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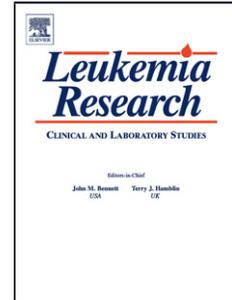
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Inhibition of NAMPT sensitizes MOLT4 leukemia cells for etoposide treatment through the SIRT2-p53 pathway

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Highlights (3 to 5 bullet points; maximum 85 characters, including spaces, per bullet point)

- NAMPT inhibitor FK866 potentiated the effects of chemotherapeutic agent etoposide
- FK866 and etoposide led to a strong induction of cell death *in vitro*
- FK866 and etoposide acted via SIRT2 downregulation in p53 wildtype Molt-4 cells

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.

Keywords: NAMPT inhibition, NAD salvage, FK866, APO866, SIRT1, SIRT2, p53, p21, BAX, caspase, NMN, chemotherapy, apoptosis

Introduction

Leukemia is the most common cancer diagnosed in children and represents approximately 30% of cancer diagnoses among children younger than 15 years (Belson et al., 2007). The main treatment for childhood leukemia is chemotherapy (Cooper and Brown, 2015). Children with high-risk leukemia or relapse generally receive more intense and aggressive chemotherapy, e.g. treatment with etoposide (Bhojwani et al., 2009; Locatelli et al., 2009). 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 (Gervasini and Vagace, 2012). 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 (Cea et al., 2013; Chiarugi et al., 2012). 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 (Audrito et al., 2015) and lymphomas (Olesen et al., 2011). 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 (Luo et al., 2001; Peck et al., 2010).

FK866 (APO866) is a specific inhibitor for NAMPT (Khan et al., 2006). 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 (Thakur et al., 2013). Furthermore, FK866 had minor toxic effects on normal hematopoietic progenitor cells (Nahimana et al., 2009). 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 (von Heideman et al., 2010).

However, combining NAD depletion with chemotherapeutic agents might enhance their therapeutic efficacy (Thakur et al., 2013). 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 (Travelli et al., 2011). In neuroendocrine tumors, the NAMPT inhibitor GMX1778 enhanced the efficacy of ^{177}Lu -DOTATATE treatment and induced a prolonged antitumor response (Elf et al., 2016).

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.

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Materials&Methods

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).

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% CO₂. Cell number was measured using a hemacytometer after trypan blue staining.

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 (155mM NH₄Cl; 10mM KHCO₃; 0.1mM 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.

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 [500µM] either alone or in combination for 24h.

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 μ L of PBS with 0.5% FBS. Cell suspension was transferred into round bottom tubes. 2 μ L 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.

Protein extraction and Western blot analyses

Cells were lysed in modified RIPA buffer as previously described (Schuster et al., 2014). 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).

NAMPT enzymatic activity

NAMPT activity was measured by the conversion of ¹⁴C-labeled nicotinamide to ¹⁴C-NMN using a method previously described (Garten et al., 2010). Radioactivity of ¹⁴C-NMN was quantified in a liquid scintillation counter (Wallac 1409 DSA, PerkinElmer). 1 x 10⁶ cells were seeded in dishes (152 cm²) and cultured as described above. NAMPT activity (counts per minute, cpm) was normalized to total protein concentration as measured by BCA protein assay.

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-minute 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 (Schuster et al., 2014).

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 20,000x 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.

Statistical analyses

Data are presented as mean \pm 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.

Results

Cancer cell lines have higher NAMPT protein abundance and activity

We compared the leukemia cell lines Jurkat (mutated p53) and Molt-4 (wildtype p53) (Cheng and Haas, 1990) with peripheral blood mononuclear cells (PBMCs) from healthy donors in regard to NAMPT protein abundance, NAMPT activity and NAD levels. Jurkat and Molt-4 cancer cell lines showed higher NAMPT protein abundance (25-fold in Jurkat cells, 21-fold in Molt-4 cells) compared to PBMCs (Fig. 1A). Furthermore, NAMPT activity (94.1 ± 18.2 cpm/ μ g total protein x h in Jurkat cells; 24.6 ± 2.6 cpm/ μ g total protein x h in Molt-4 cells) (Fig. 1B) and NAD levels were elevated in cancer cell lines (9.5 ± 1.6 μ mol NAD/g protein in Jurkat cells, 0.9 ± 0.2 μ mol NAD/g protein in Molt-4 cells) compared to PBMCs (Fig. 1C).

