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The role of the NMNAT-1 enzyme in tumor cells

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I. INTRODUCTION

1.1. Osteosarcoma

Osteosarcoma is a tumor of the skeletal system, which is a very aggressive type of the cancers. It mainly affects younger people, with most cases diagnosed between the 10 and 30 years old people. It is estimated that 3-4 in a million people develop this tumor every year. The tumor occurs predominantly in long bones (e.g. femur, tibia and humerus), but can also occur in flat bones (e.g. jawbone). In addition to the primary tumor, metastases may also develop, most of are localized in the lungs. Radiotherapy for other tumors may also lead to osteosarcoma in the long term.

Treatment of osteosarcoma involves both surgery and chemotherapy. Surgical treatment alone is often unsuccessful, due to the highly micrometastatic nature of osteosarcoma. It is therefore usually preceded by preoperative or neoadjuvant chemotherapy to reduce the size of the tumor. The aim of this procedure is to safely remove the entire tumor with as little excision as possible during surgery.

The drugs used in chemotherapy for osteosarcoma include cisplatin, methotrexate and doxorubicin. The recently adopted synthetic muramyl dipeptide analogue mifamurtide may also be used. In addition, actinomycin D is commonly used to treat Ewing's sarcoma, a type of tumor affecting the bone and surrounding soft tissue.

A high level of resistance of osteosarcoma to currently known treatments accounts for 90% of failed cases. The rapid emergence of resistance is reflected in the rescue rate of relapsed patients, which is only 20%. Osteosarcoma is therefore a highly resistant and aggressive tumor type, and new perspectives in its treatment are clearly needed to improve patient survival.

1. 2. Structure and functions of NAD^+

1.2.1 Structure and synthesis of NAD^+

Nicotinamide adenine dinucleotide (NAD^+) is an important biological cofactor, found in all living cells. Structurally, a nicotinamide is linked to a D-ribose by a β-N-glycosidic bond; an adenosine is linked to the 5' carbon atom of the D-ribose through a pyrophosphate.

NAD^+ can be synthesized through three different pathways: the Preiss-Handler pathway (PHP) from nicotinic acid; de novo synthesis (DNP) from tryptophan; and the salvage pathway (SP) from nicotinamide (NAM). The final step in NAD^+ synthesis is catalyzed by NMNAT enzymes, which synthesizes NAD^+ from ATP and NMN.

1. 2. 2. Role of NAD^+ in metabolism

NAD^+ is a coenzyme that plays several vital roles in the metabolism of the human body. The main function of the NAD^+ is the electron transport. It serves as an electron carrier in the glycolysis and tricarboxylic acid (TCA) cycle, and it transfers electrons to the electron transport chain. This enables eukaryotes to efficiently produce adenosine triphosphate (ATP). NAD^+ can also be phosphorylated to form $NADP^+$, which acts as a hydride acceptor to form NADPH and is involved in protection against oxidative stress and in anabolic pathways such as fatty acid synthesis.

In addition to its function as a metabolic cofactor, NAD^+ also functions as a signaling molecule. Cytopathological processes involve a fundamental rearrangement of metabolic pathways. One of the main features of the metabolism of rapidly dividing cells is increased glycolytic activity, even when sufficient oxygen is available. NAD^+ in this case serves not only the energy needs of the cells, but also the increased nucleotide requirements due to increased cell division via the pentose phosphate pathway. This is called the Warburg effect.

NAD^+ is involved in the mechanism of mono- and poly-ADP ribosylation, deacetylation of SIRT-dependent proteins, production of cyclic ADP-ribose and nicotinic adenine dinucleotide phosphate (NAADP). In addition, NAD^+-dependent signaling pathways are involved in a number of essential biological processes, such as the regulation of transcription, cell cycle, and cellular metabolism, DNA repair, and the maintenance of circadian rhythms.

