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Romidepsin induces G2/M phase arrest via Erk/cdc25C/cdc2/cyclinB pathway and apoptosis induction through JNK/c-Jun/caspase3 pathway in hepatocellular carcinoma cells

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ABSTRACT

The aim of the study is to demonstrate the effect of Romidepsin in hepatocellular carcinoma (HCC) by inducing G2/M phase arrest via Erk/cdc25C/cdc2/cyclinB pathway and apoptosis through JNK/c-Jun/caspase3 pathway in vitro and in vivo. Human HCC cell lines were cultured with Romidepsin and DMSO (negative control) and 5-fluorouracil (positive control). Then the cells' viability and apoptosis were determined by cell proliferation assay and flow cytometry. Protein concentrations and expression changes were measured by Western blot. Subsequently, Huh7 cells were subcutaneously inoculated into the nude mice, which were employed to further probe the tumor-suppressive effect of Romidepsin in vivo. Romidepsin treatment led to a time- and dose-dependent induction of cell cycle arrest in the G2/M phase and apoptosis. G2/M phase arrest inhibited the proliferation of HCC cells by alterations in p21/cdc25C/cdc2/cyclinB proteins. Increased concentrations of Erk and JNK phosphorylations were observed in a dose-dependent manner in the Romidepsin group, but p38 phosphorylation was not affected. G2/M phase arrest and the apoptosis of HCC cells induced by Romidepsin were mediated by the activation of Erk/MAPK pathways and JNK/MAPK pathways. The tumor size was significantly larger in the negative control group compared to Romidepsin group and no significant loss in body weight was observed in the Romidepsin group. Our findings offer proof-of-concept for use of Romidepsin as a novel class of chemotherapy in the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer and is the third leading cause of cancer-related deaths worldwide [1,2]. Surgical resection and liver transplantation are widely used to control its pathology, despite these still

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the prognosis is very poor in HCC patients, which might be due to their development of resistance to chemotherapy [3,4]. Hence, novel therapeutic agents are required for the treatment immediately [3,5].

Histone deacetylase inhibitor (HDACi) possesses potent and significant inhibitory effects on the growth of human solid tumor implant in mice [6]. Several HDACis have been well studied previously, including (FK228, Depsipeptide) [7]. Romidepsin, shown in Fig. 1A, is one of the HDACis which was isolated from a Gram negative bacterium, called Chromobacterium violaceum No. 968. Romidepsin is one of the most successful and widely used and an approved HDACi in clinical applications against refractory cutaneous and peripheral T cell lymphoma [8]. However, its role in the treatment of solid tumors, including HCC remains still unclear and is under investigation [7,9,10].

HDACis exert their antitumor activities through the induction of differentiation and cell cycle arrest [9,11]. An essential step to exert cell cycle transition includes the activation of cdc2-cyclin B complex. P-cdc2 (Tyr15) and Cyclin B1 are the proteins that activate specific cyclin-dependent kinases (CDKs) required for the progression of cell cycle [12]. The critical regulatory step in activating cdc2 during cell cycle progression into mitosis is by cdc2 dephosphorylation at Tyr15, and dephosphorylation of cdc2/cdk1 at Tyr15 by cdc25C [13]. Cdc25C is a protein phosphatase responsible for dephosphorylating and activating cdc2, which is a crucial step in regulating the cell cycle transition in eukaryotes [14]. Moreover, traversing the G2/M checkpoint to initiate mitosis requires cyclin B [15,16]. At the end of mitosis, cyclin B1 is degraded by the anaphase-promoting complex, finally allowing for cell cycle progression [16].

HDACi exerts its antitumor activity via induction of apoptotic cell death in a variety of cancer cells [9,17]. Apoptosis is an active form of cell death that occurs in response to several anticancer chemotherapeutic drugs [18]. Caspase-9 is an important member of the cysteine aspartic acid protease (caspase) family [19]. Cleaved caspase-9 which further activates other caspase members, including caspase-3, to initiate a caspase cascade mechanism, in turn leads to apoptosis [19]. Caspase-3 is a critical executioner of apoptosis, as it partially or totally functions in the proteolytic cleavage of many key proteins such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP) [20].

Extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs functions in protein kinase cascades play a critical role in the regulation of cell growth, differentiation, and apoptosis [21]. The Erk signaling pathway can be activated in response to a diverse range of extracellular stimuli such as growth factors, mitogens, and cytokines and is an important target in the diagnosis and treatment of cancer [22]. JNK is potently and preferentially activated by a variety of environmental stressors including UV, ceramides and inflammatory cytokines [23]. JNK, when activated as a dimer, can translocate to the nucleus and regulate transcription through its effects on c-Jun [24].

