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# Romidepsin Induces G2/M Phase Arrest and Apoptosis in Cholangiocarcinoma Cells

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## Abstract

**Background:** Cholangiocarcinoma (CCA) is an extremely intractable malignancy since most patients are already in an advanced stage when firstly discovered. CCA needs more effective treatment, especially for advanced cases. Our study aimed to evaluate the effect of romidepsin on CCA cells *in vitro* and *in vivo* and explore the underlying mechanisms. **Methods:** The antitumor effect was determined by cell viability, cell cycle and apoptosis assays. A CCK-8 assay was performed to measure the cytotoxicity of romidepsin on CCA cells, and flow cytometry was used to evaluate the effects of romidepsin on the cell cycle and apoptosis. Moreover, the *in vivo* effects of romidepsin were measured in a CCA xenograft model. **Results:** Romidepsin could reduce the viability of CCA cells and induce G2/M cell cycle arrest and apoptosis, indicating that romidepsin has a significant antitumor effect on CCA cells *in vitro*. Mechanistically, the antitumor effect of romidepsin on the CCA cell lines was mediated by the induction of G2/M cell cycle arrest and promotion of cell apoptosis. The G2/M phase arrest of the CCA cells was associated with the downregulation of cyclinB and upregulation of the p-cdc2 protein, resulting in cell cycle arrest. The apoptosis of the CCA cells induced by romidepsin was attributed to the activation of caspase-3. Furthermore, romidepsin significantly inhibited the growth of the tumor volume of the CCLP-1 xenograft, indicating that romidepsin significantly inhibited the proliferation of CCA cells in vivo. **Conclusions:** Romidepsin suppressed the proliferation of CCA cells by inducing cell cycle arrest through cdc2/cyclinB and cell apoptosis by targeting caspase-3/PARP both *in vitro* and *in vivo*, indicating that romidepsin is a potential therapeutic agent for CCA.

## Keywords

romidepsin, cholangiocarcinoma, cdc2/cyclinB, caspase-3/PARP, proliferation

## Abbreviations

CCA, cholangiocarcinoma; iCCA, intrahepatic; pCCA, perihilar; TACE, transarterial chemoembolization; HATs, histone acetyltransferases; HDACs, histone deacetylases; HDAC, Histone deacetylase; HDACis, HDAC inhibitors; PTCL, peripheral T-cell lymphomas; FBS, fetal bovine serum; IHC, immunohistochemistry; SD, standard deviation

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## Introduction

Cholangiocarcinoma (CCA) is a malignant epithelial cancer with a high mortality rate. There are 3 types of CCA divided by the anatomical location, namely, intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA).[1] In recent decades, the incidence and mortality of CCA have increased, mainly because CCA is insensitive to traditional therapies such as systemic chemotherapy, transcatheter arterial chemoembolization (TACE), chemoradiotherapy and targeted therapy.[2]Furthermore, patients with advanced CCA are not suitable for complete surgical resection and liver transplantation.[3] For CCA patients, the optimal treatment option is locoregional systemic chemotherapeutics. Therefore, some studies suggest that locoregional systemic chemotherapeutics should be used as a first-

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line treatment for patients with unresectable advanced CCA, and this method could significantly extend the overall survival of patients.[4,5] Currently the combination of gemcitabine and cisplatin is usually applied to patients with inoperable disease.[5] However, the mean total survival time of patients with advanced CCA is only 11.7 months, and the prognosis remain poor.[5]

It is well acknowledged that histone modifications can alter the chromatin structure and dynamically influence transcriptional regulation.[6] There are many modifications, such as acetylation, phosphorylation, methylation and ubiquitination, that exhibit synergistic or antagonistic interaction affinities with chromatin-associated proteins.[7] The most studied modifications are histone acetylation and deacetylation, and these modifications are controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs).[8] Histone deacetylase (HDAC) inhibitors represent a clinically validated therapeutic strategy with a promising future in cancer treatment. Moreover, it has been reported that HDAC inhibitors (HDACis) can inhibit angiogenesis and induce cell cycle arrest, differentiation and programed cell death in vitro and in patients in phase I and II clinical trials.[9]