1.2.3 .The role of NAD^+ in the PARylation process

The PARP1 enzyme uses large amounts of NAD^+ during its catalytic activity. It is involved in the regulation of important cellular processes such as DNA replication, gene expression, DNA repair, cell adhesion and migration. Its best known function is to detect DNA breaks and to facilitate repair. Following DNA damage, the enzyme is activated, binding to DNA with high affinity via its two Zn finger domains. Then synthesizes ADP-ribose polymers from NAD^+ and binds them to specific target proteins, including histones, transcription factors and PARP1 itself. This protein modification is crucial for the recruitment of DNA repair complexes and the regulation of other important cellular events, including transcription, apoptosis and cell cycle regulation. The NAD^+ utilization of poly-ADP-ribosylation depends on the extent of DNA damage. Excessive DNA damage can deplete NAD^+ through PARP overactivation, thereby slowing the rate of glycolysis, electron transport and ATP formation.

The enzyme NMNAT1 plays an important role in the production of NAD^+ for PARylation. PARP1 obtains the NAD^+ required for its activity by targeting NMNAT1 to

promoters that it regulates. Moreover, the interaction of the two proteins enhances PARP-1 enzymatic activity independent of NAD^+ production. It is hypothesized that NMNAT1 may facilitates PARP1 function during the initiation of DNA error repair.

In recent decades, inhibition of the PARP1 enzyme has emerged as a novel cancer therapeutic option. The efficacy of PARP inhibitors (PARPi) used in the clinic has been demonstrated in ovarian and breast cancer cells carrying BRCA1 or BRCA2 mutations. These proteins are involved in DNA repair by homologous recombination. Their mutations impair this function, so cells may become highly susceptible by inhibiting base repair (PARP inhibition) through synthetic lethality. PARP inhibitors, used alone or in combination with DNA-damaging treatments, can enhance the efficacy of therapy as described above. The chemosensitizing effect of PARP inhibitors has been observed in combination with the DNA-alkylation agent temozolomide or the topoisomerase inhibitor topotecan.

1.2.4 Role of NAD^+ in SIRT-dependent deacetylation

Sirtuin enzymes play a regulatory role in a number of physiological and pathological processes, including ageing, neurodegeneration, obesity, cardiovascular disease, inflammation and cancer. Seven members of the sirtuin family of enzymes are known to occur in mammalian cells (SIRT 1 - SIRT 7), all of which belong to the class III histone deacetylases (HDAC). They differ in structure, localization and function.

The p53 tumor suppressor protein is an interacting partner of SIRT1 and thus they are able to influence each other's function. For example, activation of p53 in the presence of severe DNA damage is required for the activation of SIRT1, which acts as a deacetylase to inhibit p53 acetylation. Deacetylation of p53 by SIRT1 inhibits apoptosis, thereby promoting cell survival. As SIRT1 uses NAD^+ as a substrate for its catalytic activity, SIRT1 is unable to perform its deacetylase function in the absence of sufficient NAD^+. Thus, the rate of acetylation of p53 is increased. High p53 acetylation levels can lead to cell cycle arrest and the induction of apoptosis. In the absence of p53 protein deacetylation, the induction of pro-apoptotic genes such as PUMA, NOXA and BAX can be observed.

1.3. NMNAT enzymes

NMNAT enzymes are involved in the production of NAD^+ via the salvage pathway and the Preiss-Handler pathway. In the salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT) converts nicotinamide (Nam) to nicotinamide mononucleotide (NMN). Alternatively, nicotinamide riboside (NR) is converted into NMN by nicotinamide riboside kinases. Then, NMNAT enzymes generate NAD^+ by hydrolyzing an adenyl group from ATP onto NMN.

Three different human NMNAT isoenzymes were discovered, which differ in tissue distribution and subcellular localization. The NMNAT1 enzyme is localized in the nucleus, NMNAT2 in the cell cytoplasm and Golgi bodies, and the NMNAT3 isoform in the mitochondria. The nuclear localization of NMNAT1, and its involvement in NAD^+ synthesis, has been associated with nuclear enzymes such as Sirtuin1 (SIRT1) and poly(ADP-ribose) polymerase 1 (PARP1). NMNAT1 expression is known to be elevated in response to genotoxic stress on cells, and it is therefore likely to play an important role in the survival of damaged cells.

