer.

Thus far, a growing number of small molecule inhibitors have been used in clinical treatment. They can effectively prolong the survival of patients with colorectal cancer, especially metastatic colorectal cancer [6]. Regorafenib is the first small molecule inhibitor approved by the FDA for the treatment of metastatic colorectal cancer, and it targets the Ras/Raf/MEK/ERK signaling pathways [7]. Zhang et al. reported that regorafenib inhibited the growth of colorectal cancer by inducing PUMA-mediated apoptosis [8]. However, to date, there are only two MEK-specific inhibitors approved by the FDA for clinical use, namely, trametinib and cobimetinib.

Cobimetinib (GDC-0973) is a potent and selective small molecule inhibitor that specifically targets MEK1/2. Cobimetinib selectively inactivates MEK kinase, thereby blocking the transduction of Ras/Raf pathway signaling [9]. In 2015, the FDA approved cobimetinib for BRAF-mutated melanoma in combination with vemurafenib, a BRAF inhibitor [10]. It has been shown that cobimetinib inhibits the cell viability of many cancer cells, including colorectal cancer [11, 12]. Regarding safety, cobimetinib treatment had a well-tolerated profile, even at the maximum administered oral daily doses of 60 mg (Clinical Trial: NCT01988896) [13]. However, its effect and mechanism of action on colon cancer is still uncertain.

In this study, we found that cobimetinib could induce G1 arrest and apoptosis in HCT116 colorectal cancer cells, which is consistent with a previous study [12]. To further investigate the mechanisms of cobimetinib on colorectal cancer, RNA-seq technology was employed to explore differentially expressed genes (DEGs) after cobimetinib treatment. Through a combined analysis with Gene Expression Omnibus (GEO) data from colorectal cancer tissues and normal colonic epithelial tissues, we found that cobimetinib can induce the activation of PKC and PI3K pathways and enhance the efficacy of 5-fluorouracil (5-FU) by decreasing TYMS expression. Our results reveal a high potential for using cobimetinib in colorectal cancer treatment.

## Materials and Methods

*Cell culture and treatment*

The human colorectal cancer cell lines HCT116, RKO, HT-29, SW480, and DLD-1 were obtained from the Cell Library of the Chinese Academy of Sciences. HCT116 p53$^{-/-}$ and HCT116 p21$^{-/-}$ were described previously [14] and obtained from Dr. Bert Vogelstein (Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD, USA). All cell lines were grown in high-glucose Dulbecco's modified Eagle medium(HyClone, South Logan, UT, USA) supplemented with 10% (*v/v*) defined fetal bovine serum (Gibco, Gaithersburg, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone) and then maintained in a humidified incubator with 5% $CO_2$ at 37°C. Cobimetinib, VS-5584, and Go 6983 were

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purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at stock concentration. Then, 5-FU (Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS, pH 7.4) at stock concentration. Drugs at stock concentrations were stored in the dark at -20°C. For drug treatment assays, the cells were seeded into 6-well or 12-well plates, and then treated with concentrations prepared by appropriate dilution with the complete culture medium when cells reached about 30% confluence.

### Cell viability assay (MTT assay) and combination index determination

Cell viability was evaluated by MTT assays. HCT116 colorectal cancer cells were trypsinized and seeded into 96-well plates at a density of 8, 000 cells per well. After overnight incubation, cells were treated with different concentrations of the agents for 48 h, and the DMSO-treated (0.1%) cells were regarded as the control group. Then, 10 μL MTT solution (5 mg/mL) was added to each well and incubated for 4 h. Afterwards, the supernatant was replaced with 100 μL DMSO to dissolve the insoluble formazan crystals. Finally, the absorbance was detected at 490 nm or 570 nm. The growth inhibition (%) was determined using the following equation: Growth inhibition (%) = 1 - (Mean of absorbance of treated group) / (Mean of absorbance of control group)] × 100%. A combination index (CI) was calculated via the Chou–Talalay method [15]. The synergism of the two drugs was evaluated by the CI, where CI > 1 indicates antagonism, CI = 1 indicates additivity, and CI < 1 indicates synergism.

### Colony formation assay

After drug treatment, the cells were seeded into 6-well plates and then incubated at 37°C with 5% $CO_2$ for 2 weeks until visible clones appeared. Then, the cells were carefully washed twice with PBS, fixed with methyl alcohol for 20 min, and stained with 0.1% crystal violet solution for 30 min.

### Western blot analysis

After drug treatment, the cells were gathered and lysed with 2× sample buffer (10% sodium dodecyl sulfate [SDS], Tris-HCl pH 6.8, glycerol, 0.5% bromophenol blue, and β-mercaptoethanol). The protein samples were boiled at 96°C for 10 min and stored at -20°C. Then, the samples were isolated by SDS polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The non-specific sites of the membrane were blocked using 5% skim milk (*w/v*) in tris-buffered saline with 0.1% Tween-20 (TBST) for 1.5 h at room temperature and incubated at 4°C with primary antibodies overnight. The primary antibodies were anti-p21 (1:2000, Proteintech, Chicago, IL, USA), anti-p53 (1:2000, Proteintech), anti-cyclin D1 (1:2000, Zen BioScience, Chengdu, China), anti-cyclin E (1:2000, Zen BioScience), anti-TYMS (1:2000, Zen BioScience), anti-β-actin (1:5000, Proteintech), and anti-PARP (1:2000, Zen BioScience). The membranes were washed with TBST three times and then incubated with corresponding secondary antibodies for 1.5 h at room temperature. Finally, the blots were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

### Quantitative real-time PCR (qRT-PCR)

Total RNA of the cells was isolated using the RNAiso Plus kit (TaKaRa, Dalian, China) according to the manufacturer's protocol and was stored at -80°C. The PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa) was used to synthesize first-strand cDNAs. Subsequently, target gene expression was examined using EvaGreen 2× qPCR MasterMix-No Dye (abm, Richmond, BC, Canada) by qRT-PCR. The relative expression levels of the target genes were analyzed according the $2^{-\Delta\Delta Ct}$ method. The *GAPDH* gene was used as an internal control to standardize variance among the target gene expression levels. All sample measurements were conducted in triplicate. qRT-PCR primers were synthesized by BGI TECH (Beijing, China), and the primer sequences are listed in Table 1.