Romidepsin is a potent HDACi FDA-approved for patients with relapsed peripheral T-cell lymphomas (PTCL).[10] In addition, it has been reported that romidepsin(FK228), an HDAC1/2 specific inhibitor, could increase $p19^{INK4d}$ and $p21^{Waf1/Cip1}$ expression, decrease CDK expression and suppress hepatocellular carcinoma (HCC) growth.[11] Furthermore, romidepsin (FK228) exert an antitumor effect on endometrial cancer by activating the p53-p21 pathway.[12] However, the role of romidepsin in the treatment of solid tumors, including CCA, remains unclear and warrants further investigation.

In our study, we demonstrate that romidepsin inhibits CCA cell proliferation by inducing cell cycle arrest and apoptosis in vitro and in vivo, indicating that romidepsin is a promising therapeutic agent for CCA.

## Materials and Methods

### Reagents

The Cell Counting Kit-8 reagent was purchased from Dojindo (Japan). The cell cycle detection kit and Annexin-FITC apoptosis detection kit were purchased from KeyGen Biotech (Nanjing, China). Romidepsin (FK228) was purchased from Selleck Chemicals (Houston, TX). Romidepsin was dissolved in DMSO for the following in vitro and in vivo experiments. The primary antibodies against cleaved-caspase3, cleaved-caspase9, cleaved-PARP, PARP, CyclinB1, p-cdc2, Ki67 and GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA).

### Cell Lines and Cultures

Two CCA cell lines, i.e. CCLP-1, and HCCC-9810, were used in the study. The CCLP-1 cell line was obtained from DSMZ (Braunschweig, Germany). The HCCC-9810 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Both the CCLP-1 and HCCC-9810 cells were cultured in RPMI-1640 (Gibco, USA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and streptomycin. All cells were cultured in a humidified incubator with 5% CO2 at 37°C

### Cell Proliferation Assay

The cell viability was measured by a CCK-8 assay according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). Various concentrations of romidepsin were incubated with CCA cells for 24 h, 48 h and 72 h, respectively. The absorbance was detected by a microplate reader (DTX880; Beckman Coulter, Inc., Brea, CA, USA) at a wavelength of 450 nm.

### Cell Cycle and Apoptosis Assays

Briefly, the CCA cells were inoculated into a 6-well plate for the cell cycle analysis. After treatment with romidepsin or DMSO, the cells were collected and fixed in ice-cold 75% ethanol and incubated at -20°C overnight. Then, the fixed cells were washed with PBS, followed by incubation with 100 μL RNase A for 30 min. Then, the cells were resuspended in 400 μL PI at room temperature in the dark for 30 min. Cell cycle analysis was performed using a BDLSR II flow cytometer. For the analysis of cell apoptosis, the cells were pretreated with romidepsin or DMSO, followed by trypsinization. Then, the cells were washed with cold PBS. The washed cells were pelleted and stained with 5 μL Annexin V and 5 μL PI at room temperature for 15 min in the dark. The BD LSR II flow cytometer (BD Biosciences, San Diego, CA, USA) was used to analyze the above cells.

### Western Blot Analysis

CCA cells plated in 6-well plates were treated with romidepsin or DMSO for 48 h, and then the cells were lysed in lysis buffer. A BCA Protein Assay Kit was used to measure the protein concentrations. The proteins were heated with LDS sample buffer at 90°C for 10 minutes, separated by 10% SDS–PAGE and transferred to a polyvinylidene fluoride membrane. After blocking for 1 hour, the membrane was incubated overnight with primary antibodies at 4°C. Subsequently, the membrane was probed with a secondary antibody at room temperature for 1 hour.