The role of NMNAT1 has been studied mainly in the nervous system. It is known that the presence of NMNAT-1 protects against hypoxia-induced dendrite degeneration and has also been shown to play a significant role in slowing axonal degeneration. Furthermore, overexpression of NMNAT1 in the process of Wallerian degeneration has been shown to promote regeneration of damaged axons.

There is a limited information about the role of NMNAT-1 in tumors. T. Song et al. found a correlation between heterozygous deletion of NMNAT1 and increased susceptibility to DNA damage in lung tumor cell lines after treatment with doxorubicin. This effect is likely to be due in part to protein-protein interactions and in part to NAD^+ produced by the catalytic activity of the enzyme. As mentioned earlier, changes in the concentration and availability of NAD^+ affect both its metabolic and signaling roles. Changes in local NAD^+ concentration in individual cellular compartments are equalize over time, but unexpected NAD^+ demand at the nucleus is expected to be difficult for cells to compensate for in the face of acute genotoxic stress. Currently, little is known about the role of NMNAT1 in tumor cells. However, our results suggest that inhibition of NMNAT1 sensitizes osteosarcoma cells to several genotoxic chemotherapeutic agents, and therefore the development of specific NMNAT1 inhibitors may be a new direction for the effective treatment of osteosarcoma.

II. AIMS

In the treatment of cancer, chemotherapy is used in a significant proportion of cases, but it is not always sufficiently effective. Moreover, chemotherapy has an effect not only on tumor cells but also on healthy cells, especially those that are growing rapidly. Therefore, it is of high importance to increase the sensitivity of tumor cells, which may lead to increased treatment efficacy and reduced side effects. NMNAT proteins are enzymes, involved in the synthesis of NAD^+, and thus can influence a number of biochemical processes. However, little is known about their role in tumor cells. Therefore, our aims in this work were:

- To study NMNAT1 expression in human tumor cell lines
- Generation and characterization of an NMNAT1 gene-deficient (knock-out) osteosarcoma cell line
- Comparison of cisplatin treatment effects in wild-type and NMNAT1 KO osteosarcoma cells

The results of the first part of our work support the important role of this enzyme in tumor cells after cisplatin treatment. It is likely that cisplatin is not the only compound showing enhanced cytotoxicity on NMNAT1 KO cells. Therefore, in the second part of our work we set the following objectives:

- A high-throughput screening assay to identify drugs causing enhanced cell death in the absence of NMNAT1
- Validation of compounds identified by screening
- Characterization of the cytotoxic effect of a compound identified by screening on wild-type and NMNAT1 KO osteosarcoma cells.

III. MATERIALS AND METHODS

III.1. Cell culturing

For the culture of human U2OS osteosarcoma cells, we used Dulbecco's modified Eagle's medium (DMEM) with high glucose content (4500 mg/l) supplemented with 10% FBS, 5% L-glutamine and 5% penicillin-streptomycin solution. Cells were cultured under standard cell culture conditions: 37 °C, 5% CO_2 in a cell culture incubator. The number of cultures used for the experiments was below 25 passages, as recommended in the literature. Cells were regularly tested for mycoplasma contamination.

III.2 Generation of NMNAT1 KO cells using Crispr-cas9 technique

NMNAT1 KO U2OS cells were generated by Crispr-Cas9 technique. Reagent and cuvette for transfection were ordered from Lonza. The protocol recommended by the manufacturer was used to generate KO cells. The cells were grown to 40-80 % confluence, then digested with trypsin and plated in antibiotic-free medium. 10^6 cells per sample were collected and after centrifugation (100 ×g, 10 min), they were resuspended in 100 µl of transfection reagent. Then 2.5 µl NMNAT1 CrisPR plasmid and 2.5 µl HDR plasmid were added to the cells. The cells were immediately transferred into transfection cuvettes and transfected with an Amaxa Nucleofector II, using the protocol (X-001), recommended by the manufacturer for U2OS cells. The suspension of transfected cells was then pipetted into a 6-well plate containing pre-warmed, antibiotic-free cell culture medium. Cells were incubated for 24 h under general cell culture conditions, and after 24 h, the medium (antibiotic-free cell culture medium) was replaced. As a visual confirmation of transfection efficiency, red fluorescent protein (RFP) was used as part of the transfection system and could be detected by fluorescence microscopy on the following day after transfection. The transfected cells were then subjected to puromycin selection (2.5 µg/ml) for 3 weeks and sorted by RFP fluorescence using a BD LSR II Cell Sorter flow cytometer. The resulted single-cell clones were then subcultured. The absence of NMNAT1 expression was demonstrated at the mRNA level by qPCR technique and at the protein level by western blot.