### Cell cycle arrest assay

Colorectal cancer cells were plated into 6- or 12-well plates and treated with various concentrations of reagents for 48 h. Then, the cells were harvested, washed with PBS, centrifuged and fixed in cold 70% ethanol overnight at 4°C. After fixation, the samples were washed with PBS. Afterward, the cells were resuspended in PBS containing 10 mg/mL propidium iodide (PI; Sigma-Aldrich) and RNase A (100 mg/mL, Solarbio, Beijing, China) and incubated for 30 min under darkness at room temperature. Finally, the

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samples were analyzed using the BD FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

*Cell apoptosis assay*

Cell apoptosis was examined using the annexin V-fluorescein isothiocyanate (FITC) staining (Annexin-FITC Apoptosis Detection Kit, Biotool, Houston, TX, USA) with flow cytometry. Colorectal cancer cells were seeded into 6- or 12-well plates and treated with various concentrations of reagents for 48 h. Then, the cells were harvested, washed with PBS, centrifuged, and resuspended in 1× binding buffer. Next, the cells were stained with annexin V-FITC and PI for 10 min in the dark at room temperature. Finally, the samples were analyzed using the BD FACScalibur flow cytometer (BD Biosciences).

Alternatively, Hoechst 33258 staining was also employed to evaluate cell apoptosis. Briefly, the cells were harvested, washed with PBS, and centrifuged. Then, the cells were incubated with Hoechst 33258 staining solution (300 μL PBS, 20 μL 10% NP-40, 36 μL 37% formaldehyde, 4 μL 1 mg/mL Hoechst 33258) for 30 min in the dark at room temperature. Finally, apoptosis was calculated under fluorescence microscopy.

*RNA-Seq and bioinformatics analysis*

The HCT116 cells were seeded into 6-well plates and treated with 1 μM cobimetinib for 24 h or 36 h. Then, the cells were collected by centrifugation and resuspended in RNAiso Plus (TaKaRa). Transcriptomes were sequenced on

**Table 1.** Primer sequences for qRT-PCR

| Gene Names | | Primers sequences (5'-3') |
|---|---|---|
| GAPDH | Forward | TACCAAGCTTATGGACGGGTCCGGGGAGCA |
| | Reverse | ATAGAATTCCGCCCATCTTCTTCCAGATGGTG |
| CCNA2 | Forward | TCACCAGACCTACCTCAAAG |
| | Reverse | CATCTTAGAAAACAAAGGCAGTC |
| CCNB1 | Forward | ACACCAACTCTACAACATTACCT |
| | Reverse | GGCTAAATCTTGAACTAGTGCAG |
| CCNE2 | Forward | ATACTGACTGCTGCTGCCTTG |
| | Reverse | ACATTCTGAAATACTGTCCCACT |
| CDC25C | Forward | CCCATCGTCCCTTTGGACACCCA |
| | Reverse | CCTCTTCACGCAGACAGCGGCAC |
| CHEK1 | Forward | GGGATACCAGCCCCTCATACATT |
| | Reverse | TTCCATTGATAGCCCAACTTCTC |
| E2F1 | Forward | CTCATCCCTCACCACAGATCCCA |
| | Reverse | AGAAGTCCTCCCGCACATGCTCC |
| MYC | Forward | CCCTGGTGCTCCATGAGGAGACA |
| | Reverse | GGGCTGTGAGGAGGTTTGCTGTG |
| PCNA | Forward | GTAATGTCGATAAAGAGGAGGAAG |
| | Reverse | ATACTGAGTGTCACCGTTGAAGA |
| PDCD4 | Forward | GAGACATATCTGAAGCTGAACAT |
| | Reverse | TGTACTTTCTCCAGTTGACTCTA |
| PRCKA1 | Forward | CAGCCACCATTCAAGCCCAAAGT |
| | Reverse | TACGAGAACCCTTCAAAATCAGA |
| RB1 | Forward | CCAGAAGCCATTGAAATCTACCT |
| | Reverse | AGGGTCCAGATGATATGTTCTAATT |
| TYMS | Forward | TGGAGGAGTTGCTGTGGTT |
| | Reverse | TGTCGTCAGGGTTGGTTTT |

the Illumina HiSeq platform (Illumina, San Diego, CA, USA). Thresholds of Q value < 0.05 and | $\log_2$(fold change) | > 1 were set for screening DEGs. Gene functional annotations were based on the genome database and mapped onto Gene Ontology (GO) terms in the GO database (http://www.geneontology.org/). Pathway analysis was based on the pathways available within the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The gene expression data for colorectal cancer tissues and normal colonic epithelial tissues were downloaded from the GEO database with the accession number GSE21815. We explored the intersection of genes between DEGs from RNA-seq and DEGs from CEO data. Then, the functions of these intersectional genes were annotated by KEGG. The raw reads are available from the NCBI Sequence Read Archive under accession numbers SRR5934059–SRR5934061, and the BioProject accession number is PRJNA398173.

*Statistical analysis*

All experiments were performed in triplicate and data were expressed as the mean ± standard deviation (SD). Student's *t*-tests were used to compare those data. Differences with $p < 0.05$ were regarded as statistically significant.