In the present study, we demonstrate that Romidepsin inhibited the growth of HCC cells by inducing cell cycle arrest and apoptosis in vivo. Romidepsin may inhibit cell proliferation with the help of ERK/MAPK signaling and c-Jun N-terminal kinase (JNK)/c-Jun signaling pathway induction which consequently may act as a potential anticancer agent for HCC.

2. Materials and methods

2.1. Reagents

Romidepsin (FK228, Depsipeptide, $C_{24}H_{36}N_4O_6S_2$) and 5-fluorouracil (5-FU) were purchased from Selleck Chemicals (Houston, TX, USA). BCA protein assay kit, the Cell Counting Kit-8, the cell cycle detection kit and the Annexin V-FITC apoptosis detection kit were purchased from KeyGen Biotech (Nanjing, China). SCH772984 (Erk inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and the Caspase inhibitor Z-VAD-FMK (L-Alaninamide,N-[(phenyl methoxy)carbonyl]-L-valyl-N-[(1S)-3-fluoro-1-(2-methoxy-2-oxoethyl)-2-oxopropyl]) were purchased from Selleck Chemicals (Houston, TX, USA). The EdU Apollo567 In Vitro Imaging Kit was purchased from Guangzhou, People's Republic of China. DAPI and Paraformaldehyde (PFA) were purchased from GuGe Biotech Co. Ltd. (Wuhan, China). Triton X-100 was purchased from ShenGong Biotech. Co. Ltd. (Shanghai, China). Methanol and Ethanol were purchased from Shanghai LingFeng Chemical Reagent Co. Ltd. DMSO, Tween 20 and Glycine was purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Skim Milk was purchased from Becton, Dickinson and Company (San Diego, CA, USA). Primary antibodies against $p21^{Cip1}$, CyclinB1, p-cdc2, cdc2, p-cdc25C, $p27^{Kip1}$,c-caspase-3, caspase-3, c-caspase-9, c-PARP, PARP, p-ERK, ERK, p-P38, p-JNK, JNK, p-c-Jun and p-AKT(Ser473)were purchased from Cell Signaling Technology (Danvers, MA, USA); Ki67 and GAPDH purchased from Abcam Company (Cambridge, MA, USA).

2.2. Cells and animal model

Human HCC cell lines (Huh-7, HCC-LM3, SK-Hep-1, and HepG2) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Huh-7, HCC-LM3, SK-Hep-1, and HepG2 were cultured in minimum essential medium (MEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

Animal experiments were performed in accordance with the National Institutes of Health guideline. 2×10^6 cells were resuspended in 100 ml PBS, and then were injected subcutaneously into the flanks of nude mice (n = 20). After 10 days, the mice were randomised into four groups, 3 groups were treated with Romidepsin (0.5 and 1 mg/kg) and DMSO (20 mg/kg, dissolved in sodium chloride, negative control) every 3 days for 21 days. One group was treated with 5-FU (100 mg/kg, positive control) every 6 days for 21 days. Tumor volume and body weight were measured every 4 days for 21 days. After 20 days, tumors were harvested. Tumor volumes were calculated using the equation: V (cm^3) = width^2 (cm^2) × length (cm)/2.

2.3. Cell proliferation assay

Cell proliferation assay was determined using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). HCC cells were treated with various concentrations of Romidepsin or 5-FU for 12, 24, 48 and 72 h, respectively. The optical density (OD) values were quantified by Versamax microplate reader at 450 nm. Furthermore, the effect of Romidepsin on cell viability was assessed by colorimetric immunoassay (Cell-Light™ EdU Apollo567 In Vitro Imaging Kit; Ribobio, Guangzhou, People's Republic of China) according to the manufacturer's instructions. 1×10^5 cells were cultured in 6-well plates then treated with varied concentrations of Romidepsin (15nM and 30nM) or DMSO. The immunoassay was performed 48 h after Romidepsin incubation. The final concentration of DMSO was kept at <0.05% in all the wells. The experiments were performed with three replicates for each condition.

2.4. Colony-forming assay

Huh7 and HCC-LM3 cells were seeded into 6-well plates in triplicates at a density of 1000 cells/well, and were maintained with or