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Original Article

Bruceine D inhibits hepatocellular carcinoma growth by targeting β-catenin/jagged1 pathways

Ziying Cheng [a, 1], Xing Yuan [a, 1], Yi Qu [b, 1], Xia Li [a], Guozhen Wu [a], Chenwei Li [c], Xianpeng Zu [a], Niao Yang [a], Xisong Ke [b], Juan Zhou [a], Ning Xie [d], Xike Xu [a], Shanrong Liu [e], Yunheng Shen [a], Huiliang Li [a, **], Weidong Zhang [a, b, *]

[a] Department of Phytochemistry, School of Pharmacy, Second Military Medical University, Shanghai 200433, China
[b] Interdisciplinary Science Research Institute, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, PR China
[c] Shanghai Sunstem Biotechnology Co. Ltd, Shanghai 200439, China
[d] State Key Laboratory of Innovative Natural Medicine and TCM Injections, Jiangxi province 341008, China
[e] Changhai Hospital, Second Military Medical University, Shanghai 200433, China

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ABSTRACT

Hepatocellular carcinoma (HCC) is known for high mortality and limited available treatments. Aberrant activation of the Wnt and Notch signaling pathways is critical to liver carcinogenesis and progression. Here, we identified a small molecule, bruceine D (BD), as a Notch inhibitor, using an RBP-Jκ-dependent luciferase-reporter system. BD significantly inhibited liver tumor growth and enhanced the therapeutic effects of sorafenib in various murine HCC models. Mechanistically, BD promotes proteasomal degradation of β-catenin and the depletion of its nuclear accumulation, which in turn disrupts the Wnt/β-catenin-dependent transcription of the Notch ligand Jagged1 in HCC. Our findings provide important information about a novel Wnt/Notch crosstalk inhibitor that is synergistic with sorafenib for treatment of HCC, and therefore have high clinical impact.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide [1]. Despite improvements in detection and clinical treatment strategies, the 5-year survival rate for HCC is less than 17% for all stages combined [2]. Sorafenib remains the first-line treatment for advanced HCC [3], with 2.8 months increased survival compared with placebo [4]. Hence, there is an urgent need for more effective therapies or synergistic agents for HCC treatment.

Mounting evidence links the Wnt and Notch pathways to the development of HCC. Wnt/β-catenin signaling is an evolutionarily well-conserved pathway that is important in liver health and repair [5]. Aberrant activation of the Wnt/β-catenin pathway has been implicated in multiple hepatic diseases, especially HCC [5]. β-catenin is the chief downstream effector of the canonical Wnt signaling pathway; its cytosolic level is tightly controlled by a multiprotein destruction complex composed of adenomatous polyposis coli (APC), AXIN, and glycogen synthase kinase-3β (GSK3β). This complex phosphorylates β-catenin at serine at residue 37 (Ser37) or threonine at residue 41 (Thr41) and subsequently induces its ubiquitination and proteasome-mediated degradation [6–9]. Wnt signaling induces translocation of β-catenin into the nucleus, where it regulates specific oncogenes, including c-MYC, cyclin D1 and survivin, and activates transcription together with the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors [10,11].

The Notch signaling pathway is involved in multiple cell differentiation processes during embryonic development and

Abbreviations: HCC, hepatocellular carcinoma; BD, bruceine D; APC, adenomatous polyposis coli; GSK3β, glycogen synthase kinase-3β; LEF1, lymphoid enhancer binding factor 1; TCF4, T-cell-specific transcription factor 4; APH-1, anterior pharynx-defective 1; PEN-2, presenilin enhancer 2; NICD, Notch intracellular domain; ABC, active β-catenin; CHX, cycloheximide.

* Corresponding author. Department of Phytochemistry, School of Pharmacy, Second Military Medical University, Guohe road 325, Shanghai 200433, China.
** Corresponding author. Department of Phytochemistry, School of Pharmacy, Second Military Medical University, Guohe road 325, Shanghai 200433, China.
E-mail addresses: faranli@hotmail.com (H. Li), wdzhangy@hotmail.com (W. Zhang).
[1] These authors contributed equally.