### Results

*Cobimetinib inhibited the cell viability of different colorectal cancer cells*

To investigate the effect of cobimetinib on colorectal cancer, five different colorectal cancer cell lines were treated with different concentrations of cobimetinib, and then cell

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viability was evaluated using the MTT assay. The results showed that cobimetinib decreased the cell viability of all five colorectal cancer cell lines (Fig. 1A). Furthermore, colony formation assays showed similar long-term effects (Fig. 1B and C).

*Cobimetinib induced G1 arrest in HCT116 cells*

Next, we explored how cobimetinib inhibited the cell viability of colorectal cancer cells. First, HCT116 cells were treated with cobimetinib at the $IC_{50}$ concentration (1 μM). Then, cell cycle changes were evaluated using flow cytometry after staining with PI. Cobimetinib was observed to induce cell arrest in the G1 phase (Fig. 2A). Moreover, western blots indicated that the expression levels of cyclin D1 and cyclin E were decreased and p21 levels were increased after cobimetinib treatment (Fig. 2B). Similarly, qRT-PCR results revealed that the mRNA expression of some important cell cycle-related genes, including CCND1, CCNA2, CCNE1, CCNB1, CDC25C, CHK1, E2F, Myc, PCNA, and Rb1, were decreased after cobimetinib treatment (Fig. 2C). These results suggest that cobimetinib induced G1 phase arrest by altering the expression of cell cycle-related genes in HCT116 cells.

*Cobimetinib induced G1 arrest in a p53/p21 independent manner in HCT116 cells*

Cobimetinib can significantly increase the expression level of p21 (Fig. 3A and B). p53 can initiate cell cycle arrest by regulating its target gene p21 or the transcription of a series of molecules. To demonstrate whether p53 was required for cobimetinib-induced upregulation of p21, p53-deficient HCT116 cells were treated using cobimetinib. The results showed that cobimetinib can increase both the mRNA and protein levels of p21 in HCT116 p53$^{-/-}$ cells (Fig. 3C and D). Thus, p53 expression was independent of cobimetinib-induced upregulation of p21 in HCT116 cells.

To explore whether p21 or p53 was necessary for cobimetinib-induced G1 arrest, p21 and p53-deficient HCT116 cells were treated with cobimetinib. Flow cytometry revealed that cobimetinib induced G1 arrest in the absence of p21 or p53. In addition, cobimetinib can also downregulate the expression of important cycle-related genes, including CCND1, CCNA2, CCNB1, CDC25C, CHK1, E2F, MYC, PCNA, and RB1, in p21-deficient cells (p21$^{-/-}$ HCT116), excepted for CCNE1 (Fig. 2C). These results suggest that cobimetinib induced G1 arrest in a p53/p21-independent manner in HCT116 cells.

**Fig. 1.** Cobimetinib inhibited the cell viability of different colorectal cancer cell lines. (A) HCT116, SW480, DLD-1, HT-29, and RKO cells were treated with different concentrations of cobimetinib (0–1 μM) for 48 h. Then cell viability was measured by MTT assays. (B and C) A colony formation assay was used to determine the long-term effect of cobimetinib. HCT116, SW480, DLD-1, HT-29, ad RKO cells were treated with 1 μM cobimetinib for 48 h, and then 1,000 cells were seeded into 6-well plates and incubated for 2 weeks. Colonies were then counted.

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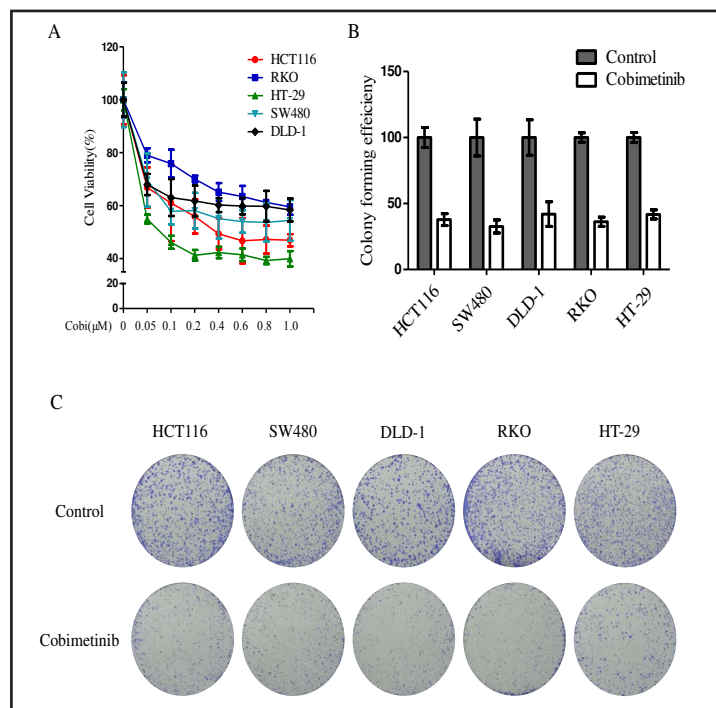
**Fig. 2.** Cobimetinib induced G1 arrest in HCT116 cells. (A and B) HCT116 cells were treated with 1 μM cobimetinib for 24 h, and then the cell cycle was evaluated using flow cytometry. The histogram provides statistical results of three independent experiments. (C) Western blots showing protein changes of p21, p53, cyclin D1, and cyclin E in HCT116 cells after treatment with 1 μM cobimetinib over different timespans. (D) mRNA expression levels of cycle-related genes were evaluated by qRT-PCR in parental HCT116 or p21$^{-/-}$ HCT116 cells. (**p<0.01)
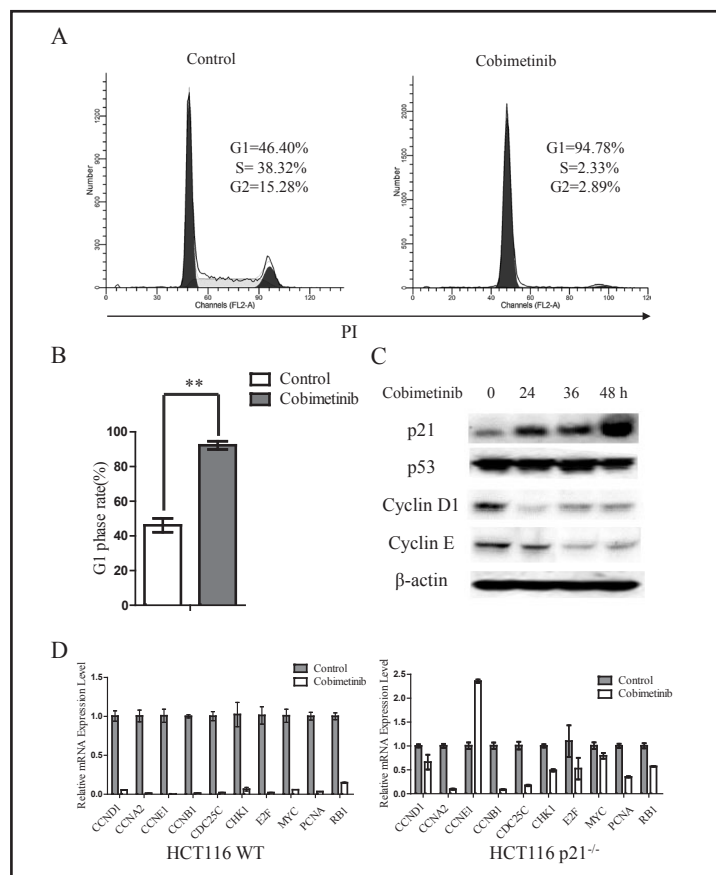