FK866 decreases viability, NAMPT activity and NAD levels

We tested the sensitivity of Jurkat and Molt-4 cell lines to inhibition of NAMPT using the specific inhibitor FK866. Incubation of cells with FK866[10nM] for 24h reduced viability ($-59.5 \pm 3.3\%$ in Jurkat cells, $-46.1 \pm 4.6\%$ in Molt-4 cells) (Fig. 2A,B)(Supporting Information 1 A,B). NAMPT activity was decreased in both cells lines significantly at a concentration of 1 nM FK866 and was almost completely blocked after using a concentration of 10 nM FK866 in both cell lines. (Fig. 2 C,D).-NAD concentrations were lowered (by 80% in Jurkat and Molt-4 cells) (Fig. 2 E,F)(Supporting Information 1 C,D) compared to control cells. The enzymatic product of NAMPT, NMN [500 μ M], was able to rescue viability (Fig. 2A,B) and NAD levels (Fig. 2E,F) of both Jurkat and Molt-4 cells, whereas it had no effect alone.

FK866 increases etoposide-induced cell death

To evaluate whether NAMPT inhibition could enhance the effects of chemotherapeutic agents, Jurkat and Molt-4 cells were incubated with the anti-leukemia drugs etoposide (eto), methotrexate (mtx) and doxorubicin (doxo) alone or in combination with FK866 for 24h. Etoposide was able to induce cell death (4.0-fold \pm 0.6 [10 μ M]) in Jurkat cells compared to control cells (Fig. 3A). Molt-4 cells were more sensitive to etoposide, which induced 5.6-fold \pm 1.0 [5 μ M] cell death (Fig. 3B). FK866 treatment alone did not induce cell death in both cell lines after 24h. However, the combination of etoposide and FK866 increased the number of dead cells compared to etoposide treatment alone (5.4-fold \pm 1.4 in Jurkat cells, 1.8-fold \pm 0.1 in Molt-4 cells) (Fig. 3A,B). NMN was able to reverse the FK866-induced effects. Methotrexate (mtx) and doxorubicin (doxo) only induced cell death in Molt-4 cells ($+17.8\% \pm 2.0\%$ and

65.8% \pm 8.2%, respectively). Combining mtx or doxo with FK866 did not increase cell death in Jurkat or Molt-4 cells (Supporting Information 2).

Etoposide treatment induced cleavage and activation of caspase-3 and its downstream target poly (ADP-ribose) polymerase (PARP)-1, but this cleavage was not further increased by the addition of FK866 to etoposide (Fig. 3 C, D). A combination of etoposide and FK866 did not alter cell cycle distribution after 24h (Supporting Information 3).

Etoposide-induced downregulation of SIRT2 is enhanced by NAD depletion

Next, we aimed to determine whether the increased cell death provoked by etoposide and FK866 co-treatment could be caused by a further decrease in NAD levels. The NAD depletion induced by FK866 was not further enhanced by etoposide (Supporting Information 4). NAMPT protein abundance was not changed. However, etoposide treatment was able to decrease the amount of NAD-dependent deacetylases SIRT1 and SIRT2 dose-dependently in p53 wildtype Molt-4 cells. After etoposide treatment of NAD-depleted cells SIRT2 was even further decreased (Fig. 4A). The acetylation and activation of the SIRT1 as well as SIRT2 target p53 was enhanced after co-treatment of etoposide with FK866, which was associated with an increased abundance of its downstream target p21 (Fig. 4B). Additionally, etoposide treatment induced cleavage of p53 downstream target BAX which was further enhanced by the combination with FK866. (Fig. 4B). Jurkat cells which harbour a mutated p53 did not show p53 acetylation or altered levels of p21 or cleaved bax (Supporting Information 5).

Discussion

Cancer cells have a high NAD turnover rate due to their increased proliferation, DNA repair, cell cycle progression and metabolism (Cea et al., 2013; Chiarugi et al., 2012). We therefore tested the efficacy of NAD depletion to sensitize leukemia cells for chemotherapeutic treatment with the aim to provide novel treatment options with reduced doses and consequently less adverse effects for patients.

