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NAMPT inhibition synergizes with NQO1-targeting agents in inducing apoptotic cell death in non-small cell lung cancer cells

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[ABSTRACT] Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the first rate-limiting step in converting nicotinamide to NAD^+, essential for a number of enzymes and regulatory proteins involved in a variety of cellular processes, including deacetylation enzyme SIRT1 which modulates several tumor suppressors such as p53 and FOXO. Herein we report that NQO1 substrates Tanshione IIA (TSA) and *β*-lapachone (*β*-lap) induced a rapid depletion of NAD^+ pool but adaptively a significant upregulation of NAMPT. NAMPT inhibition by FK866 at a nontoxic dose significantly enhanced NQO1-targeting agent-induced apoptotic cell death. Compared with TSA or *β*-lap treatment alone, co-treatment with FK866 induced a more dramatic depletion of NAD^+, repression of SIRT1 activity, and thereby the increased accumulation of acetylated FOXO1 and the activation of apoptotic pathway. In conclusion, the results from the present study support that NAMPT inhibition can synergize with NQO1 activation to induce apoptotic cell death, thereby providing a new rationale for the development of combinative therapeutic drugs in combating non-small lung cancer.

[KEY WORDS] FK866; NQO1-targeting agents; Synergy; NAD^+; SIRT1

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Introduction

NAD(P)H: quinone oxidoreductase 1 (NQO1), a flavin protease enzyme prevalent in most eukaryotic cells, has recently attracted considerable attention because of its important role in cancer chemoprevention and chemotherapy [1-2]. The discovery and development of NQO1-targeting agents has attracted much attention from scientists in academic and industrial fields. *β*-lapachone (*β*-lap) is a novel NQO1 targeting anticancer drug, which has been demonstrated to possess strong cytotoxicity toward various cancer cell lines *in vitro* and *in vivo* [3-5]. We have recently identified that Tanshinone IIA (TSA), a natural compound contained in Danshen, elicit its anti-tumor efficacy via targeting NQO1 [6]. However, a narrow therapeutic window and its toxicity against erythrocytes limit the clinical use of *β*-lap [7], while poor dissolubility limits the use of TSA. Combination therapy may be a possible strategy to reduce the dose of NQO1 substrates to either decrease toxicity or improve therapeutic effect. Various combinatione therapies of *β*-lap with various chemotherapeutic drugs, such as FDA-approved non-steroidal anti-inflammatory drug sulindac, paclitaxel and cisplatin, have been reported in previous studies [8-10]. TSA has also been reported to be used in combination with clarithromycin or arsenic trioxide [11-12].

The cancer cells treated with NQO1-taregting agents are characterized by extensive depletion of NAD^+ pool [13]. NAD^+ plays a critical role in an increasingly diverse range of cellular

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processes, including signal transduction, DNA repair, and post-translational protein modifications, and apoptosis [14]. Thus, it is reasonable to propose that the inhibition of NAD^+ biosynthesis may sensitize the cancer cells to NQO1-targeting agents. A two-step salvage pathway that converts nicotinamide (NAM) to NAD^+ represents the major route to NAD^+ biosynthesis in mammals [15-16]. Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the first rate-limiting step in converting NAM to NAD^+ and has crucial roles in many cellular functions by regulating NAD^+-dependent SIRT1 deacetylase [17-19]. Inhibition of NAMPT significantly suppresses cell growth in culture, soft agar colony formation, cell invasion, and growth of xenografted cancer cells in mice [18]. FK866, a specific competitive inhibitor of NAMPT, is currently in clinical trials in patients with several kinds of advanced solid tumors [20-22]. A recent report has noted that FK866 sensitizes pancreatic adenocarcinoma cells to ROS-induced, μ-calpain-mediated programmed cell death induced by *β*-lap [23]. However, the specific mechanisms of action remain to be explored.

SIRT1 is an evolutionarily conserved NAD^+-dependent deacetylase, which senses changes in intracellular NAD^+ levels and uses this information to adapt to certain circumstances [24]. It regulates cell survival, replicative senescence, inflammation, and metabolism through the deacetylation of histones (the major protein components of chromatin) and other cellular factors including the transcription factors p53, NF-*κ*B, and FoxO family [25]. SIRT1 may contribute to tumorigenic potential; it is overexpressed in many cancers, and inhibition of its enzymatic activity by small molecules kills tumor cells [26].

In the present study, we demonstrated that NAMPT inhibition may synergize with TSA and *β*-lap in inducing apoptotic cell death of non-small lung cancer cells, thus providing a rationale for the development of combination therapy using NAMPT inhibitors and NQO1 substrates to enhance sensitivity and/or reduce toxicity in therapy of non-small lung cancer (NSCLC).