**Fig. 3.** Cobimetinib induced G1 arrest in a p53/p21-independent manner in HCT116 cells. (A) Relative mRNA levels of p21 were determined by q-PCR after treatment with cobimetinib (1 μM) for 48 h in HCT116 parental and p53$^{-/-}$ cells. (B) Western blots showing the p21 level in HCT116 parental and p53$^{-/-}$ cells. (C) HCT116 p53$^{-/-}$ and p21$^{-/-}$ cells were treated with cobimetinib (1μM), and the cell cycle distributions were determined by flow cytometry.
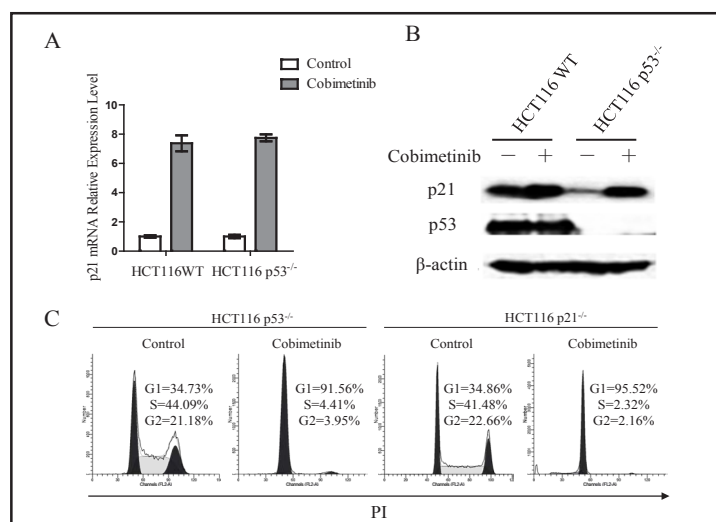


*Cobimetinib induced apoptosis in HCT116 cells*

It has been reported that cobimetinib can induce cell apoptosis in neuroblastoma cells [16]. To investigate whether cobimetinib could induce apoptosis in colorectal cancer cells, HCT116 was treated with cobimetinib and then stained with annexin V/PI. Flow cytometry was used to detect apoptosis induced by cobimetinib. As shown in Fig. 4A, more apoptotic cells were found after cobimetinib treatment compared with the control. The western blot result also showed that c-PARP (cleaved PARP, activated form) was increased gradually by cobimetinib (Fig. 4B). In addition, we also found that cobimetinib significantly induced apoptosis in both p21 and p53 deficient cells (p21$^{-/-}$ HCT116 or p53$^{-/-}$ HCT116; Fig. 4C).

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The results further suggested that cobimetinib can induce apoptosis in a p53- or p21-independent manner in HCT116 cells.
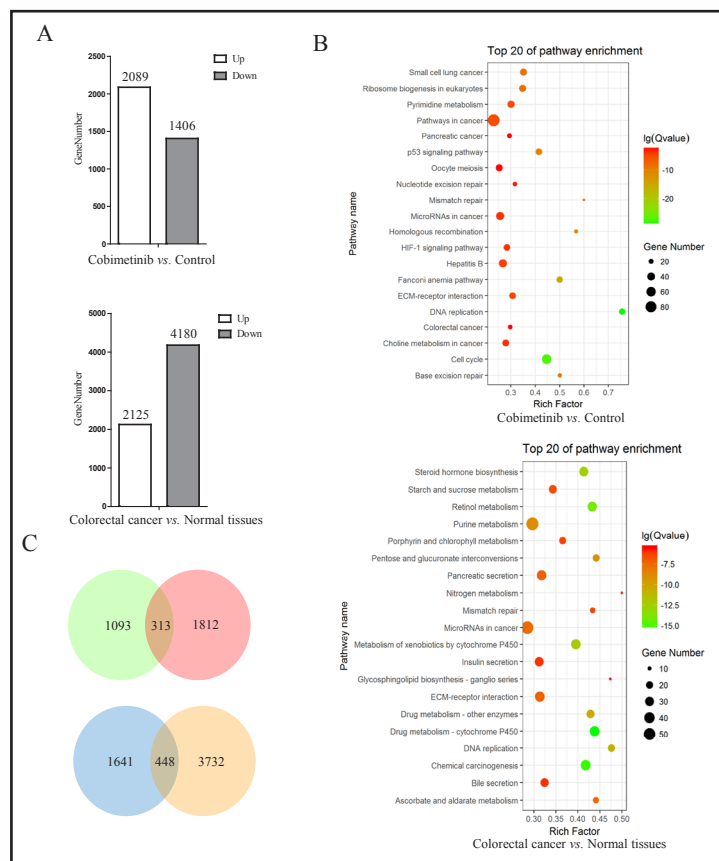
### Comparative analysis of transcriptome data and GEO data

