Abstract. The combined antitumor effects of 1,25-dihydroxy vitamin D3 [1,25(OH)2D3] and the Notch inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, a synthetic γ secretase inhibitor) in liver cancer cells remain to be fully elucidated. In the present study, HepG2 cells were divided into six groups and different treatments were applied: Control, 10^{-10} M 1,25(OH)2D3, 10^{-8} M 1,25(OH)2D3, 10^{-6} M 1,25(OH)2D3, 1 µM DAPT, 5 µM DAPT, 10 µM DAPT, and 10^{-6} M 1,25(OH)2D3 + 10 µM DAPT. The proliferation, cell cycle, apoptosis, migration and invasion of the cells were then examined. The expression levels of Notch and its ligand Jagged were detected by reverse transcription-quantitative polymerase chain reaction and western blot analyses. The results revealed that 1,25(OH)2D3 inhibited cell proliferation, migration and invasion; arrested cell cycle at the G1 phase, and promoted apoptosis in a concentration-dependent manner between 10^{-10} and 10^{-6} M. DAPT inhibited cell proliferation, migration and invasion, arrested cell cycle at the G1 phase, and promoted apoptosis in a concentration-dependent manner between 1 and 10 µM. Additionally, 1,25(OH)2D3 and/or DAPT reduced the expression of Notch1, Notch2, Jagged1 and Jagged2. The co-application of 10 µM DAPT further increased the anti-cancer effect of 10^{-6} M 1,25(OH)2D3. Collectively, these results indicated that the treatment of HepG2 cells with 1,25(OH)2D3 inactivated Notch signaling, prevented proliferation, migration and invasion, and promoted apoptosis. The combined application of 1,25(OH)2D3 with DAPT may be a useful treatment for preventing the onset or progression of liver cancer.

Introduction

The incidence of primary liver cancer continues to increase in numerous countries, in part due to an increase in the number of individuals infected with hepatitis C virus (1). Liver cancer is a malignant tumor with a high mortality rate. Treatment modalities include surgery, cryotherapy, hepatic arterial chemoembolization, radiofrequency ablation, biological therapy, radiotherapy and radioactive seed implantation (2), however, each of these approaches has limitations. It is of clinical significance to identify reasonable and effective treatments for patients with advanced liver cancer (3,4).

1,25-Dihydroxy vitamin D3 [1,25(OH)2D3] is an important vitamin required to sustain physiologic functions (5). It is important in cell proliferation, differentiation and immune function, in addition to regulating bone development and calcium and phosphorus metabolism (6). 1,25(OH)2D3 can induce apoptosis in a variety of cells, with particularly pronounced effects in tumor cells (7); however, the specific mechanism of action remains to be fully elucidated.

Notch is a conserved cell membrane surface receptor, which interacts with Notch ligands expressed on adjacent cell surfaces (8). Normally, the ligand can activate Notch signaling by inducing proteolysis, which releases the intracellular region of Notch (ICN) from the cell membrane. Upon entering the nucleus, the ICN activates the suppressor of hairless/C-promoter binding factor 1 family of transcription factors and regulates cell growth and development (9). Notch signaling can either promote or prevent cell differentiation, and it is critical in tumor stem cell development (10). The Notch pathway increases the activity of cancer stem cells, resulting in tumor formation (11). N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) is a synthetic γ secretase inhibitor, also known as a Notch signal pathway inhibitor. DAPT does not induce toxicity or cause side effects at a certain dose range, and has antitumor effects (12,13), particularly with regard to preventing hepatocellular carcinoma (HCC) invasion (14).

The present study applied 1,25(OH)2D3 in conjunction with the Notch signaling pathway inhibitor DAPT to examine the anticancer effects of these two drugs in liver cancer. The results may provide experimental evidence supporting their use in the clinical treatment of liver cancer.
Materials and methods

Cell culture. The HepG2 liver cancer cell line was purchased from the Shanghai Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA) in 5% CO₂ at 37°C.