### Xenograft Model Analysis

The protocol for the *in vivo* studies was approved by the Animal Experimental Ethical Inspection of Laboratory Animal Centre, Wenzhou Medical University (No. wydw 2019-0017). The experiment followed the Guide for the Care and Use of Laboratory Animals. Five-week-old male athymic nude mice were inoculated with CCLP-1 cells ($5 \times 10^6$). In total,

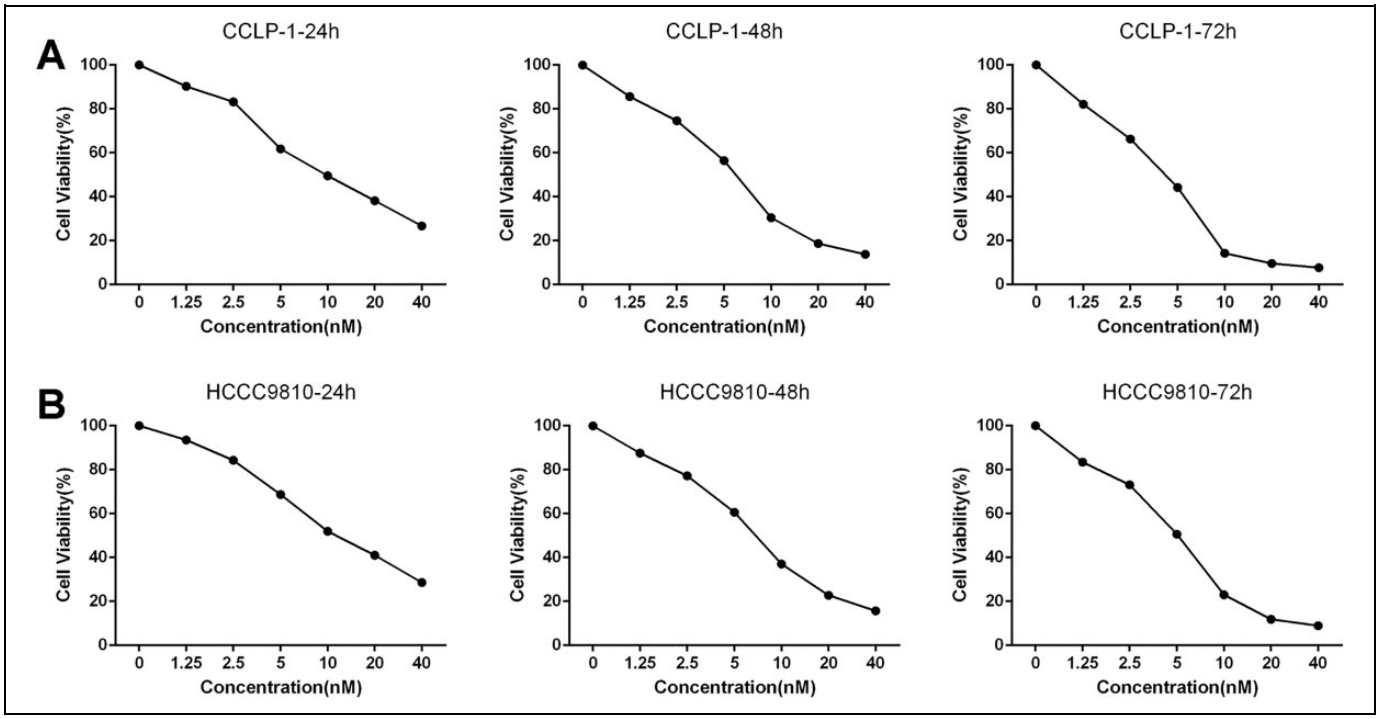


**Figure 1.** Effect of romidepsin on CCA cell proliferation. CCLP-1 cells (A) and HCCC9810 cells (B) were treated with increasing doses of romidepsin (0-40 nM) for 24 h, 48 h and 72 h. Then, CCA cell proliferation was assessed by a CCK-8 assay. Data are shown as the mean ± SD.

12 mice were randomly divided into the vehicle control and romidepsin (0.5 mg/kg) groups once the tumor volume reached ~100 $mm^3$ [volume ($mm^3$) = $(width)^2 \times length \times 0.5$, n = 6], and all mice were treated every 3 days for 28 days. The tumor volume and body weight were recorded every 4 days. After treatment for 4 weeks, all mice were sacrificed, and the tumors were isolated.