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throughout adulthood [12]. Notch is essential to coordinate biliary fate and morphogenesis in the liver, and abundant evidence indicates that the Notch pathway has similar functions in HCC progression [13,14]. To date, four Notch receptors (Notch1–4) and two types of Notch ligands (Jagged1/2 and Delta1/3/4) have been discovered in mammals. Notch signaling is initiated when a ligand interacts with the notch transmembrane receptor [15], resulting in a 3-step proteolysis process that involves three proteolytic cleavage sites known as S1, S2, and S3 [16–18]. The γ-secretase complex, which is made up of four proteins, presenilin, nicastrin, APH-1 (anterior pharynx-defective 1), and PEN-2 (presenilin enhancer 2), is essential for S3 site cleavage [19]. S3 site cleavage releases the Notch intracellular domain (NICD), which in turn translocates into the nucleus of the cells and functions as co-activator for the translation factor Recombining Binding Protein Suppressor of Hairless (RBPSUH), also termed RBP-Jκ or CSL. RBP-Jκ is the DNA-binding component of the canonical Notch signaling transcription complex, and activates target genes, such as genes coding hairy and enhancer of split (Hes) and Hes-related with YRPW motif (Hey) family proteins [20], which have been implicated in various cancers, including HCC [21].

Genetic interactions or the association of common cofactors indicates crosstalk between Wnt and Notch signaling in Drosophila [22,23]. Moreover, in mammalian cells, Notch1 physically binds to and antagonizes β-catenin [24,25], and β-catenin activates Jagged1 transcriptions, resulting in Notch activation in colorectal [26] and breast cancer [27]. These physical interactions establish reciprocal interactions between Wnt and Notch signaling.

The present study was conducted to discover therapeutic agents for HCC by screening natural products that inhibit the Notch signaling pathway. Bruceine D (BD) was identified as a Notch inhibitor and showed strong inhibition of liver tumor growth in various murine models.

Materials and methods

Compounds and reagents

Bruceine D (BD) which was firstly reported by Xie JX and Ji Z [28], was previously designed and synthesized in our laboratory; its identity was confirmed based on nuclear magnetic resonance (NMR) and mass spectrometry (MS) data. The purity of BD was >95% based on high-performance liquid chromatography (HPLC) analysis. For *in vitro* experiments, all drugs were dissolved in 100% DMSO at a final stock concentration of 10 mM and stored in single-use vials at −20 °C. Cycloheximide (CHX), proteasome inhibitor MG132, dimethyl sulfoxide (DMSO), GSK3β inhibitor BIO, and Wnt3a were purchased from Sigma (St. Louis, MO, USA); CHIR99021 was purchased from Selleck (Houston, USA). Tetracycline was procured from Applichem (Darmstadt, Germany). X-tremeGENE HP DNA Transfection Reagent was obtained from Roche (Basel, Switzerland), while lipofectamine 2000 transfection reagent was obtained from Invitrogen. Glo lysis buffer was procured from Promega (Madison, WI, USA). The BCA protein assay kit was purchased from PierceChemical (Rockford, IL, USA). The primary antibodies against Notch 1, Notch2, Notch3, Jagged1, cleaved Notch1 (NICD), Hes1, β-catenin (rabbit), non-phosphorylated β-catenin, c-Myc, cyclinD1, survivin, and GAPDH were obtained from Cell Signaling Technology (CST, Danvers, MA). The primary antibody against β-catenin (mouse) was procured from Santa Cruz (Oregon, USA). TCF7L2 and histone H2A were purchased from Abcam (Cambridge, UK).

Plasmids

The human shJAG1 gene and shβ-catenin gene were purchased from GeneChem Co., Ltd. (Shanghai, China). The Notch1 NEXT (Notch 1 extracellular truncation, the amino acid residues 1721-2555) gene, pcDNA3, and pcDNA4/TO-N100-GV were gifts provided by the WUFANG Research fellow at Shanghai Jiao Tong University. pGLD4.31[Luc2P/Gal4UAS/Hygro], which contains a synthetic firefly luciferase sequence for high expression in mammalian cells and a protein degradation sequence (PEST) for faster response to transcriptional regulation, was purchased from Promega. pTOPflash and pFOPflash were products of Millipore (Temecula, CA, USA). The Renilla luciferase reporter vector pRL-TK was a product of Promega (Madison, WI, USA).

