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2′-hydroxycinnamaldehyde inhibits cancer cell proliferation and tumor growth by targeting the pyruvate kinase M2

Yae Jin Yoon[a], Young-Hwan Kim[a,d], Yena Jin[a,c], Seung-Wook Chi[b,c], Jeong Hee Moon[b], Dong Cho Han[a,c,*], Byoung-Mog Kwon[a,c,**]

[a] Laboratory of Chemical Biology and Genomics, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahakro, Daejeon, 34141, Republic of Korea
[b] Medical Proteomics Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahakro, Daejeon, 34141, Republic of Korea
[c] Korea University of Science and Technology in Korea, Daejeon, 34113, Republic of Korea
[d] Department of Biology, Chungnam National University, Daejeon, 34134, Republic of Korea

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ABSTRACT

It is reported that 2′-hydroxycinnamaldehyde (HCA), isolated from cinnamon, has anti-tumor effects through the modulation of multi-target molecules. In this study, we identified pyruvate kinase M2 (PKM2) as a direct target of HCA by use of biochemical methods including affinity chromatography, drug affinity responsive target stability, and cellular thermal shift assay. PKM2 is up-regulated in multiple cancer types and is considered as a potential target for cancer therapy. HCA binds directly to PKM2 and selectively decreases the phosphorylation of PKM2 at Tyr105, indicating a potential anti-proliferative effect on prostate cancer cells. As a PKM2 activator, HCA increases pyruvate kinase activity by promoting the tetrameric state of PKM2. However, HCA suppresses protein kinase activity of PKM2 by decreasing the phosphorylation at Tyr105. Moreover, this leads to a decrease of PKM2-mediated STAT3 phosphorylation at Tyr705 and a down-regulation of target genes, including MEK5 and cyclin D1. Furthermore, HCA suppresses tumor growth and the release of tumor extracellular vesicles *in vivo* by inhibiting the phosphorylation of PKM2. Collectively, our results suggest that HCA may be a potential anticancer agent targeting PKM2 in cancer progression.

1. Introduction

Cancer cells with highly proliferative phenotypes require increased glucose uptake and enhanced lactate production regardless of oxygen availability [1]. To supply the energy needed for anabolic reactions, cancer cells use aerobic glycolysis, known as the Warburg effect [2]. Compared to normal cells, cancer cells display a considerably different metabolism to support cell growth and proliferation [1,3]. Cancer cells balance the synthesis of macromolecules as well as sufficient ATP production [3]. Targeting the metabolic enzyme has gained attention as a potential therapeutics for cancer, but there are currently few molecules that target the central carbon metabolism in clinical trials [4,5]. Because rapidly proliferating cells rely on the same metabolic pathways, targeting cancer cell metabolism shows adverse effects on normal cells such as gut epithelium and bone marrow [4]. To reduce unwanted toxicity, drugs that target metabolic enzymes with metabolic differences between cancer cells and normal cells, should be explored.

Pyruvate kinase (PK) is a rate-limiting enzyme during glycolysis, and it has four isoforms (M1, M2, L, and R) with unique tissue expression patterns in mammals [6]. PKM2 is expressed in cancer cells, embryonic tissues, and adult stem cells, whereas PKM1 is expressed in many differentiated cells such as brain and muscle [7]. As a key regulator of the Warburg effect, enhanced expression of PKM2 has been reported in various cancer cell lines and samples from cancer patients [8,9]. PK and protein kinase activity of PKM2 are determined by regulating the conformational states of PKM2 [10,11]. The tetrameric state of PKM2 has high PK activity, whereas the dimeric state of PKM2 has low PK activity but high protein kinase activity [11]. While normal proliferating cells express the tetrameric form, cancer cells predominantly express the dimeric form of PKM2 [9,12]. Additionally, the conversion of tetrameric to dimeric PKM2 is regulated by oncoproteins, tyrosine kinase-mediated phosphorylation, and oxidative stress [13].