### *Tumor Histology and Immunohistochemistry*

Immunostaining (IHC) was performed on formalin-fixed, paraffin-embedded xenograft tumors. The protocol for the *in vivo* studies was in accordance with the animal welfare guidelines andapproved by the Institutional Animal Care and Use Committee. Xenograft tumor sections were incubated with a primary antibody against Ki-67 at 4°C overnight, followed by incubation with a secondary antibody at 37°C for 30 min.

### *Statistical Analysis*

Statistical analyses were performed using Student's t-test. All analyses were performed with SPSS 21.0 software. Differences were considered significant at $P < 0.05$. Data from representative experiments are presented as the means ± standard deviation (SD).

## Results

### *Romidepsin Inhibited the Proliferation of CCA Cells*

After incubation with romidepsin, the viability of CCLP-1 and HCCC-9810 cells was investigated by a CCK-8 assay. As shown in Figure 1, romidepsin clearly inhibited the viability of both the CCLP-1 and HCCC-9810 cells. Moreover, we found that romidepsin significantly suppressed the proliferation of the CCLP-1 cells when the concentration was 0 ~ 40 nM and incubation time was 24 h ~ 72 h (Figure 1A). Meanwhile, HCCC-9810 cells revealed time- and dose-dependent effects under the condition (Figure 1B). In summary, the results showed that romidepsin significantly inhibited the proliferation of CCA cells.

### *Antitumor Effect of Romidepsin on CCA Cell Lines by Inducing G2/M Cell Cycle Arrest*

The flow cytometry analysis revealed that romidepsin could induce G2/M phase arrest in a dose-dependent manner, as shown in Figure 2A-B. Moreover, we detected several G2/M phase-associated proteins and measured the expression levels of p-cdc2 and CyclinB1 in CCLP-1 and HCCC-9810 cells (Figure 2C-D). The results suggested that romidepsin could induce higher expression levels of p-cdc2 and lower expression levels of CyclinB1 in these 2 CCA cell lines. Collectively, the results revealed that romidepsin exhibited an antitumor effect on the CCA cells by inducing G2/M cell cycle arrest.

### *Antitumor Effect of Romidepsin on CCA Cell Lines by Promoting Cell Apoptosis*

The flow cytometry analysis showed that romidepsin markedly induced cell apoptosis in a dose-dependent manner after 48 h. As shown in Figure 3A-B, romidepsin remarkably increased