Cell lines and cell culture

Huh7 and Hep3B HCC cell lines and L-02 immortalized hepatic cell line were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (SIBS, CAS). Cells were authenticated using GoldeneyeTM20A STR Complex Amplification Kit (Goldeneye, China) by Beijing Microread GeneticsCo., Ltd in November 2013 and were routinely tested for Mycoplasma using a Mycoplasma Detection Kit (Bimake, USA) in July 5th, 2016. The HEK293 cell line stably expressing RBP-Jκ Reporter was provided by the medical school Affiliated Hospital of Qingdao University. HEK293 and Huh7 cells were cultured in 10% FBS in DMEM containing 1000 units of penicillin and 1 mg/ml streptomycin (1% P/S). Hep3B were cultured in monolayers in 10% FBS in MEM containing 1000 units of penicillin and 1 mg/ml streptomycin. L-02 was cultured in RPMI-1640 medium supplemented with 10% FBS. T-REx-HeLa cells were purchased from Invitrogen and cultured in DMEM supplemented with 10% FBS, 1% P/S, and 5 μg/ml blasticidin. All of these cell lines were cultured at 37 °C under 5% CO_2. All cell lines used in this manuscript were thawed from the authenticated cell stock and used within 4 passages.

Cell viability assay

Cells were cultured overnight in 96-well plates (3000 cells/well) and were then treated with BD or sorafenib at the indicated concentration for 24, 48, or 72 h. Then, cells were treated with 10 μl Cell Counting Kit-8 (CCK8) reagent (Dojindo, Japan) for 1 h on each well. Optical density was measured at 450 nm and normalized to background absorbance of the medium in the absence of cells. All samples were assayed in triplicate.

Soft agar colony formation assay and clonogenic assay

Cells (1000/well) in 6-well plates were incubated with or without BD (0–1 μM) in 1 ml 0.33% basal medium eagle agar containing 10% FBS or in 3.5 ml 0.5% basal medium eagle agar containing 10% FBS. The cultures were maintained at 37 °C in a 5% CO_2 incubator for 10–14 days. Cell colonies were counted under a microscope using the Image-Pro Plus software program (Media Cybernetics, Silver Spring, MD).

Apoptosis analysis by flow cytometry

Cell apoptosis was examined with an Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson Biosciences, San Jose, CA, USA). Cells were treated for 24 h with BD or sorafenib alone or combined before staining with Annexin V-FITC and propidium iodide (PI). After incubation at room temperature for 5 min in the dark, the apoptosis was analyzed by flow cytometry (Becton Dickinson FACS Vantage SE, San Jose, CA, USA).

Trypan blue exclusion assay

Huh7 cells were cultured overnight in 6 well-plates, incubated with or without BD for 24 h. The cells were stained with trypan blue dye (Sigma, USA) according to the recommended protocol.

Protein preparation and western blot analysis

Nuclear and cytoplasmic extract isolation of Huh7 and Hep3B cells was performed using the Nuclear and Cytoplasmic Extraction Reagent kit (Beyotime, Haimen, China). HCC cells or tumors from xenograft mice were lysed with RIPA buffer (150 mM·NACl, 1% NP-40, 50 mM Tris, 1 mM EDTA, 1 mM·Na_3VO_4) supplemented with 1x protease inhibitors (Roche Applied Sciences, Germany). The concentration of protein in the supernatant fractions was measured using the PierceTM BCA Protein Assay Kit (Beyotime, Haimen, China), according to the manufacturer's instructions. Equal amounts of protein were subjected to 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE), and were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membrane was blocked in 5% fat-free milk for 1 h at room temperature, and was then incubated with a specific primary antibody at 4 °C overnight, followed by incubation with a donkey anti-rabbit or goat anti-mouse second antibody (IRDye 800, LI-COR, Biosciences). Images were captured with the Odyssey Infrared Imaging System (LI-COR, Biosciences), according to the manufacturer's instructions. Protein densitometry was performed using Quantity One imaging software (Bio-Rad).

RNA extraction and real-time PCR

Total RNA was extracted from HCC cell lines with the Trizol reagent (Thermo Fisher) according to the protocol provided by the manufacturer. RNA was reversely transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). The quantitative RT-PCR analysis of mRNA expression was performed on a Stratagene Mx3005P RT-PCR system (Applied Biosystems) using the PowerUp™ SYBR® Green Master Mix (Thermo Fisher), according to protocol. The PCR primers used for the amplification of the indicated genes are listed in Supplementary Table 1. The relative expressions of the samples were normalized to the internal controls.