The cells were divided into the following six groups: Control, 10⁻¹⁰ M 1,25(OH)₂D₃, 10⁻⁸ M 1,25(OH)₂D₃, 10⁻⁶ M 1,25(OH)₂D₃, 1 µM DAPT, 5 µM DAPT, 10 µM DAPT, and 10⁻⁶ M 1,25(OH)₂D₃ + 10 µM DAPT. Treatments were applied when the cell confluence had reached 70%. Following treatment for 48 h at 37°C, Cell Counting Kit-8 (CCK-8) assays, flow cytometry, Transwell assays and wound-healing assays were performed to assess cell proliferation, cell cycle, apoptosis, migration and invasion. The expression of Notch and Jagged were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses.

CCK-8 assay. The HepG2 cells were seeded in 96-well plates (3x10³ cells/ml) and treated with 1,25(OH)₂D₃ and/or DAPT for 48 h. Following treatment, 10 µl medium with CCK-8 (Gibco; Thermo Fisher Scientific, Inc.) was added into each well. Following an additional incubation period of 4 h in a CO₂ incubator at 37°C, the absorbances were detected on a microplate reader (Thermo Fisher Scientific, Inc.) at 560 nm. Cell viability was defined by the optical density values.

Flow cytometry. The HepG2 cells were seeded in 6-well plates and treated with 1,25(OH)₂D₃ and/or DAPT for 48 h. The cells were collected following digestion by trypsin (Gibco; Thermo Fisher Scientific, Inc.) and incubated with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; cat. no. C1062; Beyotime Institute of Biotechnology, Ningbo, China) for 30 min in the dark. Apoptosis and cell cycle distribution were detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) and data were analyzed with FlowJo 10 software (FlowJo LLC, Ashland, OR, USA). The Cell Cycle platform in FlowJo 10 software was used to analyze the cell cycle distribution.

Transwell assay. The HepG2 cells were seeded in 6-well plates and treated with 1,25(OH)₂D₃ and/or DAPT for 48 h. The cells were then digested, collected, seeded into the upper chamber of Transwells, and subjected to serum starvation for 1 day. The lower chamber contained DMEM with 10% FBS. The cells were cultured in a CO₂ incubator for 24 h, following which the lower chamber was removed, fixed with polyformaldehyde for 20 min and stained with crystal violet (0.1%). Light microscopy was used to capture images in at least five fields. The counted cell numbers represented the cell invasion capacity.

Cell migration. The HepG2 cells were seeded in 6-well plates and treated with 1,25(OH)₂D₃ and/or DAPT for 48 h. A pipette tip was used to create an even line across the center of the confluent cell mass. Following incubation in a CO₂ incubator at 37°C for 48 h, images were captured under a light microscope. The width at 0 h was divided by the width at 48 h to calculate cell migration.

RT-qPCR analysis. The HepG2 cells were seeded in 6-well plates and treated with 1,25(OH)₂D₃ and/or DAPT for 48 h. Total mRNA was extracted using a TRIzol assay kit (Baosheng Science and Technology Innovation Co., Ltd., Shanghai, China). Subsequently, the mRNA was transcribed into cDNA using a Reverse Transcription kit according to the manufacturer’s protocol (Takara Biotechnology, Co., Ltd., Dalian, China). Fluorescence RT-qPCR analysis was utilized to detect expression levels of the targeted genes using cDNA as a template. mRNA was transcribed into cDNA using a reverse transcription kit (cat. no. 639522; Takara Biotechnology) at 37°C and qPCR was used to detect the expression level of the target genes by using SYBR Green. The amplification reactions were performed with initial denaturation at 95°C for 10 min, followed by 35 cycles of a two-step PCR at 95°C for 14 sec and 60°C for 1 min. The levels of Notch1, Notch2, Jagged1 and Jagged2 were normalized to GAPDH using 2⁻ΔΔCq method as previously described (15). The primers were as follows: Notch1, forward 5'-AAT GTG GAT GCC GCA GTT G-3' and reverse 5'-ATCCGTGATGTCGGCGTTG-3'; Notch2, forward 5'-AGCTGCTACTCACAGGTGAACGAA-3' and reverse 5'-CCAGCCTGCATCACAGACAC-3'; Jagged1, forward 5'-CCAGGGCTTTGGAGAATCCAGATG-3' and reverse 5'-TGACCAGACGCCCCGATGAA-3'; GAPDH, forward 5'-CAATGGACCCCTTCACTGACC-3' and reverse 5'-GAGAAGCTTCCGTCTCAG-3'.