III.3. Western blot

U2OS cells were resuspended in 100 µl RIPA lysis buffer (50 mM Tris-Hcl (pH 7.4), 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 150 nM NaCl, 2 mM EDTA and 50 mM NaF) containing a protase inhibitor cocktail (PIC, 1:100) and a phosphatase inhibitor (PMSF, 1:100). Cell lysis was performed by sonication. Cell debris was then removed by centrifugation (16100 ×g, 10 min, 4 °C). Protein concentration was determined from the supernatant using a Direct Detect infrared spectrometer. Subsequently, the lysates were supplemented with 5 x sample buffer (50 % glycerol, 10 % SDS, 100 mM DTT, 0.31 M Tris-HCl, 0.01 % bromophenol blue and sterile H2O) and β-mercaptoethanol (5 %) were added and incubated at 95 °C for 10 min. Samples (20 µg/well) were loaded onto the gel (15 comb, 10 %, 1.5 mm, 1.5 mm, home-cast SDS-PAGE gel), based on the determined total protein concentration (15 comb, 10 %, 1.5 mm, SDS-PAGE gel was used for the experiments. Composition of the separating gel: H2O, 30% acrylamide, 0.8% bis-acrylamide, 1.5 M Tris-HCl (pH 8.8), 0.4% SDS, 10% AMPER and TEMED. Composition of the collecting gel: H2O, 30% acrylamide, 0.8% bis-acrylamide, 0.5 M Tris-HCl [pH 6.8], 0.4% SDS, 10% AMPER and TEMED).

Electrophoresis was carried out in SDS-Tris-glycine running buffer (composition, per 1 liter, dissolved in dH2O: 144.1 g glycine, 30.3 g Tris-Hcl, 10 g SDS (pH 8.3)) at 100 V. The proteins were then transferred onto nitrocellulose membrane in transfer buffer (5x transfer buffer: pH 8.3, 15.5 g Tris-HCl and 72.05 g Glycine dissolved in 1 L dH2O), supplemented with 20% methanol (99.9%). The membranes were blocked for 1 h in 5% milk powder, dissolved in 0.01% PBS, supplemented with 1% Tween20 (1x TBS-T). Primary antibodies were diluted into the blocking solution and incubated at 4°C, overnight. Secondary antibodies conjugated with horseradish peroxidase were also used in blocking solution prepared as above, for 2 hours at room temperature. Detection of the results was performed by chemiluminescence using ECL reagent. Bio-Rad ChemiDoc Imager was used to document the blot images. ImageLab 6.0 software was used to evaluate the results.

III.4. High Content Analysis (HCA)

III.4.1. γH2AX detection

Cells (2×10^4, 100 µl/well) were pipetted into a HCA microplate and grown for 24 h. Cells were then treated according to the experimental parameters. After the treatment period, cells were fixed in a 3 % formaldehyde/PBS solution for 15 min at room temperature. Then washed 3 times with PBS and incubated with blocking solution (5% BSA in PBS) for 15 min at room temperature. Anti-phospho-H2AX antibody was diluted in the blocking solution and

incubated for 2 hours. Secondary antibodies were diluted in the blocking solution and incubated for 1 hour. Cells were then washed 2 times with PBS and stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) diluted in PBS on room temperature for 5 minutes. Subsequently, cells were washed 3 times with PBS and then kept in 100 µl PBS until detection. Microscopic images were acquired using an Opera Phenix High Content Analyzer with a 10x objective (NA 0.3). The images were evaluated using the built-in Harmony software, which allowed us to distinguish three different cell morphologies: normal cells that did not contain a phospho-H2AX (-H2AX) signal, spotted cells that contained -H2AX foci, and fragmented cells that showed a condensed morphology with a diffuse -H2AX signal. Harmony Analyzer software was used to determine the number and relative proportions of cells in each group in an automated manner.

