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Vitamin K_3 and K_5 are inhibitors of tumor pyruvate kinase M2

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ABSTRACT

Pyruvate kinase M2 (PKM2) is a rate-limiting enzyme of aerobic glycolysis in cancer cells and plays important roles in cancer metabolism and growth. Here we show that vitamin K_3 and K_5 (VK_3 and VK_5) are relatively specific PKM2 inhibitors. VK_3 and VK_5 showed a significantly stronger potency to inhibit PKM2 than to inhibit PKM1 and PKL, 2 other isoforms of PK dominantly expressed in most adult tissues and liver. This study combined with previous reports supports that VK_3 and VK_5 have potential as adjuvant for cancer chemotherapy.

1. Introduction

Most malignant cancer cells use glycolysis as a major metabolic pathway to generate ATP even in the presence of ample oxygen. Since glycolysis is much less efficient to generate ATP than oxidative phosphorylation, cancer cells exhibit abnormally high glycolytic rate to sustain energy homeostasis. This phenomenon is called Warburg Effect or aerobic glycolysis, which is essential for cancer cells to survive and to proliferate [1–5]. The concept of Warburg effect has influenced significantly the strategy of cancer diagnosis and therapy. Excess glycolysis is the fundamental biochemical basis for FDG-PET. Targeting aerobic glycolysis is an important approach to treat cancer [6]. The metabolic reprogramming – shift from oxidative phosphorylation to aerobic glycolysis – has been listed as one of the 10 hallmarks of cancer [7].

Pyruvate kinase (PK) is the last rate-limiting enzyme of glycolysis. Different isoforms of PK express in different tissues [8,9]: pyruvate kinase M1 (PKM1) in most adult tissues, pyruvate kinase M2 (PKM2) in embryonic tissues, pyruvate kinase L and R (PKL and PKR) in liver and erythrocytes. Cancer cells, regardless of their origin, predominantly express PKM2 [10]. Thus, PKM2 is also called tumor-type PKM2. The shift from other isotypes of PK to PKM2 occurs during tumorigenesis [10] and the underlying mechanism may involve mTOR signaling [11]. PKM2 not only partially contributes to the metabolic shift from oxidative phosphorylation to glycolysis in cancer cells, but also may divert glycolytic intermediate to biosynthesis of amino acid, nucleotide, lipid, etc. [5,10]. The importance of PKM2 in cancer metabolism and growth has been confirmed by numerous reports [10–14].

Due to the importance of PKM2 for cancer cells [12–14], it is proposed that inhibitors of PKM2 could be used as adjuvant for cancer treatment. N-(3-carboxy-4-hydroxy)phenyl-2,5-dimethylpyrrol (compound 3) was the most potent small molecule inhibitor of PKM2 screened from more than 100,000 chemicals by Vander Heiden et al. [15]. Prompted by the proposal, we carried out a study to screen naturally occurring compounds for PKM2 inhibitor and identified that shikonin and its analogs were potent and specific PKM2 inhibitors [16].

Vitamin Ks (VKs) are essential fat-soluble naphthoquinones (Fig. 1), which have very important physiological roles [17–19]. Vitamin K_1, K_2 and K_3 (VK_1, VK_2 and VK_3) are efficient coagulants [20–22]. Vitamin K_5 (VK_5) is an anti-fungal agent [23,24] and an insulin mimetics [25,26]. In addition, VKs were reported to be important in bone metabolism [27–29]. In the last 30 years, in vitro and in vivo experiments demonstrated that VK_3 and VK_5 were promising anti-cancer adjuvant [30–39]. VK_3 combined with vitamin C showed a synergistic anticancer activity [31,36–38]. VK_3 enhanced the efficacies of anticancer drugs including doxorubicin [31,39]. A clinical trial demonstrated that VK_3 sensitized inopera-