To explore the application of cobimetinib on colorectal cancer and its underlying mechanisms, we employed RNA-seq technology to analyze the expression changes induced by cobimetinib. HCT116 cells were treated with cobimetinib for 24 h or 36 h, and then

**Fig. 4.** Cobimetinib induced apoptosis in HCT116 cells. (A) Percentage of apoptotic cells was detected by annexin-V and PI staining after cobimetinib (1 μM) treatment of HCT116 cells for 48 h. (B) Western blots showing the level of c-PARP was upregulated by cobimetinib (1 μM) treatment. (C) HCT116 p53$^{-/-}$ and p21$^{-/-}$ cells were treated with cobimetinib (1 μM), and cell apoptosis was evaluated by flow cytometry.
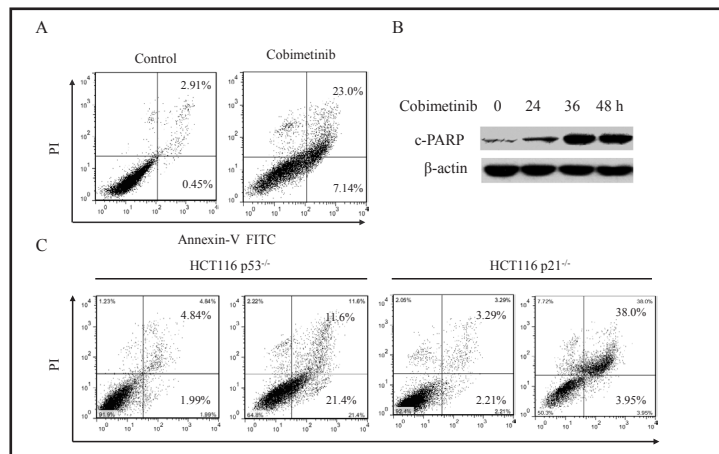


**Fig. 5.** Comparative analysis of transcriptome data and GEO data. (A) Number of downregulated and upregulated DEGs; upper panel, cobimetinib treatment vs. control; lower panel, colorectal cancer vs. normal tissues). (B) According to p-values, the top 20 enriched pathways for DEGs (upper panel, the pathways of cobimetinib vs. control; lower panel, pathways of colorectal cancer vs. normal tissues). (C) Venn diagrams showing the overlapping DEGs between transcriptome data and GEO data. Upper diagram, 313 genes highly expressed in colorectal cancer tissues were downregulated by cobimetinib in HCT116 cells (green, 1,406 downregulated DEGs after cobimetinib treatment in HCT116; red, 2,125 upregulated DEGs in colorectal cancer tissues compared to normal tissues). Lower diagram, 448 genes with lower expression in colorectal cancer tissues were upregulated by cobimetinib in HCT116 cells (blue, 2,089 upregulated DEGs after cobimetinib treatment in HCT116; orange, 4,180 downregulated DEGs in colorectal cancer tissues compared to normal tissues).

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the transcriptomes were sequenced. We found that expression patterns of HCT116 after cobimetinib treatment for 24 or 36 h showed the same trend. Therefore, RNA-seq data after cobimetinib treatment for 24 h was used for the next study. After cobimetinib treatment for 24 h, 3, 495 differentially expressed genes (DEGs) were found, including 2, 089 upregulated genes and 1, 406 downregulated genes (Fig. 5A, upper panel), and most of these DEGs were mapped to the DNA replication, cell cycle, Fanconi anemia, p53 signaling, mismatch repair, and base excision repair pathways (Fig. 5B).

We downloaded the data set corresponding to GEO series accession number GSE21815 [17, 18] from the GEO database, which includes gene expression profiles of 132 colorectal cancer tissues and nine normal human intestinal epithelial tissues. DEGs between normal tissues and tumors were analyzed using GeneSpring software. Finally, by comparisons with normal tissues, 6, 305 DEGs were obtained from among colorectal tumors, including 2, 125 upregulated genes and 4, 180 downregulated genes (Fig. 5A, lower panel; Table, 2). GO and KEGG analyses showed that these DEGs were enriched in the pathways of DNA replication, mismatch repair, drug metabolism-other enzymes, drug metabolism-cytochrome P450, and pentose and glucuronate interconversions (Fig. 5B).