III.4.2. Caspase activation assay

Cells (2×10^4/mL, 100 µL/well) were pipetted into 96-well plates and grown for 24 hours. The next day, cells were pretreated with 50 µM z-DEVD-FMK caspase inhibitor for half an hour, followed by treatment with 5 µM cisplatin (medium for control cells). After the treatment time, cells were incubated in a final volume of 50 µl with CellEvent™ Caspase-3/7 Green Detection Reagent at a final concentration of 7 µM. The plates were placed in an Opera Phenix High-Content Analyzer, using the instrument's ability to control the environment (5% CO_2, 37°C), and the measurement was performed for 11 hours, detecting the appearance of the fluorescent signal at 502/530 nm every hour. Analysis was performed using Harmony software.

III.4.3. Cell proliferation assay

Cells (10^3 cells /100 µl/well) were plated in Cell Carrier ultra 96 ultra microplates, and grown for 24 hours. The next day, ActD (1.25 nM) was added and cells were incubated for 4 days. For experiments with PARP inhibitors, olaparib (at 10 µM final concentration) or PJ34 (at 10 µM final concentration) was added to the cells 30 min before ActD treatment. After four days, the plates were placed in an Opera Phenix HCA analyzer and counted using phase-contrast microscopy images acquired using Harmony software.

III.5. Viability testing

Calcein AM viability assay was used to test viability. Calcein, also known as fluorescein, is a fluorescent dye with an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Cells were grown in 96-well cell culture dishes (2×10^4/ml cells, 100 µl/well) for 24 hours. On the following day, the cells were treated according to the experimental parameters. After the treatment period, the cell supernatant was removed and stained with 50 µl of Calcein-AM solution at a final concentration of 1 µM for 1 h at 37°C. The fluorescence signal released from the cells (EX/EM: 485 nm/530 nm) was measured using a Tecan Spark multimode reader. Viability was expressed as a percentage of untreated control.

III.6. Clonogenic activity

U2OS cells were plated in 6-well plates (1 x 10^3 cells/mL). After 24 h, cells were pretreated with 10 µM PJ34 or 10 µM olaparib for 30 min, followed by treatment with 5 µg/mL cisplatin. Cells were incubated for 6 days, and colonies were counted manually after staining with 0.5% crystal violet dissolved in 20% ethanol.

III.7. LDH release

LDH assay kit was used to determine the amount of lactate dehydrogenase (LDH) released into the cell supernatant. Cells were grown in 96-well cell culture plates (2×10^4/ml cells, 100 µl/well) for 24 hours. The next day, cells were treated with 5 µg/mL cisplatin or 40 nM ActD, following 50 µM DEVD-fmk or 1 µM Nec1 pretreatment (30 min) for 24 hours. Positive controls were prepared using the LDH lysis buffer supplied with the kit, which was added to the supernatant on the cells at a ratio of 1:10. The plate was centrifuged (800 ×g, 5 min) and the supernatant was collected into an empty 96-well plate. We used cell culture medium for blank sample. The LDH buffer was diluted 1:20 with distilled water. The prepared assay buffer was added to the samples in a 1:1 ratio. Incubated in the dark for 20 min and then detected absorbance at 490 nm using a Tecan Spark multimode reader.