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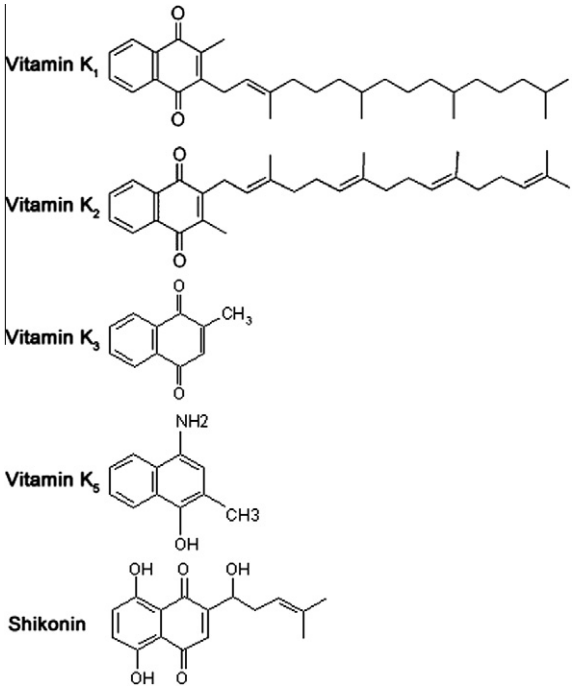

Fig. 1. The chemical structures of vitamin Ks and shikonin.

ble bronchial carcinoma patients to radiotherapy and increased their survival time [35].

VKs and shikonin share similar chemical structures (Fig. 1), implicating that VKs might be PKM2 inhibitors. In this study, we determined the inhibitory activity of VKs toward PKM2, PKM1, and PKL.

2. Materials and Methods

2.1. Reagents and enzymes

VK_1, VK_2 and VK_3 were purchased from Sigma, St Louis, MO, USA; VK_5 was purchased from Wako Pure Chemical Industries Ltd., Chuo-ku, Okasa, Japan. Trypan blue was from Amresco Inc., Solon, OH, USA. β-nicotinamide adenine dinucleotide (β-NADH), adenosine diphosphate (ADP) and phosphoenolpyruvic acid (PEP) were from Roche, Basel, Switzerland. Dimethylsulfoxide (DMSO), fructose 1,6-bisphosphate (FBP), lactate dehydrogenase (LDH) and pyruvate were from Sigma. Mammalian protein extraction reagent (M-PER), protease inhibitor cocktail kit, BCA protein assay kit and non-reducing lane marker sample buffer were from Thermo Fisher Scientific Inc., Waltham, MA, USA; EZ-ECL kit (Chemiluminescence Detection Kit for HRP) was from Biological Industries, Kibbutz Beit-Haemek, Israel; Molecular Imager FX was from Bio-Rad Laboratories, Hercules, CA, USA.

2.2. Purification of recombinant human PKM1, PKM2, and PKL

The cDNA of PKM1, PKM2 and PKL were cloned into pQE-30 with an N-terminal 6xHis-tag and purified from *E. coli.* using Ni-Sepharose column (GE, Piscataway, NJ, USA) as described previously [16]. Briefly, when *E. coli.* grew to an OD (600 nm) of 0.7, the expression was induced by 1 mM IPTG (Gibco, Grand Island, NY, USA) for 6 h at room temperature. Cells were collected and lysed by freeze/thaw cycle and sonication. Lysate was passed through Ni-Sepharose column, the protein not bound to Ni-Sepharose was washed away with washing buffer (0.1 M Tris–HCl, pH 7.8, containing 0.5 M NaCl and 40 mM imidazole), and pyruvate kinase was eluted by 250 mM imidazole. The purity and kinetic characteristics of pyruvate kinases were determined according to our previous report [16].

2.3. Preparation of compound 3

N-(3-Carboxy-4-hydroxy)phenyl-2,5-dimethylpyrrole (compound 3) was prepared from 5-amino-2-hydroxybenzoic acid and hexane-2,5-dione via a modified procedure as described by us previously [16]. 5-aminosalicylic acid (5 mmol), hexane-2,5-dione (6 mmol) and 4-methylbenzenesulfonic acid (0.05 mmol) were added to a dried round bottom flask. Toluene (10 ml) was added and the mixture was refluxed for 1 h. After cooling to room temperature, the solvent was removed in vacuum and the residue was subject to a flash chromatography on silica gel with petroleum ether/ethyl acetate (1:1) as eluent to give pure product, which was recrystalized from petroleum ether/ethyl acetate. The qualification of compound 3 was described previously [16]: Orange solid; mp. 167–169 °C (Ref. 169–171 °C); ^{1}H NMR ($CDCl_3$) δ 10.45 (s, 1H), 7.83 (d, J = 2.4 Hz, 1H), 7.42–7.39 (m, 1H), 7.13 (d, J = 8.8 Hz, 1H), 5.93 (s, 2H), 2.06 (s, 6H) ppm; MS (ESI) m/z 232.0 ([M + H] $^+$).