Western blot analysis. The HepG2 cells were seeded in 6-well plates and treated with 1,25(OH)₂D₃ and/or DAPT for 48 h. Protein (20 µg) was extracted from the treated cells by a protein isolation kit (cat. no. 28-9425-44; ReadyPrep; GE Healthcare Life Sciences) and protein levels were quantified with a bicinchoninic acid protein assay kit. Protein (25 µg/lane) was run on
sodium-dodecyl sulfate-polyacrylamide gels (10%), and transferred onto membranes. The membranes were blocked with 5% de-fat milk for 2 h at room temperature. The following primary antibodies were used for overnight incubation at 4˚C: Anti-Notch1 (dilution 1:1,000; cat. no. ab44986; Abcam, Cambridge, UK), anti-Notch2 (dilution 1:2,000; cat. no. ab8926; Abcam), anti-Jagged1 (dilution 1:1,000; cat. no. ab7771; Abcam) and anti-Jagged2 (dilution 1:1,000; cat. no. ab109627; Abcam). Following washing with PBST (0.2% Tween-20), the membranes were incubated with the secondary antibody (dilution 1:100; cat. no. ab131368; Abcam) for 2 h at room temperature. An Enhanced Chemiluminescence kit (cat. no. RPN2133; GE Healthcare Life Sciences) was added to the membrane prior to visualization with a gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The data are presented as mean ± standard error of mean (SEM). Statistical significance was calculated by one-way analysis of variance (ANOVA) with Newman-Keuls as the post hoc test (SPSS 17.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

DAPT and 1,25(OH)2D3 inhibit HepG2 cell proliferation. As shown in Fig. 1, 1,25(OH)2D3 inhibited cell proliferation in a concentration-dependent manner at a range of 10-10-10-6 M. Following statistical analysis, 10-10, 10-8 and 10-6 M 1,25(OH)2D3 were found to significantly inhibit cell proliferation compared with the control (P<0.05). Similarly, DAPT inhibited cell proliferation in a concentration-dependent manner at the range of 1-10 µM. Following statistical analysis, 5 and 10 µM DAPT significantly inhibited cell proliferation compared with the control (P<0.05). In addition 10-6 M 1,25(OH)2D3 and 10 µM DAPT were co-applied. The results showed that 10 µM DAPT further increased the anti-proliferative effect of 10-6 M 1,25(OH)2D3.
DAPT facilitates 1,25(OH)₂D₃-induced cell cycle arrest in HepG2 cells. As shown in Fig. 2A and B, 1,25(OH)₂D₃ increased and decreased the numbers of cells in the G1 and S phases, respectively, in a concentration-dependent manner at the range between 10⁻¹⁰ and 10⁻⁶ M. Statistical analysis showed that the effects of 10⁻¹⁰, 10⁻⁸ and 10⁻⁶ M 1,25(OH)₂D₃ were significant compared with the control (P<0.05). It was also observed that DAPT increased the number of cells in the G1 phase and decreased the number of cells in the S phase in a concentration-dependent manner between 1 and 10 µM. Statistical analysis showed that 5 and 10 µM DAPT significantly increased the number of cells in the G1 phase and decreased those in the S phase compared with the control (P<0.05). Notably, the co-application of 10 µM DAPT further increased the cell cycle arrest observed following treatment with 10⁻⁶ M 1,25(OH)₂D₃.

DAPT facilitates 1,25(OH)₂D₃-induced apoptosis. As shown in Fig. 3A and B, 1,25(OH)₂D₃ induced HepG2 cell apoptosis in a concentration-dependent manner between 10⁻¹⁰ and 10⁻⁶ M. Statistical analysis showed that 10⁻¹⁰, 10⁻⁸ and 10⁻⁶ M 1,25(OH)₂D₃ significantly induced apoptosis compared with that in the control (P<0.05). Similarly, DAPT concentration-dependently induced apoptosis at a range of 1-10 µM. Following statistical analysis, 1, 5 and 10 µM DAPT were found to significantly induce HepG2 cell apoptosis compared with that in the control (P<0.05). The co-application of 10⁻⁶ M 1,25(OH)₂D₃ and 10 µM DAPT revealed that inhibiting γ-secretase further increased the apoptosis induced by 10⁻⁶ M 1,25(OH)₂D₃.