III. 8. Total cellular NAD^+ levels

Cells were plated in 6-well cell culture plates and grown for 24 hours. The next day, cells were treated with 5 µg/mL cisplatin or 40 nM ActD for 24 h, while for basal NAD^+ content determination, cells were collected in NAD^+ extraction buffer without treatment after 24 h of culture. The supernatant was extracted from the cell lysate by centrifugation at 13000 ×g for 10 min at 4°C. Protein concentration was determined from the supernatant using a Direct Detect spectrometer. Standards were diluted from the NAD^+ standard supplied with the kit. The blank

sample was the NAD^+ extraction buffer. The NAD^+-cycling enzyme was diluted 1:50 with NAD^+-cycling buffer. The samples and the diluted NAD^+-cycling enzyme were mixed in a 1:1 ratio in a 96-well half-area plate. NAD^+-developer solution was added to the samples at a ratio of 1:10. The samples were then incubated on a plate shaker at 37°C for 2 hours with continuous stirring (300 RPM). Fluorescence was measured at 450 nm using a Tecan Spark multimode reader. Fluorescence intensities were normalized to protein content.

III.9. Total cellular ATP levels

Cells were pipetted into 6-well plates (10^6 cells/well). The next day, cells were treated with the appropriate treatments (50 µM DEVD-FMK, 1 µM Necrostatin1, 5 µg/mL cisplatin or 40nM ActD). At the end of the treatment period, cells were washed with PBS and then plated in ATP-assay buffer. Cells were then centrifuged for 5 min at 4°C at 13,000 ×g and the supernatant was transferred to new tubes. Samples were stored on ice until assay assembly.

ATP content was determined with an ATP assay kit according to the manufacturer's instructions (#110M6101, Merck, Darmstadt, Germany). 96-well microplates were used for the measurement. The reagent blank was ATP-assay buffer. To prepare the standard series, the 10mM standard solution was diluted to a concentration of 0.2 - 1 nmol/well in 50 µL/well in plate. Samples diluted with the buffer solution were also measured in 50 µL/well in plate. 50 µL of the ATP reaction mix was added to the blank, standards and samples. The plate was incubated in the dark for 30 min. Fluorescence was measured at Ex/Em= 535/587 nm using a fluorimeter. ATP content was normalized to protein content.

III.10. Cellular metabolism assay with the Seahorse metabolic analyser

U2OS WT and NMNAT1 KO cells ($1x10^5$ cells/well) were plated in DMEM in XF96 cell culture microplate and grown overnight at 5% CO2 and 37°C.

Sensors were rehydrated with dH_2O overnight and then replaced with Seahorse Bioscience XF96 calibrant solution (pH 7.4). The sensors were incubated in the calibration solution for 2 hours at 37°C in the absence of CO2. Measurements were performed with a Seahorse XF96 Analyzer. XF Cell Mito Stress analysis was performed according to the manufacturer's instructions with the following modifications: the final concentration of mitochondrial inhibitors was changed to 2 µM oligomycin, 0.5 µM FCCP, and 1 µM antimycin-A.

III.11. Sulforhodamine B (SRB) essay

Cells were fixed with 10% trichloroacetic acid overnight at 4°C, washed with distilled water and stained with 0.4% SRB stain, and incubated for 10 min at room temperature. The cells were then washed with 1% acetic acid and the bound SRB stain was dissolved in 10 mmol/l unbuffered Tris. Optical density was measured at 540 nm using a microplate reader.

III.12. RNA isolation from cell culture

RNA was isolated using TRIzol reagent (Tri-RNA reagent, #FATRR001, Amplicon, Odense, Denmark) according to the manufacturer's instructions.

III.13. Real-time quantitative PCR (RT-QPCR)

Measurements were performed using the LightCycler 480 thermocycler with SYBR Green solution according to the manufacturer's protocol. Samples were triplicated and data were normalized to the mathematical mean of the housekeeping genes (36B4 and cyclophilin).

III.14. Viability measurement by high-throughput screening

A US Food and Drug Administration (FDA) approved compound library containing 774 compounds was used for screening: Screen-Well® FDA approved Drug Library. Each plate contained four untreated wells (CTL).