To further determine the key modulators for cobimetinib treatment, a comparative analysis was performed between the RNA-seq and GEO datasets. We found that a total of 313 genes that were highly expressed in colorectal cancer tissues were downregulated by cobimetinib in HCT116 cells (Fig. 5C, upper), including CCND1, E2F1, CDC25C, CCNE2, MYC, and PCNA. Furthermore, 448 genes with lower expression in colorectal cancer tissues were upregulated by cobimetinib treatment in HCT116 cells (Fig. 5C, lower panel), including PRKCA, PI3K, RTK, and PKC. Then, we conducted a KEGG pathway enrichment analysis for these up- and downregulated genes. Table 3 shows the statistically significant KEGG pathways and the correlated DEGs (Table 3). The results of overlap and pathway enrichment analysis revealed that PKC and PI3K pathways were activated after cobimetinib treatment.

*Inhibition of the PI3K or PKC pathway could increase cobimetinib cytotoxicity in HCT116 cells*

Through bioinformatics analyses, we found that PI3K and PKC pathways were activated after cobimetinib treatment. Activation of the PI3K or PKC pathway has been reported to be associated with tumorigenesis [19–21]. Therefore, activation of the PI3K and PKC pathways may attenuate the effects of cobimetinib. To explore the possibility of combination therapy with other inhibitors, we used Go 6983 (a Pan PKC inhibitor) and VS-5584 (a PI3K-mTOR dual inhibitor) alone or in combination with cobimetinib to treat HCT116 cells. MTT assays showed that both Go 6983 and VS-5584 can inhibit the viability of HCT116 cells and also have synergistic effects with cobimetinib (with a CI of less than 1) in HCT116 cells (Fig. 6A and B). Furthermore, treatment with Go 6983 or VS-5584 alone induced moderate G1 arrest and apoptosis in HCT116 cells, and Go 6983 or VS-584 significantly induced cell cycle arrest and apoptosis in combination with cobimetinib (Fig. 6C and D). These results revealed that inhibition of the PI3K or PKC pathway could increase the cytotoxicity of cobimetinib in HCT116 cells.

*Cobimetinib enhances 5-FU cytotoxicity by decreasing TYMS expression in HCT116 cells*

Intriguingly, we found that thymidylate synthetase (TYMS), an important direct target of

**Table 2.** Top 10 most significant differentially expressed genes upregulated and downregulated in colorectal cancer tissues compared with normal tissues

| Upregulated genes | | Downregulated genes | |
|---|---|---|---|
| Gene | log FC | Gene | log FC |
| TMEM97 | -1.149511 | ZG16 | 9.3678875 |
| SP5 | -2.9767313 | CA1 | 8.558522 |
| HSPD1 | -1.1093872 | GCG | 7.6726413 |
| PAQR4 | -1.1977377 | CLCA1 | 7.436072 |
| IL2RA | -1.2920417 | SLC4A4 | 7.203979 |
| DYNLT3 | -1.7005748 | ITLN1 | 6.9722238 |
| POLE2 | -1.2588199 | CLCA4 | 6.8729124 |
| CCND1 | -1.3114972 | GUCA2B | 6.8429737 |
| BRCA2 | -1.4148383 | CA4 | 6.5177035 |
| CENPN | -1.3245826 | CA2 | 6.4782157 |

**Table 3.** Top five statistically significant KEGG pathways and correlated DEGs among colorectal cancer tissues. Note: The top half of the table shows the top five statistically significant KEGG pathways enriched among 448 upregulated DEGs in colorectal cancer tissues compared to normal tissues. The lower half of the table shows the top five statistically significant KEGG pathways enriched among 313 downregulated DEGs in colorectal cancer tissues compared to normal tissues

| | Pathway ID | Pathways | Gene Counts | P value | Genes |
|---|---|---|---|---|---|
| Upregulated genes | ko05032 | Morphine addiction | 7 | 0.002918 | PRKCA, ADCY9, PDE3A, PDE8B, PDE4D, GNG7, GNG13 |
| | ko04713 | Circadian entrainment | 7 | 0.003085 | PRKCA, ADCY9, CAMK2B, PRKG1, CACNA1I, GNG7, GNG13 |
| | ko04360 | Axon guidance | 8 | 0.004132 | SEMA3B, CXCR4, UNC5B, SEMA6A, EPHA4, SEMA4G, SLIT2, ROBO3, |
| | ko04151 | PI3K-Akt signaling pathway | 15 | 0.004952 | SGK2, THBS1, PRKCA, CHAD, ITGB7, FGFR2, EPOR, PIK3R5, COL4A5, COL4A6, NR4A1, FGFR3, GNG13, LAMA3, GNG7, |
| | ko04810 | Regulation of actin cytoskeleton | 11 | 0.005664 | ACTN2, SLC9A1, ITGB7, SCIN, NCKAP1L, FGFR2, PIK3R5, ITGAX, FGFR3, IQGAP2, GSN |
| Downregulated genes | ko04110 | Cell cycle | 22 | 1.31E-15 | CCND1, RBL1, CHEK1, ORC6, RB1, E2F1, CDC25C, MAD2L1, CCNE2, PLK1, MYC, BUB3, PCNA, ORC3, MCM4, CCNA2, CDC6, BUB1, CDK1, CCNB1, TTK, TFDP1 |
| | ko03030 | DNA replication | 12 | 2.47E-12 | PRIM1, RNASEH2A, RPA3, PCNA, POLE2, PRIM2, RFC5, MCM4, RFC2, RFC3, RFC4, RNASEH1 |
| | ko03430 | Mismatch repair | 7 | 5.05E-07 | RPA3, PCNA, EXO1, RFC5, RFC2, RFC3, RFC4 |
| | ko04914 | Progesterone-mediated oocyte maturation | 9 | 4.21E-05 | HSP90AA1, HSP90AB1, CDK1, CCNA2, CDC25C, MAD2L1, PLK1, BUB1, CCNB1 |
| | ko03420 | Nucleotide excision repair | 7 | 0.000202 | RPA3, PCNA, POLE2, RFC5, RFC2, RFC3, RFC4 |