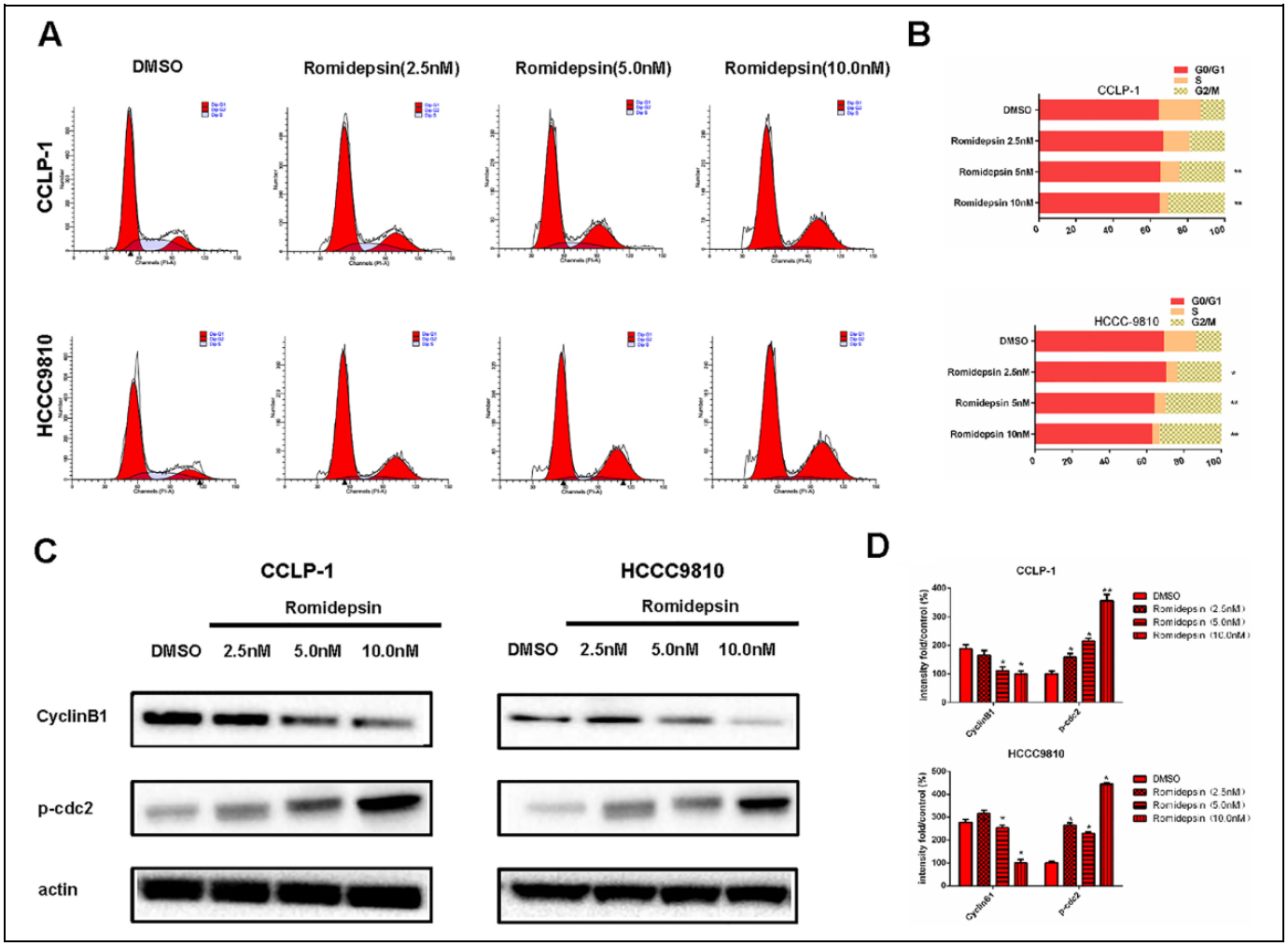


**Figure 2.** Romidepsin induced G2/M cell cycle arrest in CCA cells. (A-B) After 48 h of treatment with romidepsin (0-20 nM), the CCA cells were examined using PI staining, and the cell cycle distribution was measured by flow cytometric analysis. (C-D) Western blot analysis of G2/M-related proteins after romidepsin treatment. The data are shown as the mean ± SD.*$P < 0.05$ vs. the control group; **$P < 0.01$ vs. the control group.

the percentage of cell apoptosis in the CCLP-1 and HCCC-9810 cells, indicating that romidepsin could induce cell apoptosis. Furthermore, apoptosis-associated proteins were also detected. After 48 h treatment by romidepsin, the expression levels of cle-Caspase-3 and cle-PARP in the 2 CCA cell lines are shown in Figure 3C-D. Western blot assay verified that caspase-3 may be involved in the process by which romidepsin promot cell apoptosis. In brief, the results confirmed that romidepsin exerts an antitumor effect on CCA cells by promoting cell apoptosis.

### *Romidepsin Reduced CCA Growth in a CCA Xenograft Model*

To further explore the effects of romidepsin in CCA cells in vivo, we employed male athymic nude mice bearing palpable tumors (approximately 100 $mm^3$) of CCLP-1 xenografts. The mice were randomly assigned to 2 treatment groups, i.e. vehicle control and romidepsin (0.5 mg/kg) and were treated every 3 days for 28 days (n = 6 animals in each group). Romidepsin significantly inhibited tumor growth in CCLP-1 xenografts compared to the control group (Figure 4A-C). To assess the potential toxicity of romidepsin, the weight of the mice was measured. There was no significant difference in weight between the experimental group and the control group, implying that romidepsin was very safe (Figure 4D). Consistent with*in vitro* data, the tumor proliferation marker Ki67 was measured by immunohistochemistry staining, revealing that romidepsin effectively suppressed the expression of Ki67, thus confirming that romidepsin exerted antitumor effects on the xenografts (Figure 4E).