The NAD salvage enzyme NAMPT was shown to be overexpressed in hematologic malignancies (Audrito et al., 2015; Cagnetta et al., 2015; Olesen et al., 2011). We could confirm these findings and furthermore showed that basal NAMPT activity and NAD levels were also increased in leukemia cells in comparison to PBMCs from healthy volunteers.

We used FK866 (APO866) to specifically target NAMPT (Khan et al., 2006). Thakur *et al.* proposed that leukemia cell lines containing nonfunctional p53 were relatively unaffected by FK866 (Thakur et al., 2013), whereas FK866 was able to induce cell death in p53-deficient hepatocarcinoma cells (Schuster et al., 2015). In our study, FK866 decreased viability, NAMPT activity and consequently depleted NAD levels in both p53 mutated (Jurkat) and p53 wildtype (Molt-4) cells.

Given the influence of NAD depletion on regulators of cellular survival and stress responses, such as PARPs and sirtuins, we postulated that FK866 could synergize with chemotherapeutic agents that target the same pathways. Travelli *et al.* showed that the etoposide-specific effect of inducing DNA damage was potentiated by FK866 (Travelli et al., 2011). Contrary to their findings, however, we did not see an additive effect on total cellular NAD depletion (Supporting information 4). We could show that inhibition of NAMPT with FK866 in combination with etoposide led to increased cell death compared to etoposide alone. Interestingly, this effect was not mediated by the caspase cascade. This result stands in contrast to a study performed in HEK293 cells, where a knockdown of NAMPT made cells more sensitive to etoposide treatment and levels of cleaved caspase-3 were increased, whereas the overexpression of NAMPT led to a resistance of cells to etoposide and reduced levels of cleaved caspase 3 (Yang et al., 2007). However, apoptosis, autophagy as well as necrosis were proposed as mechanisms of cell death induction by FK866 (Cea et al., 2013; Gehrke et al., 2014; Nahimana et al., 2009) or combination treatments (Cagnetta et al., 2015; Nahimana et al., 2014; Travelli et al., 2011). The lack of apoptosis induction by FK866 could also be explained by the shorter treatment period we used compared to other studies (Schuster et al., 2015; Thakur et al., 2013).

SIRT1 and SIRT2 are overexpressed in leukemia cells, both are involved in disease development and drug resistance (Deng et al., 2016; Li and Bhatia, 2015; Xu et al., 2015). In

our study, combining etoposide with FK866 decreased SIRT2 protein abundance in Molt-4 cells more than etoposide alone. Possible mechanisms leading to selective SIRT2 depletion could involve the up regulation of microRNAs targeting SIRT2 (Li et al., 2016), as has been reported for the effects of etoposide on SIRT1 protein (Fan et al., 2014).

P53 is a target of both SIRT1 and SIRT2 (Luo et al., 2001; Peck et al., 2010). Blocking SIRT1 activity was shown to increase acetylation of p53 at lysine 382 in combination with DNA damaging agents (Audrito et al., 2011; Solomon et al., 2006). In the p53 wildtype cell line Molt-4, we found an accumulation of p53 after treatment with etoposide (Golubovskaya and Cance, 2013). Furthermore, the combined effect of FK866 and etoposide on SIRT2 was mirrored by an even stronger induction of p53 acetylation, higher expression of p53 downstream target p21 and cleavage of BAX. The cleavage of BAX during apoptosis was shown to increase the intrinsic cytotoxic properties of this proapoptotic factor (Wood and Newcomb, 2000). This activation of p53-p21 signalling, as well as the increased cell death seen after co-treatment with etoposide and FK866, were reversible by supplementation with NMN, which confirmed the effects to be specific for NAD depletion (Espindola-Netto et al., 2017; LU et al., 2014; Yoshino et al., 2011). In Jurkat cells, which harbour a mutated p53, we did not detect p53 dependent changes in p21 or cleaved bax and no reduction in SIRT2 levels. A failure of p21 induction upon etoposide exposure has been shown before (Davies et al., 2011). Potentially, cell death in Jurkat cells could be induced via c-Myc. SIRT2 inhibits c-Myc ubiquitination and degradation supporting tumorigenesis. Vice versa, reduced SIRT2 activity due to NAD depletion leads to cell death by c-Myc ubiquitination and degradation (Jing et al., 2016). We therefore conclude that there are at least two pathways mediating the increased cell death seen upon combined treatment with etoposide and FK866 and that p53 is dispensable for cell death induction in this scenario.