* Corresponding author. Laboratory of Chemical Biology and Genomics, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahakro, Daejeon, 34141, Republic of Korea.
** Corresponding author. Laboratory of Chemical Biology and Genomics, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahakro, Daejeon, 34141, Republic of Korea.
E-mail addresses: dchan@kribb.re.kr (D.C. Han), kwonbm@kribb.re.kr (B.-M. Kwon).

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Recent studies show that the phosphorylated or acetylated PKM2 translocates into the nucleus and binds to STAT3 or β-catenin, leading to cell proliferation-related gene expression, such as MEK5, cyclin D1, and c-Myc [2,14–16]. Thus, inhibiting the phosphorylation of PKM2 can suppress cell proliferation and tumor growth. Several groups have reported that PKM2 inhibitors and activators suppress cancer cell proliferation and tumor growth [17–19]. Because therapies targeting PKM2 expression can have toxic effects towards some normal tissues, small molecules that activate PK activity of PKM2 are considered therapeutic modalities [19,20].

Cinnamon, a spice used daily by people all over the world, has beneficial effects when used as a treatment for cancer, allergies, bacterial/viral infections, and Alzheimer's disease [21]. Cinnamon extracts consist of active compounds, including essential oils (cinnamaldehydes), tannins, mucus, and carbohydrates [22]. One of active components, isolated from stem bark of *Cinnamomum cassia* named 2′-hydroxycinnamaldehyde (HCA), was screened as an anticancer drug candidate due to its inhibitory effects on farnesyl protein transferase (FPTase) [23]. HCA and its derivatives showed anti-tumor activities in various types of cancer cells [21]. To understand the molecular mechanisms of HCA, molecular targets of HCA have been reported: the proteasome subunits in colon cancer cells, the low-density lipoprotein receptor-related protein 1 (LRP1) in microglial cells and breast cancer cells, and the Pim-1 kinase in human leukemia cells [24–26]. Although HCA and its derivatives have various biological activities in different cell types, the molecular mechanism associated with the binding target of HCA is not completely understood.

In this study, we identified PKM2 as a direct target of HCA, and determined its anti-proliferative and anti-tumor activities in prostate cancer cells. HCA suppressed cell proliferation by inhibiting tyrosine phosphorylation of PKM2 and STAT3 as well as promoting tetramer formation of PKM2. In addition, the amount of extracellular vesicle (EV) harboring PKM2 was reduced in tumor-bearing mice treated with HCA compared to the vehicle-treated mice. Thus, HCA significantly suppressed cell proliferation and tumor growth by regulating cancer cell metabolism and oncogenic molecules.

2. Materials and methods

2.1. Cell culture

All cell lines used in this study were originally obtained from the American Type Culture Collection (ATCC). DU145, LNCaP (human prostate cancer), HCT116, SW480, SW620 (human colon cancer), MDA-MB-231, MDA-MB-468 (human breast cancer) cells were maintained in RPMI 1640 medium (Gibco). PC3 (human prostate cancer) and HFF (human fibroblast) cells were maintained in Dulbecco's modified Eagle's medium (Gibco). MCF-10 A (human mammary epithelial) cells were maintained in DMEM-F12 (Gibco). All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS: Gibco), 100 U ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin (Sigma-Aldrich). Cell cultures were maintained in a 37 °C incubator under a humidified atmosphere with 5% CO_2.