## Discussion

The resistance of CCA to available chemotherapy is attributed to desmoplastic stroma and genetic heterogeneity.[13] The main causes of chemotherapeutic resistance is the significant intertemporal and intratumoral heterogeneity of CCA. Currently,

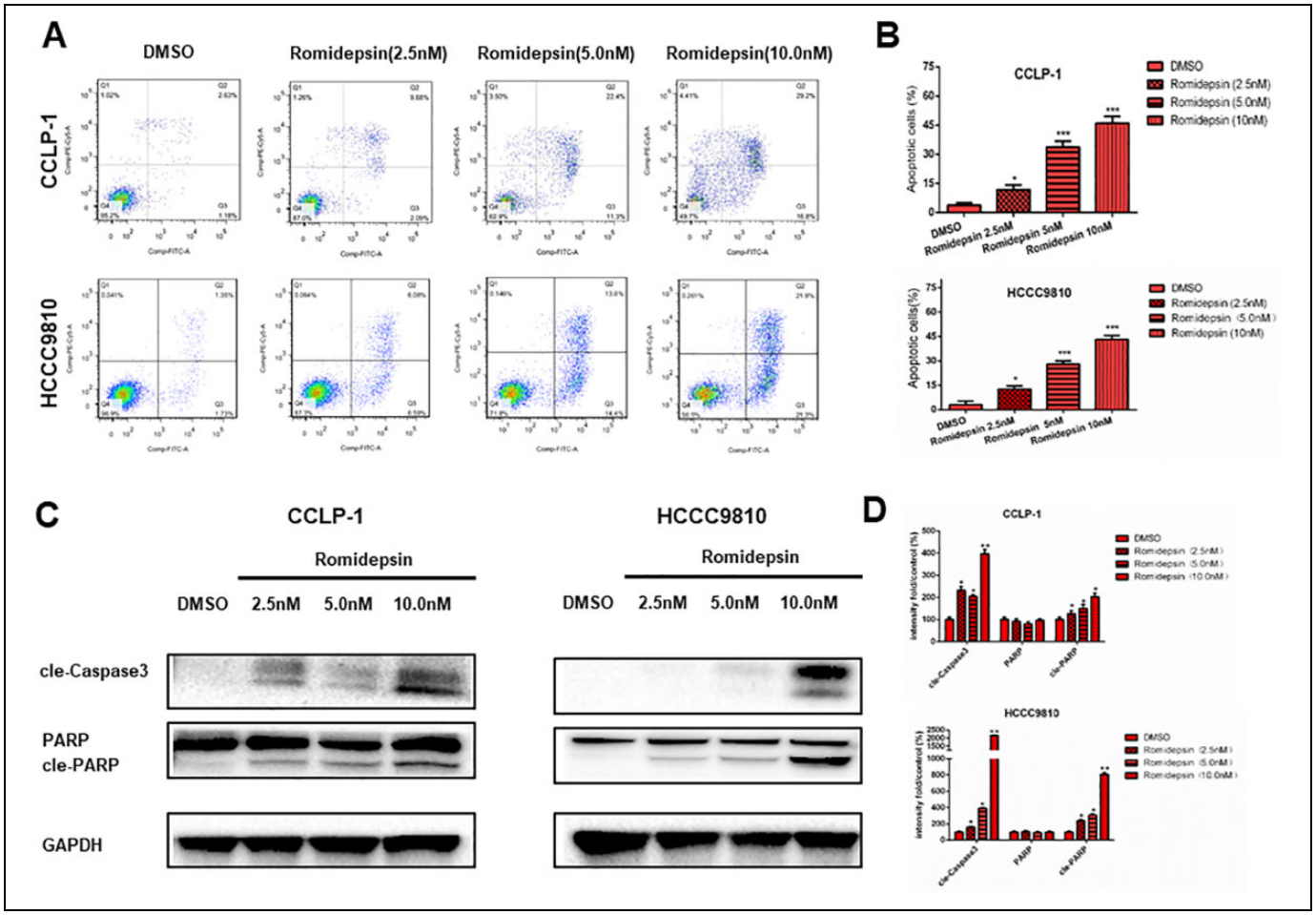


**Figure 3.** Romidepsin promoted apoptosis in CCA cells. (A-B) CCA cells were treated with increasing doses of romidepsin (0-10 nM) for 48 h. After the treatment with romidepsin, the CCA cells were examined using Annexin V/PI staining, and the distribution of apoptotic cells was measured by flow cytometry. (C-D) Western blot analysis of apoptosis related proteins after romidepsin treatment. The data are shown as the mean ± SD. *$P < 0.05$ vs. control group; **$P < 0.01$ vs. control group; ***$P < 0.001$ vs. control group.

targeted therapy, immunotherapy and radiation therapy are used to prolong patient survival.[14] Because gemcitabine has limited efficacy in treating advanced CCA, additional available systemic therapies for unresectable CCA are required for immediate treatment. It has been reported that therapeutic interventions involving HDACs mainly focus on reversing aberrant epigenetic conditions in cancer cells.[15] Moreover, HDACs have been verified to control cell cycle progression, differentiation, cell survival and programed cell death at nanomolar concentrations.[16] As a potent HDACs, romidepsin (FK228) was identified to markedly inhibit class I HDAC.[17] In addition romidepsin was approved by the FDA for the effective treatment of cutaneous and peripheral T-cell lymphomas.[18]