Conclusion

Combined treatment with the NAMPT inhibitor FK866 potentiates the effects of chemotherapeutic agent etoposide and leads to a strong induction of cell death *in vitro*, via downregulation of SIRT2 and activation of the p53-p21 pathway in the p53 competent Molt-4 cells. Targeting NAD salvage may be a useful strategy for chemo-sensitisation with the possibility to increase the efficacy of chemotherapeutic agents in the treatment of childhood leukemia. Additionally, SIRT2 inhibitors may represent interesting therapeutic options for p53 wildtype leukemia.

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Conflict of interest

All authors have nothing to disclose.

Authorship

TG, SPQ, SS, MP, SR, WK and AG conceived experiments and designed the study. TG, SPQ and SR carried out experiments. TG analysed the data. TG, SPQ, SS, MP, SR, WK and AG interpreted data and drafted the article. TG wrote the paper. All authors revised it critically for important intellectual content and had final approval of the submitted and published version.

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Figure legends**Fig. 1 NAMPT protein abundance, activity and NAD levels are higher in leukemia cell lines.**

NAMPT protein abundance (A), NAMPT activity (B) and NAD levels (C) were measured in PBMCs, Jurkat cells and Molt-4 cells. Western Blots show results of 3 independent experiments and lanes were analyzed densitometrically. Data are represented as mean \pm SEM and statistical analysis was performed using one-way ANOVA and Bonferroni post hoc test (* $p < 0.05$ compared to PBMCs). 2-column fitting image

Fig. 2 FK866 decreases viability, NAMPT activity and NAD levels.

Viability (A,B), NAMPT activity (C,D) and NAD levels (E,F) were measured in Jurkat and Molt-4 cells after stimulation with FK866 with indicated concentrations and/or NMN [500 μ M] for 24h. Data are represented as mean \pm SEM and statistical analysis was performed using unpaired Student's t-test or one-way ANOVA with Bonferroni post hoc test (* $p < 0.05$ compared to control; + $p < 0.05$ compared to FK866). 2-column fitting image

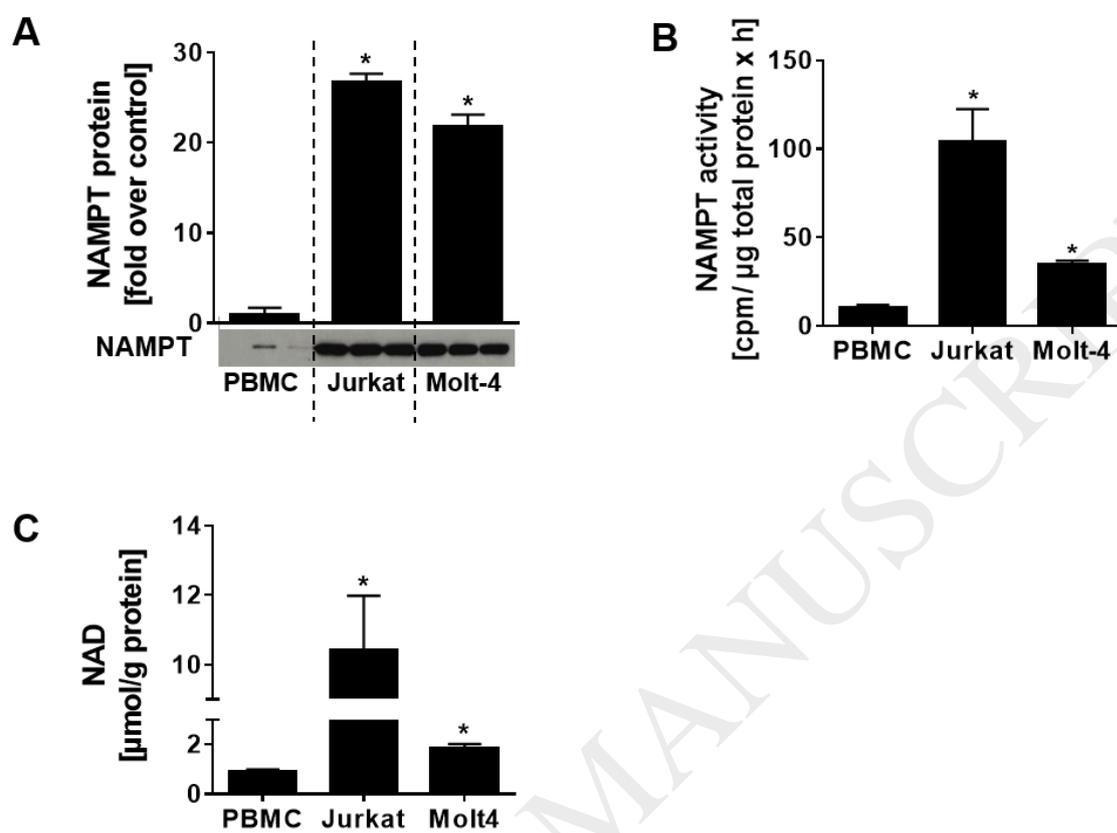
Fig. 3 FK866 increases etoposide-induced cell death.

