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Inhibition of NAMPT sensitizes MOLT4 leukemia cells for etoposide treatment through the SIRT2-p53 pathway

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ABSTRACT

NAMPT (Nicotinamide phosphoribosyltransferase) catalyses the rate-limiting step in the NAD biosynthesis from nicotinamide and thereby regulates the activity of NAD-dependent enzymes. Cancer cells are highly dependent on NAD for energy and DNA repair processes and are assumed to be more susceptible to an inhibition of NAD synthesis than non-transformed cells. We aimed to investigate whether or not inhibition of NAMPT with its specific inhibitor FK866 can sensitize leukemia cells for chemotherapeutic agents.

NAMPT protein abundance, enzymatic activity and NAD concentrations were significantly higher in Jurkat and Molt-4 leukemia cell lines compared to normal peripheral blood mononuclear cells. Combination of etoposide and FK866 caused increased cell death in leukemia cell lines compared to etoposide alone. Etoposide decreased protein abundance of NAD-dependent deacetylases SIRTUIN1. After combining etoposide and FK866 treatment SIRTUIN2 was further decreased and accumulation and acetylation of the downstream target p53 was further enhanced in MOLT4 cells. Concomitantly, protein abundance of p21 and cleaved BAX was increased.

Targeting NAMPT could be a novel therapeutic strategy to enhance the efficacy of chemotherapeutic agents such as etoposide against leukemia.

1. Introduction

Leukemia is the most common cancer diagnosed in children and represents approximately 30% of cancer diagnoses among children younger than 15 years [1]. The main treatment for childhood leukemia is chemotherapy [2]. Children with high-risk leukemia or relapse generally receive more intense and aggressive chemotherapy, *e.g.* treatment with etoposide [3,4]. Although chemotherapy is successful in up to 90% of pediatric patients with acute lymphoblastic leukemia, the toxicity of chemotherapy is a common cause of morbidity and mortality in children during treatment and later in life [5]. New strategies to reduce doses of chemotherapeutics are therefore urgently needed.

Cancer cells are characterized by metabolic adaptations such as a high nicotinamide adenine dinucleotide (NAD) turnover rate due to increased proliferation, DNA repair and metabolism [6,7]. Nicotinamide phosphoribosyltransferase (NAMPT) is the key enzyme of the NAD salvage pathway from nicotinamide and thus a regulator of the intracellular NAD pool. NAMPT was shown to be overexpressed in different types of cancer, among them hematologic malignancies such as leukemia [8] and lymphomas [9]. Given that, NAMPT may be crucial for maintaining cellular NAD levels in cancer cells to facilitate cancer proliferation and survival. It is therefore a potential therapeutic target for the treatment of cancer.

SIRTUIN1 (SIRT1) and SIRT2 are NAD-dependent enzymes that are overexpressed in leukemia and could play a causal role in leukemia development Dan et al., 2012; Kozako et al., 2012; Wang et al., 2011b. Furthermore, these two central SIRTs play an important role in tumor metabolism by deacetylating p53 and therefore regulating apoptosis [10,11].

FK866 (APO866) is a specific inhibitor for NAMPT [12]. Treatment with FK866 reduced intracellular NAD levels leading to apoptosis and reduced cell proliferation in leukemia cell lines with functional tumor suppressor p53. This effect was mediated by increased acetylation of p53 at lysine 382 with subsequently increased expression of p21 and BAX. In contrast, leukemia cell lines containing nonfunctional p53 were relatively unaffected by FK866 treatment [13]. Furthermore, FK866 had minor toxic effects on normal hematopoietic progenitor cells [14]. Although FK866 was relatively well tolerated in humans and advanced to phase II clinical trials, it did not demonstrate sufficient tumor selectivity to achieve clinical success as single agent [15].

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However, combining NAD depletion with chemotherapeutic agents might enhance their therapeutic efficacy [13]. In neuroblastoma cells, the effects of etoposide on DNA damage were potentiated by FK866 treatment, whereas the effect of FK866 on cytosolic NAD depletion was potentiated by etoposide [16]. In neuroendocrine tumors, the NAMPT inhibitor GMX1778 enhanced the efficacy of 177Lu-DOTATATE treatment and induced a prolonged antitumor response [17].

The aim of this study is to test the hypothesis that NAMPT inhibition using the specific inhibitor FK866 makes leukemia cells more susceptible for chemotherapeutic agents and to elucidate underlying signalling pathways.

2. Materials & methods

2.1. Material

Cell culture media and supplements were obtained from PAA (Cölbe, Germany) or Invitrogen (Karlsruhe, Germany). FK866 and nicotinamide mononucleotide (NMN) were obtained from Sigma Aldrich (Munich, Germany). Etoposide was purchased from Merck Millipore (Darmstadt, Germany). Primary antibodies were obtained from Cell Signaling (CST, Beverly, MA, USA) and Millipore. Secondary antibodies were purchased from DAKO (Hamburg, Germany).