In our study, we revealed that romidepsin markedly inhibited the proliferation of CCA cells both in vivo and in vitro. The antitumor mechanism of romidepsin in the CCA cell line was the induction of G2/M cell cycle arrest and promotion of cell apoptosis.

The results suggest that the antitumor effect of romidepsin on the CCA cells was mainly attributed to the induction of G2/M cell cycle arrest by targeting the cdc2/cyclinB pathway. In addition, Sun et al. demonstrated the antitumor effect of romidepsin on HCC cells and its mechanisms may target the Erk/cdc25C/cdc2/cyclinB pathway.[19] Karam et al. also reported that romidepsin treatment in T24 cells resulted in G2/M phase arrest.[20]

This paper demonstrat that romidepsin remarkably promotedapoptosis in the CCA cells, possibly by mechanisms that targeted the caspase-3 pathway. In the study conducted by Li et al, the authors reported that the antitumor effect of FK228 on EC tumor cells was possibly mediated by mechanisms that activated caspase3/PARP via the stimulation of the p53/p21 signaling axis, which is consistent with our study.[12] Furthermore, other studies have shown that the proapoptotic effect of oncogenic HRas on FK228 treatment was attributed to the activation of the caspase-3 pathway.[21] In our study, we did not further explore the factors and pathways leading to caspase-3 activation, which is a drawback of this study. Based on previous reports we speculate that romidepsin may activate caspase3 through the stimulation of the p53/p21 signaling axis, but this hypothesis needs to be verified by further studies.