DAPT facilitates the anti-migration effect of 1,25(OH)₂D₃. As shown in Fig. 4A and B, 1,25(OH)₂D₃ inhibited HepG2 cell migration at the range of 10⁻¹⁰-10⁻⁶ M. It was also shown that DAPT inhibited HepG2 cell migration at the range of 1-10 µM. The results showed that the combination of 10 µM DAPT with 10⁻⁶ M 1,25(OH)₂D₃ further inhibited cell migration.

DAPT facilitates the anti-invasion effect of 1,25(OH)₂D₃. As shown in Fig. 5A and B, 1,25(OH)₂D₃ inhibited cell invasion in
a concentration-dependent manner at the range of $10^{-10}$-$10^{-6}$ M. Statistical analysis showed that $10^{-10}$, $10^{-8}$ and $10^{-6}$ M 1,25(OH)$_2$D$_3$ significantly inhibited cell invasion compared with that in the control (P<0.05). It was also shown that DAPT inhibited cell invasion in a concentration-dependent manner at the range of 1-10 µM. Statistical analysis revealed that 1, 5 and 10 µM DAPT significantly inhibited cell invasion compared with that in the control (P<0.05). The results also showed that 10 µM DAPT further increased the anti-invasion effect of $10^{-6}$ M 1,25(OH)$_2$D$_3$.

1,25(OH)$_2$D$_3$ and/or reduces the expression of Notch1, Notch2, Jagged1 and Jagged2. Compared with the control group, the mRNA levels of Notch1, Notch2, Jagged1 and Jagged2 were significantly reduced following 1,25(OH)$_2$D$_3$ and/or DAPT treatment (Fig. 6A-D). It was also confirmed that 1,25(OH)$_2$D$_3$,
and/or DAPT reduced the protein levels of Notch1, Notch2, Jagged1 and Jagged2 (Fig. 7A-E).

Discussion

Several studies have reported the anticancer effect of 1,25(OH)\(_2\)D\(_3\) (14,16,17), including in the context of liver cancer (18,19). However, the mechanisms involved remain to be fully elucidated. HCC and hepatoblastoma are types of primary liver cancer, which occur predominantly in adults and children, respectively (20). In the present study, the HepG2 hepatoblastoma cell line was selected, which was initially considered to be a HCC cell line (19) but was later identified as a hepatoblastoma-derived cell line (21). The present study investigated the antitumor effect of 1,25(OH)\(_2\)D\(_3\), which is the active form of vitamin D, and examined its synergistic effects with DAPT. The HepG2 cell line was selected to assess 1,25(OH)\(_2\)D\(_3\) cytotoxicity over a wide concentration range to provide a therapeutic index for this agent as an anticancer drug in humans. At 1,25(OH)\(_2\)D\(_3\) of >1,000 nM cell viability was significantly reduced. The physiological range of 1,25(OH)\(_2\)D\(_3\) in human plasma is 36-150 pM. A dose of 165 µg (~2.75 µg/kg) is clinically safe, even when administered weekly (Cmax 14.9±4.78 nM) (22). As the intracellular concentration is usually higher than that in serum, the present study used 100-1,000 nM for \textit{in vitro} experiments, which was similar to the approach adopted in other studies (23,24). It was previously reported that 1,25(OH)\(_2\)D\(_3\) did not affect normal gastric cells in this dose range (25), suggesting the safety of clinical 1,25(OH)\(_2\)D\(_3\) use. In addition, DAPT co-application promoted the action of 1,25(OH)\(_2\)D\(_3\). In future investigations, a lower dose of 1,25(OH)\(_2\)D\(_3\) requires assessment to confirm the synergistic effect of DAPT.

Liver cancer is one of the most common primary malignant tumors and is the third leading cause of cancer-associated mortality globally (26). Effective treatments are required to
improve patient quality of life and prolong survival rates, therefore, it is of clinical significance to identify effective therapeutic compounds and clarify their mechanisms of action. DAPT is a synthetic secretory enzyme inhibitor, which can inhibit Notch signaling (27). As previously described, signaling dysfunction is a hallmark of multiple types of cancer (28,29). The Notch pathway is closely associated with tumor occurrence and development. Therefore, inhibitors of tumor-related signaling pathways are considered candidates for treating tumors (30). In addition, the Notch signaling pathway inhibitor has low cytotoxicity and does not induce side effects. These characteristics provide a novel approach for the investigation and development of novel antitumor drugs (31). The present study provided in vitro evidence that DAPT inhibited liver cancer cell proliferation, migration and invasion. In a previous study, long intergenic non-coding RNA-p21 was found to inhibit HCC invasion and metastasis through its effects on Notch signaling (7). The results of the present study also demonstrated that 1,25(OH)2D3 enhanced the anti-invasive effects of DAPT in liver cancer cells. However, the antitumor effects of 1,25(OH)2D3 + DAPT require validation in an in vivo model in the future to understand its antitumor effects.