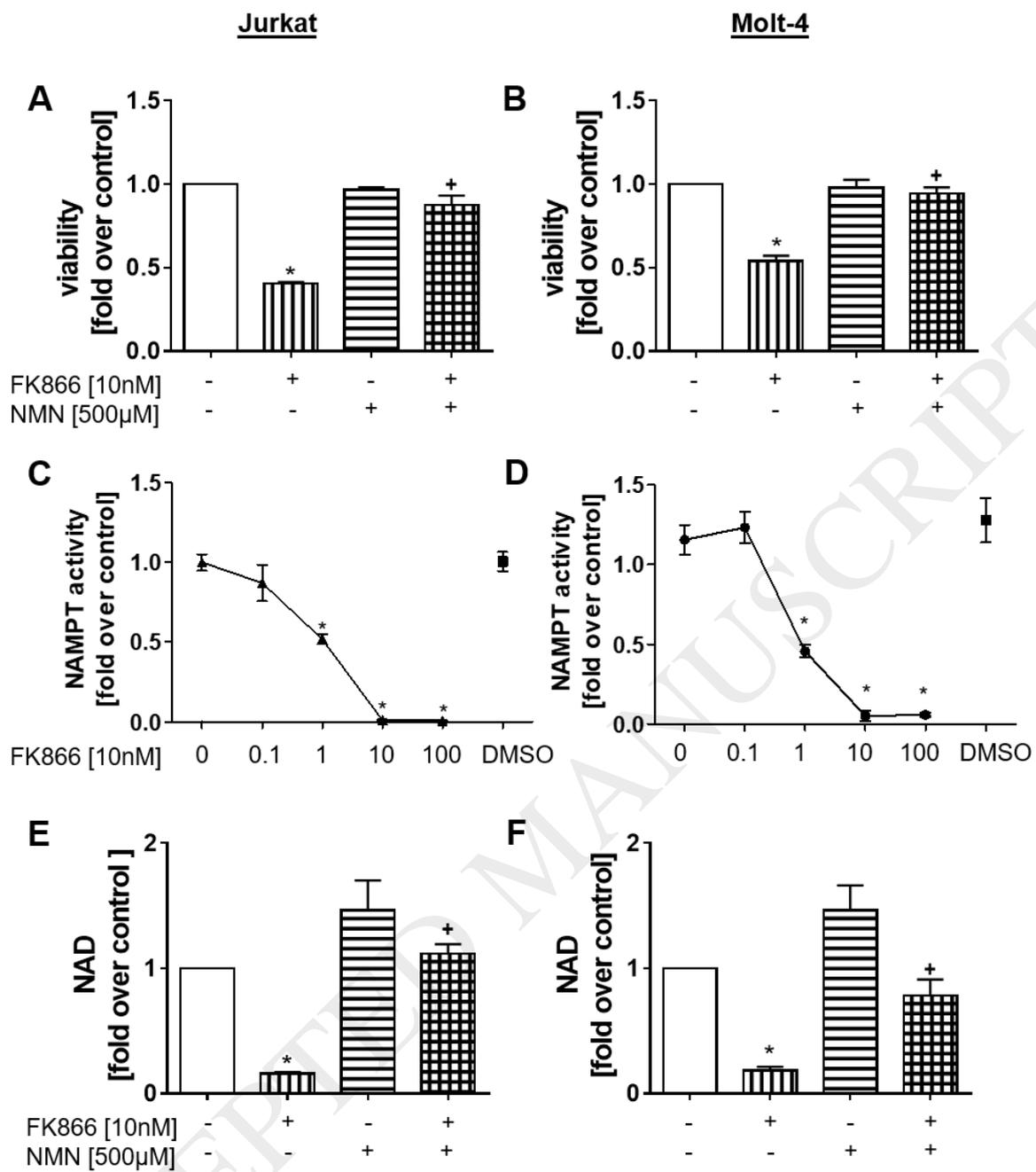
Jurkat and Molt-4 cells were stimulated with etoposide [0.1, 1, 5 or 10 μ M] alone or in combination with FK866 [10nM] and/or NMN [500 μ M] for 24h. Cell death was measured by flow cytometry using propidium iodide (A,B). Cleaved and full-length caspase-3 and PARP-1 protein abundance was detected by Western blot analysis (C,D). Western Blots show results of at least 3 independent experiments, GAPDH was used as loading control and lanes were analyzed densitometrically. Data are represented as mean \pm SEM and statistical analysis was performed using one-way ANOVA and Bonferroni post hoc test (* $p < 0.05$ compared to control; + $p < 0.05$ compared to FK866 alone; # $p < 0.05$ compared to etoposide [eto]; \$ compared to eto+FK866). 2-column fitting image