2.4. Cell culture

Hela was purchased from Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China) and maintained in RPMI 1640 (Gibco) containing 10% FCS (Gibco) and 100 U/ml Penicillin–Streptomycin (Gibco). Cells were grown in a humidified CO_2 incubator at 37 °C, and subcultured with 0.25% trypsin containing 0.02% EDTA (Gibco) [16].

2.5. Pyruvate kinase activity assay

PK activity was measured by an LDH-coupled assay according to the previous reports [15,16]. The reaction solution contained 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 2 mM ADP, 0.2 mM β-NADH and 4.8 U/ml LDH. Compound 3, vitamin K_3 and K_5 were dissolved in DMSO, and vitamin K_1 and K_2 were dissolved in ether. The dissolved chemicals were added to the PK solution to different concentrations in the presence or absence of 125 μM FBP, and the mixture were incubated for 60 min in the room temperature. To avoid reducing the enzyme activity dramatically, DMSO/Ether should not be more than 1/100 of the incubation mixture. 75 ng pyruvate kinase was added to 100 μl PK reaction solution, and PK activity was calculated by monitoring changes of absorbance at 340 nm from 0 to 5 min at 25 °C. The activity of PK was defined as the quantity of NADH oxidized by 1 mg PK per minute [15,16].

2.6. Lactate dehydrogenase activity assay

Assay of LDH activity was performed at 25 °C in a mixture containing 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 0.2 mM β-NADH and 2 mM pyruvate. 1.25×10^{-3} U LDH in the presence or absence of VKs was added to 100 μl LDH reaction solution. The absorbance of 340 nm was monitored from 0 to 2 min at 25 °C [16].

2.7. Trypan blue exclusion assay

Loss of integrity of the plasma membrane was determined by trypan blue exclusion assay. After drug treatment, cells were collected and stained with 0.4% trypan blue for 5 min. Dead cells were permeable and viable cells were impermeable to trypan blue. The viable and dead cells were counted microscopically. The viable rate of each data set was calculated.

2.8. Measurement of the metabolic rate of glucose and lactate

A total of 1×10^6 cells per well were plated in a 6-well plate. After attachment, the cells were treated with VKs or vehicle (0.1% DMSO or 0.1% Ether) for 1 h. The culture medium was collected. The concentration of glucose in the medium and the concentration of lactate in the medium were determined by using the Vitros 5,1 FS chemistry system from Johnson & Johnson Company, New Brunswick, NJ, USA. Consumption of glucose and production of lactate were calculated from the difference between the concentrations in the medium at the beginning and at an appropriate time of culture.

2.9. Transfection of Hela with PKM1 and sensitivity of transfectants toward vitamin K_3 or K_5

The cDNA of PKM1 was cloned into pCDNA 3.1 (Invitrogen Co., Carlsbad, CA, USA). PKM1-pCDNA3.1 and pCDNA 3.1 (vector-only control) were introduced into Hela cells with lipofectamine LTX (Invitrogen) according to manufacturer's instruction. Cells were subjected to western blot and vitamin Ks susceptibility assays 48 h after the transfection. Transfectant and control cells (2.5×10^5 per well in 24-well plate) were treated with 50 μM vitamin K_3 or K_5 for 6 h or with 15 μM vitamin K_3 or K_5 combining 2.5 μM doxorubicin for 24 h. Cell survival rate was determined using a trypan blue exclusion assay as described in the preceding section.

2.10. Preparation of cell extract

Cells were trypsinized and collected, and then were lysed by M-PER containing protease inhibitor cocktail. After a centrifugation at 17,000*g* for 15 min, the protein content in the supernatant was determined by BCA protein assay kit.