Material and Methods

Cell line and cell culture

Human NSCLC cell line A549 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in McCoy's 5a medium supplemented with 10% FBS, 100 $U \cdot mL^{-1}$ of penicillin, and 100 $mg \cdot mL^{-1}$ of streptomycin at 37 °C in a humidified incubator with 5% CO_2.

Determination of cytotoxicity and apoptosis

In MTT assay, A459 cells were pretreated with FK866 10 $nmol \cdot L^{-1}$ for 1 h when cell confluence was up to 80% and then treated with TSA for 48 h or *β*-lap exposure for 2 h and harvested at 24 h at indicated concentration. 20 μL of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) solution was added to each well in the 96-well plates and incubated for 4 h at 37 °C. MTT solution was removed and 150 μL of DMSO was added. Dissolved MTT crystals were quantitated using a microplate reader with 570 nm wavelength after agitating the plates for 10 min on a shaker.

A459 cells were pretreated with FK866 10 $nmol \cdot L^{-1}$ for 1 h and then exposed to 40 $\mu mol \cdot L^{-1}$ of TSA for 48 h or 5 $\mu mol \cdot L^{-1}$ of *β*-lap for 24 h. Apoptosis was quantified by usingAPO-BRDU^{TM} Kit (BD Biosciences, San Diego, CA, USA) or AnnexinV-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) . The cells were harvested and stained according to the manufacturer's instruction. The labeled cells were analyzed with flow cytometry FACS Calibur (BD Biosciences).

Semi-quantitative RT-PCR and Western blotting analysis

A459 cells were pretreated with 40 $\mu mol \cdot L^{-1}$ of TSA or 5 $\mu mol \cdot L^{-1}$ of *β*-lap for indicated time. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed according to the manufacturer's protocol (Promega, Madison, WI, USA). qRT-PCR was performed with SYBR Premix ExTaq^{TM} II (Takara Co., Ltd., Dalian, China) in a reaction volume of 10 μL. β-actin gene was used as an endogenous control. Homo-NAMPT primers were as follows: forward: AAGAGACTGCTGGCATAGGA, and reverse: ACCACAGATACAGGCACTGA; homo-ACTB primers were as follows: forward: AAGAGCTACGAGCTGCCTGAC, and reverse: TCCT GCTTGCTGATCCACAT.

For Western blot analysis, A549 cells were pretreated with FK866 10 $nmol \cdot L^{-1}$ for 1 h and then exposed to 40 $\mu mol \cdot L^{-1}$ of TSA for 48 h or 5 $\mu mol \cdot L^{-1}$ of *β*-lap for 24 h. The cellular proteins were extracted with lysis buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Proteins were detected using specific primary antibodies against SIRT1 (H-300, 1 : 200, Santa Cruz Biotechnology, CA, USA), Ac-FOXO1 (D-19, 1 : 200, Santa Cruz Biotechnology), TRAIL (C92B9, 1 : 1 000, Cell Signal Technology, Beverly, MA, USA), Bim (C34C5, 1 : 1 000, Cell Signal Technology), FasL (1 : 200, BD pharmingen^{TM}, San Diego, CA, USA) or BCL-6 (N-3, 1 : 200, Santa Cruz Biotechnology). The protein levels were normalized with GAPDH (1 : 1 000, Shengxing, Wuhan, China). After washing with TBST, the membrane was incubated with HRP-conjugated secondary antibody (1 : 10 000, KeyGen, Nanjing, China) for 1 h. The signal was detected by enhanced cheniluminescence (ECL, Millipore, Billerica, MA, USA).

SIRT1 activity assay

A549 cells were pretreated with FK866 10 $nmol \cdot L^{-1}$ for 1 h and then exposed to 40 $\mu mol \cdot L^{-1}$ of TSA or 5 $\mu mol \cdot L^{-1}$ of *β*-lap for 2 h and harvested at 12 h. SIRT1 activity was measured using a fluorometric SIRT1 assay kit (Sigma CS1040, Saint Louis, Missouri, USA), according to the manufacturer's instructions. In brief, 20 μL of whole cell extracts prepared with lysis buffer were added to a black side-clear bottom 96-well plate and incubated with 10 μL of fluorometric SIRT1 substrate and 5 μL of NAD^+ solution for 30 min at room temperature. The reactions were stopped by adding 5 μL of developer solution and incubated for 10 min. Experimental values were expressed as a percentage of the control. Filter excitation and emission wavelengths were set at 340 and 430 nm, respectively.