2.2. Synthesis of HCA-Biotin

To a mixture of N-Biotinlcarproic acid (90 mg, 1.0 equiv.) and HCA (45 mg, 1.1 equiv.) in DMSO (20 ml), DMAP (20 mg) and 72 mg of N,N′-dicyclohexylcarbodiimide (DCC) were added. The reaction mixture was stirred for 12 h at room temperature. Then, the reaction solution was diluted with methylene chloride and water. The aqueous layer was extracted three times with methylene chloride. The combined organic layer was dried over anhydrous $MgSO_4$ and filtered through a filter paper. The filtrate was concentrated *in vacuo* and purified with silica gel flash column chromatography and HPLC to provide the desired product Biotin-HCA (23 mg, yield: 15%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.67 (d, J = 7.5 Hz, 1H), 7.65 (d, J = 6.5 Hz, 1H), 7.53 (d, J = 16 Hz, 1H), 7.46 (dt, J = 7.5, 1.1 Hz, 1H), 7.51 (dt, J = 1.1, 7.5 Hz, 1H), 7.15 (d, J = 8.0 Hz, 1H), 6.72 (dd, J = 7.0, 16.0 Hz, 1H), 6.06 (m, 1H), 6.05 (s, 1H), 5.25 (s, 1H), 4.49 (m, 1H), 4.29 (m, 1H), 3.26 (m, 2H), 3.13 (m, 1H), 2.87 (m, 1H), 2.67 (m, 3H), 2.19 (m, 2H), 1.4–1.8 (m, 12). ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.74, 173.17, 171.71, 163.69, 149.39, 145.95, 132.14, 130.21, 128.25, 126.67, 126.48, 123.31, 61.75, 60.12, 55.48, 40.51, 39.15, 35.93, 34.05, 29.25, 28.09, 28.00, 26.34, 25.57, 24.42.

2.3. Pull-down assay

DU145 cells were washed with PBS and homogenized with a 26-gauge syringe in binding buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA and 0.1 mM Na_3VO_4). The cell lysate was centrifuged and the supernatant was collected. The cell lysate was precleared by incubation with Neutravidin beads (Thermo Scientific) for 1 h at 4 °C. The cleared lysate was incubated with biotin-conjugated HCA (Biotin-HCA) overnight at 4 °C. Proteins bound to Biotin-HCA were precipitated with Neutravidin beads. After 3 washes in washing buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Tween-20, 10% (v/v) glycerol, 1 mM NaF, 0.1 mM Na_3VO_4, and 1 × protease inhibitor cocktail (Roche Applied Science)), the beads were eluted with 1 × sample buffer. The samples were boiled for 10 min and separated for Coomassie blue staining or immunoblotting.

2.4. Drug affinity responsive target stability (DARTS)

The DARTS experiment was conducted as previously described with some modifications [27,28]. Cells were washed with ice-cold PBS and treated with ice-cold M-PER lysis buffer (Thermo Scientific) supplemented with a protease inhibitor cocktail, 1 mM Na_3VO_4 (Sigma-Aldrich), and 1 mM NaF (Sigma-Aldrich). After collecting the cells with a scraper, the cell lysates were centrifuged at 13,000 rpm for 10 min. The protein lysates were mixed with 10 × TNC buffer (500 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 100 mM $CaCl_2$) (Sigma-Aldrich). The lysates in 1 × TNC buffer were incubated with DMSO or HCA for 1 h at room temperature. Following the incubation, each sample was proteolyzed in various concentrations of pronase (Roche Applied Science, 10165921001) for 10 min at room temperature. After 10 min, 2 μl of ice-cold 20 × protease inhibitor cocktail was added to stop proteolysis and the samples were immediately placed on ice. Digestion was further stopped by adding 5 × sample loading dye and boiling at 95 °C for 10 min. An equal portion of each sample was then loaded onto SDS-PAGE gels for Western blotting.

2.5. Cellular thermal shift assay (CETSA)

CETSA was conducted using cell and tissue lysates as previously described [29,30]. For CETSA with cell lysates, DU145 cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.2% NP-40, 5% glycerol, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, and 1 × protease inhibitor cocktail). After centrifugation, the lysates were incubated with DMSO or HCA at room temperature. For CETSA with tissue lysates, DU145 tumor tissues were obtained from mice treated with vehicle or HCA (30 mg/kg). The tumor tissues were lysed with lysis buffer and the supernatants were aliquoted into 0.2 ml PCR tubes and heated for 5 min at the indicated temperature in a PCR machine (Applied Biosystems). The precipitated proteins were separated from the soluble fraction by centrifugation and equal portions of the supernatants were loaded onto SDS-PAGE gels for Western blotting.

2.6. Western blotting

Cell lysates were prepared in RIPA lysis buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% deoxycholic acid, 0.1% SDS, 30 mM