5-Florouracil (5-FU), was significantly downregulated by cobimetinib through bioinformatic analyses. TYMS plays a key role in DNA synthesis and its high expression is responsible for the 5-FU resistance of colorectal cancer [22, 23]. qRT-PCR and western blot analysis showed that both mRNA and protein levels of TYMS decreased significantly after cobimetinib treatment in HCT116 cells (p<0.01) (Fig. 7A). Therefore, we hypothesized that cobimetinib could enhance the cytotoxicity of 5-FU in HCT116 cells. To explore the potential synergy between cobimetinib and 5-FU, we evaluated effects of single and combination treatment with cobimetinib and 5-FU in HCT116 cells by MTT assay. Cobimetinib was thus revealed to act synergistically with 5-FU, with a CI of less than 1 (Fig. 7B). In order to further explore their combined effects, we examined the cell cycle by using flow cytometry. As shown in Fig. 7C, combination treatment with cobimetinib and 5-FU showed more G1 arrest than treatment with cobimetinib or 5-FU alone. These results suggest that cobimetinib could enhance 5-FU cytotoxicity in HCT116 cells.
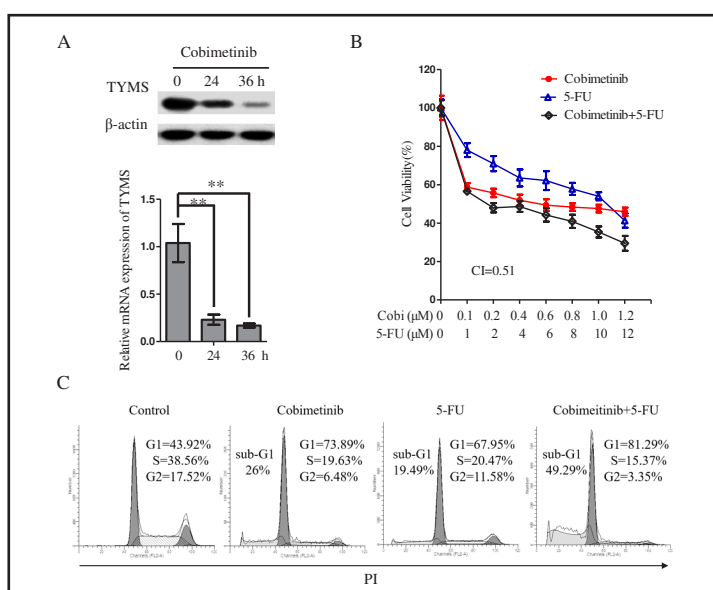
## Discussion

Cobimetinib is a drug marketed for treating BRAF-mutated melanoma. Previous studies have shown that cobimetinib can inhibit the cell viability of many cancer cells, such as hepatocellular carcinoma [16, 24]. In this study, we found that cobimetinib could inhibit cell

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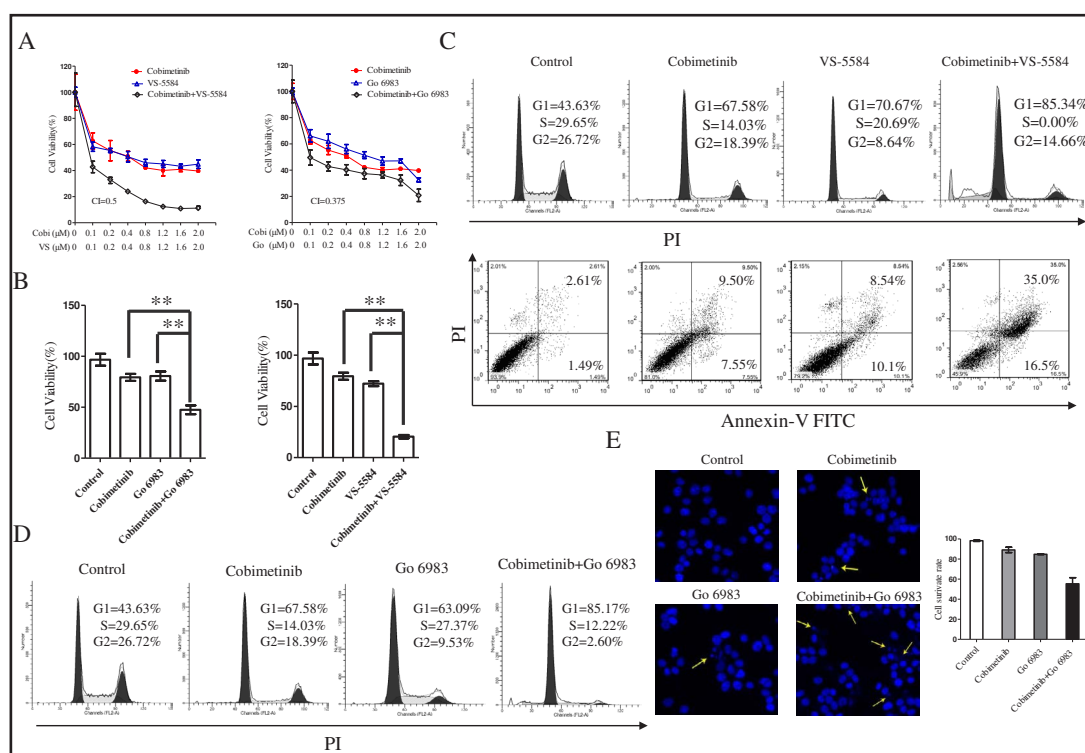
**Fig. 6.** Go 6983 and VS-5584 increased the cytotoxicity of cobimetinib in HCT116 cells. (A) HCT116 cells were treated with cobimetinib or 5-FU alone or with cobimetinib and 5-FU in combination at indicated concentrations for 48 h. Cell viability was evaluated by MTT assays. Error bars represent means ± SD. (B) HCT116 cells were treated with Go 6983 (0.75 μM) or VS-5584 (0.6 μM) alone or in combination with cobimetinib (0.15 μM) for 48 h. Then, cell viability was evaluated by MTT (C, D, and E) HCT116 cells were treated with Go 6983 (0.75 μM) or VS-5584 (0.6 μM) alone or in combination with cobimetinib (0.15 μM) for 48 h. Then, cell cycle arrest was determined by flow cytometry, and cell apoptosis was determined by flow cytometry or Hoechst 33258 staining. * *p<0.01.