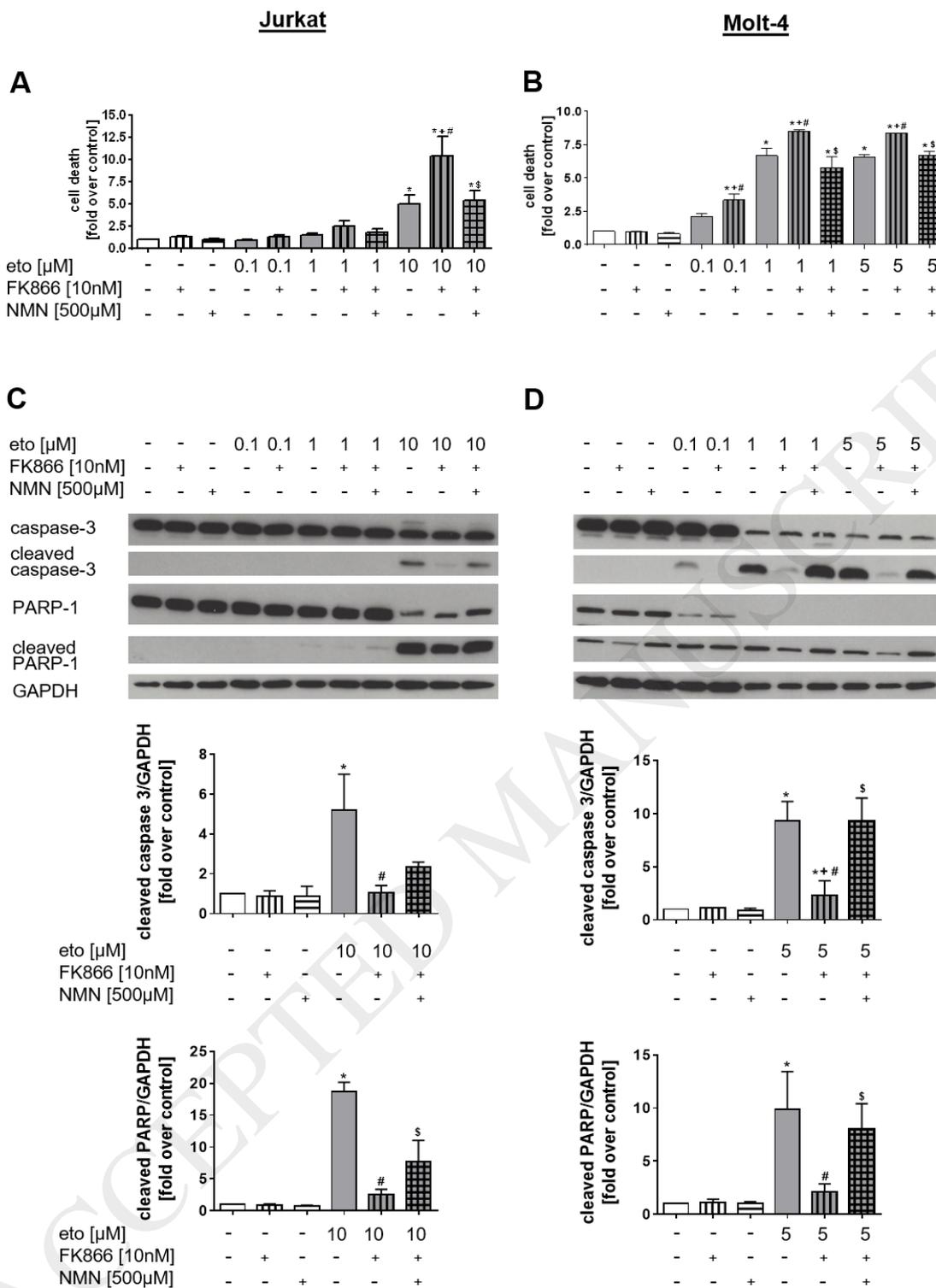
Fig. 4 Etoposide-induced downregulation of SIRT2 is enhanced by NAD depletion.