Cells (2×10^4 cells/100 µL/well) were plated in 96-well cell culture plates and grown for 24 hours. Library compounds were transferred to the plates using a Tecan Freedom EVO liquid handling robot at a final concentration of 10 µM. Cells were incubated with the compounds for 24 hours. Cell viability was determined by addition of Calcein-AM solution (50 µl/well, 1 µM final concentration). Cells were incubated for 1 hour at 37°C and the fluorescence signal (Ex/Em = 485/530 nm) was measured using a Tecan Spark 20M plate reader. Viability was expressed as percentage of untreated control and then expressed as percentage cytotoxicity.

III.15. Validation of identified compounds

Hit compounds was retested with a wide range of concentrations (1.22 nM to 40,000 nM), using a Calcein-AM assay. For this purpose, cells (2×10^4 cells/well, 100 µl/well) were cultured in 96-well plates and incubated for 24 h. Cell viability was determined as described above. Cytotoxicity was calculated from the viability data obtained.

III.16. Cell cycle analysis by flow cytometry

1. Synchronization of the cell cycle

Cell cycle synchronization was performed by serum starvation. The cells were grown in T25 cell culture flasks for 1 day and when they reached approximately 60% confluence, the cells were washed once with PBS and kept in serum-free medium for 24 hours. After washing with PBS, they were trypsinized and pipetted into Falcon tubes.

2. Preparation of samples

The cells were centrifuged at 300 x g for 5 min, the supernatant was discarded and the cells were resuspended in 3 ml PBS. The cells were centrifuged again at 300 x g for 5 min. The supernatant was discarded and the cells were resuspended in 400 µl PBS. Then 3 ml of 70% ice-cold ethanol was slowly added to the cells and the samples were placed on ice for 30 minutes. After the incubation period, the cells were centrifuged at 300 x g for 5 min. The supernatant was discarded and the cells were washed with 3 ml PBS. Afterwards, the supernatant was discarded and the cells were resuspended in 500 µl PBS, and 50 µl RNase A solution and 5 µl propidium iodide (PI) were added to the cells. Samples were incubated in the dark at room temperature for 1 h before analysis.

- *RNase A solution*: 50 mg RNase A was dissolved in 50 ml PBS and 0.1% Tween-20 and 5 mM EDTA. The solution is placed in a 95 °C water bath for 30 min. The solution is then cooled on ice for 1 hour. The precipitate of the solution was removed with a 0,2 µm filter.
- *Propidium iodide (PI) solution*: The PI stock solution was diluted in dH_2O to a final concentration of 1 mg/ml.

3. Cell cycle analysis by flow cytometry

Analysis was performed using a Novocyte 3000 flow cytometer.

III.17. Statistical analysis

The experiments were performed at least three times, and the calculated mean of the three experiments is plotted ± SEM. GraphPad Prism 9 was used for statistical analysis. The normal distribution of the data was determined by D'Agostino-Pearson test. All data were normally distributed and analyzed using a two-way ANOVA test followed by Sidak, Tukey or Dunett post-hoc tests.

IV. RESULTS

IV.1. Analysis of NMNAT1 expression in tumor cells and generation of NMNAT1 gene-deficient cell lines.

IV.1.1. NMNAT1 expression in human tumor cell lines

No much information is available in the literature on NMNAT1 mRNA expression levels in tumor cell lines. Therefore, we investigated NMNAT1 mRNA expression in eleven different human tumor cell lines. The transcript was detected in all cell lines and expressed at different levels. Compared to the average expression level, A431 cells showed significantly higher NMNAT1 mRNA expression, whereas significantly lower expression was observed in A549, Capan2, MCF7 and HepG2 cell lines. For further analysis, we chose a cell line with an average NMNAT1 mRNA expression level - U2OS osteosarcoma cell line.

IV.1.2. Effect of genotoxic stress on NMNAT1 expression

U2OS cells were treated for 24 h with the DNA-damaging chemotherapeutic agents, cisplatin (cis-diamminedichloroplatinum (II)) and doxorubicin, which are used in osteosarcoma therapy. The treatment significantly increased NMNAT1 mRNA expression in osteosarcoma cells. This correlates with previous findings in the literature that NMNAT1 expression is increased upon doxorubicin treatment. This suggests that the enzyme may play an important role in cell survival following DNA damage. Further studies were performed with cisplatin.