Although the cytotoxicity of DAPT is low, high doses can elicit adverse effects. The dose of DAPT requires strict control, however, a single low dose may not effectively prevent cancer progression. 1,25(OH)2D3 has several biological activities, including maintaining a stable calcium environment, regulating immune function and affecting tumor biological activity, which may affect cell proliferation and apoptosis (32). 1,25(OH)2D3 functions mainly through binding to a specific vitamin D receptor to inhibit myeloid leukemia cell proliferation (33). Previous studies have shown that 1,25(OH)2D3 can inhibit tumor cell proliferation, reduce tumor cell migration and invasion, and induce apoptosis of prostate cancer cells (34). The results of the present study confirmed that 1,25(OH)2D3 exerted anticancer activity in liver cancer cells. Critically, the combined application of 1,25(OH)2D3 with DAPT had more

Figure 6. 1,25(OH)2D3 and/or DAPT reduces the mRNA levels of Notch1, Notch2, Jagged1 and Jagged2. mRNA levels of (A) Notch1, (B) Notch2, (C) Jagged1 and (D) Jagged2. Results are presented as the mean ± standard error of the mean of six replicates. *P<0.05, vs. control. DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; 1,25(OH)2D3, 1,25-dihydroxy vitamin D3.
pronounced effects, compared with either 1,25(OH)₂D₃ or DAPT alone. In the present study, Annexin V-FITC /PI were applied to stain the cells and apoptosis was detected by flow cytometry. Although the combined application of 1,25(OH)₂D₃ with DAPT had more pronounced effects on apoptosis, typical apoptotic pathways require evaluation, including caspase pathways (35). The present study also quantified the cell cycle distribution following drug treatments. The combined application of 1,25(OH)₂D₃ with DAPT further arrested the cells at the G1 phase. Cyclin D1 is an important cell cycle regulatory protein (36), which requires detection to confirm the effects on cell cycle arrest.
Notch is a conserved pathway in biological evolution and reflects the complex mechanisms of intercellular communication. It is important in cell fate determination during the development of several organs (37). Notch signaling also occurs in several mature mammalian tissues (38), regulating cell growth, differentiation, tissue regeneration and intracellular environment stability. It is involved in normal and pathological states of liver development; the major molecules are Notch1-4 and their ligands are Jagged1 and δ-like 4 (39). Abnormal Notch expression and signaling are associated with various malignancies. In the present study, RT-qPCR and western blot analyses were performed, and it was found that Notch1, Notch2, Jagged1 and Jagged2 were expressed at low levels in liver cancer cells following treatment with DAPT and/or 1,25(OH)2D3. These results suggested that Notch signaling is involved in promoting liver cancer cell progression by regulating cell cycle and apoptosis. As Notch signaling is also associated with autophagy (40), whether 1,25(OH)2D3 and/or DAPT affects autophagy in liver cancer cells requires further investigation.

In conclusion, the findings of the present study demonstrated that treatment of liver cancer with 1,25(OH)2D3 inactivated Notch signaling, prevented proliferation, migration and invasion, and promoted apoptosis. The combined application of 1,25(OH)2D3 with the synthetic γ secretase inhibitor DAPT had more marked effects. However, the present study selected a type of liver cancer cell with hepatoblastoma origin. In a future study, the anti-liver cancer effect of the combination application of 1,25(OH)2D3 with DAPT on HCC also requires confirmation.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

LC, ZT and XM conceived and designed the study. LC and LL performed the experiments. LC and XM wrote the manuscript. LC, LL, ZT and XM reviewed and edited the manuscript. All authors read and approved the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board of Jiangxi Provincial People’s Hospital (Jiangxi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

References