Molt-4 cells were stimulated with etoposide [5 μ M] alone or in combination with FK866 [10nM] and/or NMN [500 μ M] for 24h. NAMPT, SIRT1 and SIRT2 (A), acetyl-p53, p53, p21 and BAX (B) protein abundance was analysed by Western blot. Western Blots show results of at least 3 independent experiments and lanes were analyzed densitometrically. GAPDH was used as loading control. Data are represented as mean \pm SEM and statistical analysis was performed using one-way ANOVA and Bonferroni post hoc test (* $p < 0.05$ compared to control; + $p < 0.05$ compared to FK866 alone; # $p < 0.05$ compared to etoposide [eto]; \$ compared to eto+FK866). 2-column fitting image

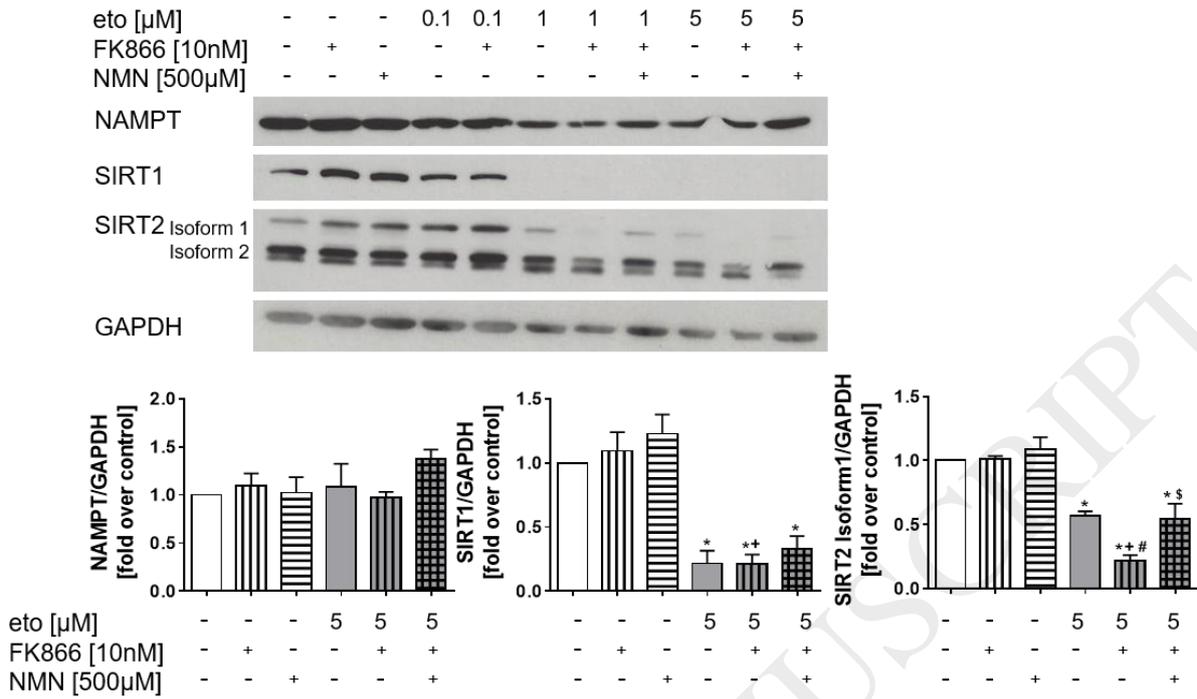
Figures







A



B