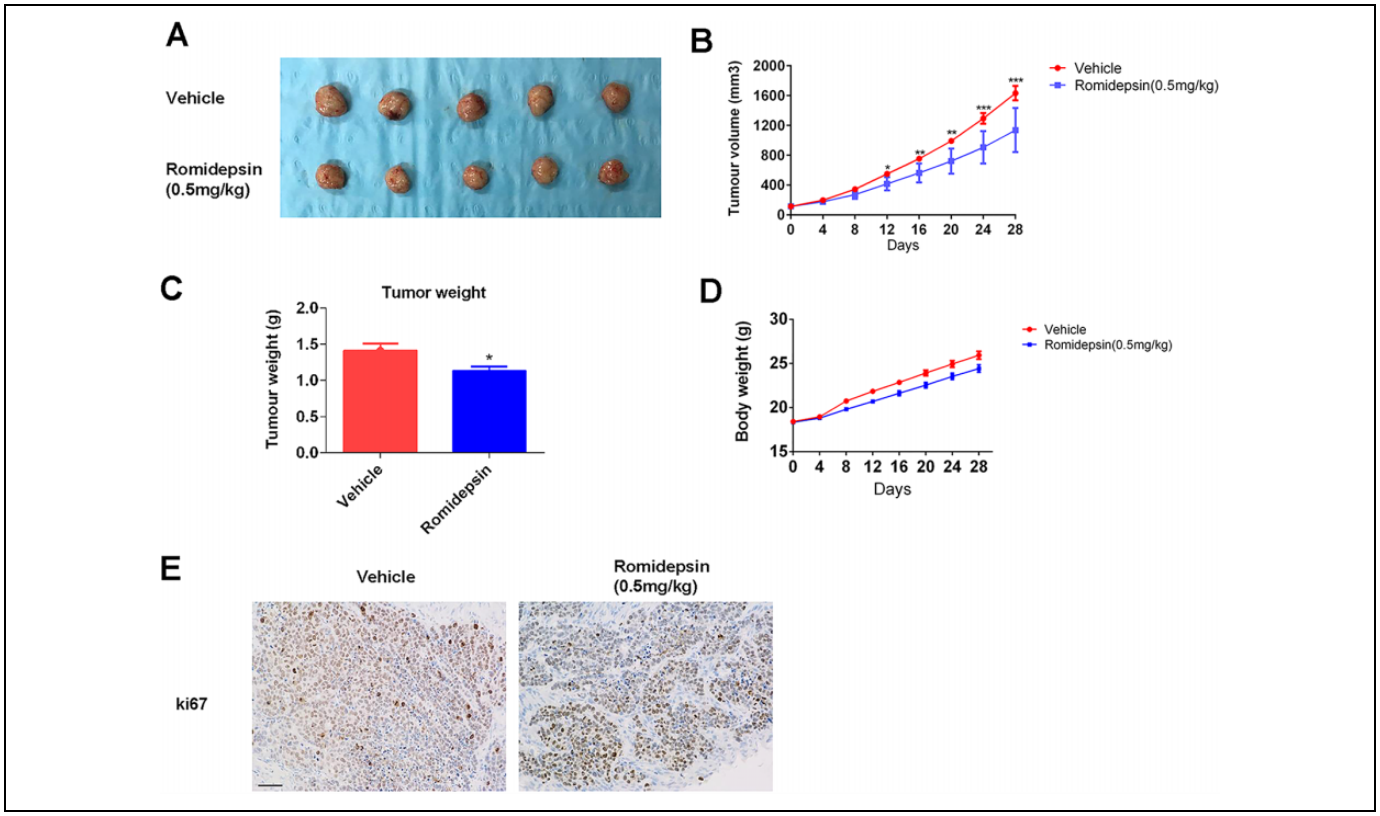


**Figure 4.** Romidepsin reduced CCA growth in a xenograft model. (A) CCLP-1 cells were implanted subcutaneously into the flank regions of nude mice. Once the tumor volume reached approximately 100 $mm^3$, vehicle or romidepsin was administered. After 4 weeks, the mice were euthanized. The tumors were isolated and shown (A). The tumor volume (B) and tumor weight (C) were measured. (D)The weight of mice was measured in each group. (E) The expression of Ki67 in the tumors was detected by IHC. Scale bar, 100 μm. The data are shown as the mean ± SD. *$P < 0.05$ vs. the control group; **P < 0.01 vs. the control group; ***P < 0.001 vs. the control group.

## Conclusion

In this study, we showed that romidepsin achieves its antitumor effect on CCA cell lines by inducing G2/M cell cycle arrest via the cdc2/cyclinB pathway and apoptosis by targeting caspase-3 *in vitro* and *in vivo*, suggesting that FK228 is a potential therapeutic agent for CCA. Therefore, our study provides new evidence suggesting that Romidepsin is a promising chemotherapy drug, that can have a significant anti-tumor effect on CCA and other human solid cancers.

### Ethical Approval statement

The protocol for *vivo* studies was approved by the Animal Experimental Ethical Inspection of Laboratory Animal Centre, Wenzhou Medical University (No. wydw 2019-0017). The experiment follows the Guide for the Care and Use of Laboratory Animals.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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