2.2. Cell culture

Jurkat and Molt-4 T-ALL cell lines were purchased from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures). The cell lines were cultured in RPMI 1640 medium with 2 mmol/L glutamine and 10% fetal bovine serum (FBS) for Jurkat cells and 20% FBS for Molt-4 cells. All cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cell number was measured using a hemacytometer after trypan blue staining.

2.3. PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from anonymised blood buffy coats of healthy donors purchased from the University Hospital Leipzig Blood Bank. Buffy coats were diluted in lysis buffer (155 mM NH4Cl; 10 mM KHCO3; 0.1 mM Na-EDTA; pH 7.29), gently mixed and kept on ice for 10 min. After centrifugation, the supernatant was removed carefully and the process was repeated until the pellet appeared clear.

2.4. Cell treatments

Etoposide was dissolved in DMSO to generate a stock solution of 42.5 mM. FK866 was dissolved in DMSO to create a stock solution of 10 mM. NMN was dissolved in the appropriate medium for a stock solution of 100 mM. Jurkat and Molt-4 cells were treated with the indicated concentrations of etoposide, indicated concentrations of FK866 or NMN [500 μM] either alone or in combination for 24 h.

2.5. Measurement of cell viability and cell death

To investigate the effects of the chemotherapeutic agents on proliferation and cell viability, Cell Proliferation Reagent WST-1 (Roche, Grenzach-Wyhlen, Germany) was used according to manufacturer's instructions. 40,000 cells/well were seeded in a 96-well plate and cultured as described above.

To evaluate the effects of etoposide the number of dead cells was measured by flow cytometry using propidium iodide (PI) (Calbiochem, Dan Diego, CA, USA). 800,000 cells/well were seeded in a 6-well plate and cultured as described above. Cells were harvested and washed with ice-cold PBS. The cell pellet was re-suspended in 100 μL of PBS with 0.5% FBS. Cell suspension was transferred into round bottom tubes. 2 μL of PI were added to the cell suspension, followed by gentle vortexing. Samples were incubated for 10 min on ice in darkness and analysed using a Beckton-Dickinson FACS LSRII. For each sample, 10,000 cells were counted. PI^+ cells were considered dead.

2.6. Protein extraction and Western blot analyses

Cells were lysed in modified RIPA buffer as previously described [18]. Protein concentration was determined using Pierce BCA protein assay (Thermo Scientific) and equal amounts of protein were separated by SDS–PAGE and transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Next, membranes were blocked in 5% non-fat dry milk in TBS buffer containing 0.1% Tween 20. Primary antibodies used for immunoblotting included anti-NAMPT (clone OMNI 379) (Cayman Chemical, Ann Arbor, MI, USA), anti-Caspase-3, anti-PARP, anti-SIRT1, anti-SIRT2, anti-Ac-p53, anti-p53, anti-p21, anti-Bax (Cell Signaling, Beverly, MA, USA) and anti-GAPDH (Merck Millipore, Schwalbach, Germany). Appropriate secondary antibodies were purchased from DAKO (Hamburg, Germany). Detection of proteins was carried out using Luminata Classico Western HRP Substrate (Merck Millipore) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

2.7. NAMPT enzymatic activity

NAMPT activity was measured by the conversion of ^{14}C-labeled nicotinamide to ^{14}C-NMN using a method previously described [19]. Radioactivity of ^{14}C-NMN was quantified in a liquid scintillation counter (Wallac 1409 DSA, PerkinElmer). 1×10^6 cells were seeded in dishes (152 cm^2) and cultured as described above. NAMPT activity (counts per minute, cpm) was normalized to total protein concentration as measured by BCA protein assay.

2.8. NAD measurement

Concentrations of total NAD from whole-cell extracts were quantified by HPLC analysis by reversed-phase HPLC using a Chromaster Purospher STAR RP-18 endcapped 3 μm Hibar RT 150-3 HPLC column (Merck). 800,000 cells/well were seeded in a 6-well plate and cultured as described above. Cells were harvested and pellet was re-suspended in 100 μL 1 M perchloric acid. After a 10-min incubation period on ice samples were centrifuged and the supernatant was neutralized with 3 M potassium carbonate. After repeated centrifugation samples were loaded onto the column as previously described [18].

The cell pellet of each sample was resuspended in 100 μL 2% SDS, shaken for 10 min at 99 °C and centrifuged for 5 min at 20,000*g* and then used for protein determination (BCA Assay, Pierce Thermo Scientific). The NAD concentration of each sample was referred to the corresponding total protein amount of the sample.

2.9. Statistical analyses

Data are presented as mean ± SEM. Data were analysed for statistical significance by either unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. All analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA). ImageJ 1.41 was used for densitometric analysis (NIH, USA). $p < 0.05$ was considered significant compared to the referred control.

3. Results

3.1. Cancer cell lines have higher NAMPT protein abundance and activity

We compared the leukemia cell lines Jurkat (mutated p53) and Molt-4 (wildtype p53) [20] with peripheral blood mononuclear cells