**Fig. 7.** Cobimetinib enhances 5-FU cytotoxicity in HCT116 cells. (A) Protein and mRNA levels of TYMS after cobimetinib (1 μM) treatment. (B) HCT116 cells were treated with cobimetinib or 5-FU alone or with cobimetinib and 5-FU in combination at indicated concentrations for 48 h. Cell viability was evaluated by MTT assay. Error bars represent means ± SD. (C) HCT116 cells were treated with cobimetinib (0.05 μM) or 5-FU (2.5 μM) alone or with cobimetinib (0.05 μM) and 5-FU (2.5 μM) in combination for 48 h, and then the cell cycle distributions were determined by flow cytometry. * *p<0.01.

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viability by inducing G1 cell arrest and apoptosis in HCT116 colorectal cancer cells. Moreover, G1 arrest induced by cobimetinib was independent of p53 or p21. We also found that cobimetinib may induce G1 arrest via downregulation of cell cycle related genes, including CCND1, E2F1, CDC25C, CCNE2, MYC, and PCNA. CCND1 and CDC25C are downstream of MEK/ERK. ERK can regulate cyclin D1 transcriptional induction via Fos family members and MYC [25–27], and cyclin D1 can promote phosphorylation and inactivation of Rb, which then activates E2F1 and induces cyclin E expression [28]. In addition, ERK also regulates the assembly of the cyclin/CDK complex [25].

In order to further understand the mechanisms of cobimetinib, we performed RNA-seq to identify DEGs between treatments with and without cobimetinib in HCT116 cells and compared these DEGs with the GEO dataset from colorectal cancer tissues and normal colonic epithelial tissues. Finally, we found that PKC and PI3K/Akt pathways were activated after cobimetinib treatment.

PKC, a family of specific serine/threonine kinases, is involved in different cellular processes via target protein phosphorylation [29]. PKCα is expressed in many tissues, particularly in transformed cells, and plays a key role in the process of cell carcinogenesis and tumor angiogenesis. PKCα can regulate cell division and promote cancer cell growth. Previous studies have shown that inhibition of PKCα can decrease cell proliferation and migration and that interfering with the expression of PKCα by siRNA can reduce multidrug resistance in cancer cells and restore their sensitivity to anticancer drugs [30, 31]. The effects of PKCα on cancer cells is related not only to the cell types but also to the conditions [32]. It has been reported that PKCα could inhibit the occurrence of intestinal cancer and the expression level of PKCα in colorectal cancer cells is low relative to other cancers [33, 34]. In contrast, some studies have shown that PKCα activation can promote cancer cell proliferation, migration, survival, and drug resistance [35–37]. In colorectal cancer, the PKCα inhibitor can reduce cell viability and inhibit cell migration [38]. In this study, we showed that the PKCα inhibitor Go 6983 could enhance the cytotoxicity of cobimetinib in HCT116 cells.

The PI3K/Akt pathway plays an important role in many cellular processes, such as cell survival, proliferation, migration, differentiation, and tumorigenesis [39–41]. Several studies have indicated that aberrant activation of the PI3K/Akt pathway is found in many human cancers, including colorectal cancer [20, 42]. Accordingly, the PI3K/Akt pathway is an ideal target for cancer therapy [41]. At present, the PI3K/Akt inhibitor has been applied to cancer therapy in preclinical trials [41, 43, 44]. In this study, the results of RNA-seq and GEO data revealed that the PI3K/Akt pathway was activated after cobimetinib treatment of HCT116 cells. Furthermore, the PI3K inhibitor VS-5584 can enhance the cytotoxicity of cobimetinib in HCT116 cells. Thus, inhibition of PKC and PI3K/Akt pathways is an effective method for enhancing the efficacy of cobimetinib in colorectal cancer cells.

5-FU is widely used in the treatment of many cancers, especially colorectal cancer. TYMS is a downstream target molecule of 5-FU and plays a key role in DNA synthesis. TYMS can catalyze the methylation of deoxyuridine monophosphate to produce deoxythymidine triphosphate, followed by synthesis of thymidylate [45]. It has been reported that the high expression of TYMS is responsible for resistance to 5-FU in patients with colorectal cancers [46, 47]. Moreover, high TYMS expression levels is a sign of poor prognosis in colorectal cancer [48, 49]. Intriguingly, we found that cobimetinib can significantly decrease TYMS expression in both mRNA and protein levels and enhance the efficacy of 5-FU. In addition, our results also suggest that cobimetinib can act synergistically with 5-FU.

Taken together, our data showed that cobimetinib not only inhibited cell proliferation but also induced G1 phase arrest and apoptosis in colorectal cancer cells HCT116. Furthermore, inhibition of the PKC or PI3K pathway can increase the cytotoxicity of cobimetinib in HCT116 cells. Notably, we found that cobimetinib could enhance the efficacy of 5-FU by decreasing TYMS expression. Considering the use of cobimetinib as a clinical drug and its synergistic action with 5-FU, our results suggest the potential utility of cobimetinib in colorectal cancer therapy.

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## Disclosure Statement

The authors declare that they have no conflict of interests.

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