IV.1.3 Generation and control of NMNAT1 gene-deficient cell lines

There is no known specific pharmacological inhibitor of NMNAT1, so to investigate its role, the NMNAT1 gene was inactivated using CRISPR-Cas9 technology. Following this procedure, puromycin-resistant cells were selected and single-cell clones were obtained from NMNAT1 knockout (KO) cells by flow cytometry sorting. NMNAT1 expression in NMNAT1 KO cells was also examined at mRNA and protein levels. No significant NMNAT1 mRNA expression was observed in any NMNAT1 KO clone. For further experiments, we used clone 1B6. Using western blot, we demonstrated that NMNAT1 protein was not detectable in the cell line derived from clone 1B6.

IV.1.4. Characterization of NMNAT1 KO cells

IV.1.4.1 Viability and colony forming activity

Limited data is available on the phenotypic consequences of NMNAT1 deficiency, we compared some basic physiological characteristics of the two cell lines (viability, proliferation,

NAD^+ and ATP content, metabolic pathway activity, chemosensitivity). Based on our studies, there is no difference in viability between wild type and NMNAT1 KO cell lines. In contrast, colony forming activity is reduced in the absence of NMNAT1 enzyme.

IV.1.4.2 Measurement of NAD^+ and ATP content

By measuring the total cellular NAD^+ levels, it can be said that the NAD^+ content of NMNAT1 KO cells was reduced by about one third compared to wild-type cell lines (WT) due to inactivation of the NMNAT1 gene. This may indicate that the NMNAT1 enzyme plays a crucial role in NAD^+ synthesis and maintenance in U2OS cells. Interestingly, lower NAD^+ levels in NMNAT1 KO cells did not reduce ATP levels significantly.

IV.1.4.3 Investigation of the role of NMNAT1 in cellular energy metabolism

We found that the absence of NMNAT1 did not affect cellular respiration, as indicated by the unchanged oxygen consumption. However, glycolytic activity was found to be significantly higher in the absence of NMNAT1 compared to the wild-type cell line.

IV.2 Investigation of the role of NMNAT1 in cisplatin-treated osteosarcoma cells

IV.2.1. Viability assay

There is an increase in NMNAT1 protein expression following treatment with doxorubicin or cisplatin. To investigate the chemosensitivity of KO cells, a range of cisplatin concentrations (0-40 μg/ml) was used to compare the sensitivity of the two cell lines to cisplatin. We found that NMNAT1 deficiency resulted in increased cell death compared to wild-type cells.

IV.2.2. Determination of DNA damage

Detection of double-stranded DNA breaks (DSB) is most commonly detected by determining the phosphorylation level of histone variant H2AX. H2AX phosphorylation (γ-H2AX) was detected by immunocytochemistry. In cisplatin-treated cells, H2AX was significantly phosphorylated, reflecting the activation of DNA repair mechanisms such as nucleotide excision repair or the non-homologous ends joining. Using a HCA assay, three different cell morphologies were determined: 'normal' cells contained no γ-H2AX signal, 'speckled' cells contained γ-H2AX foci, while 'fragmented' cells showed dense-diffuse γ-H2AX staining. Cisplatin caused H2AX phosphorylation (γ-H2AX formation) in wild-type cells, but significantly higher amounts of γ-H2AX were detectable in NMNAT1-deficient cells after

cisplatin treatment (i.e., the proportion of "fragmented" cells was significantly higher compared to their wild-type counterparts).

IV.2.3. Investigation of cell death pathways

During the DNA damage assay, in particular in the NMNAT1 KO line, a high proportion of condensed nucleated cells with "fragmented" morphology was identified, indicating apoptotic cell death. We therefore continued our work by detecting apoptosis-specific caspase-3 activity using a cell permeable, fluorogenic caspase-3/7 substrate. In addition to cisplatin treatment, cells were pretreated with a caspase inhibitor (z - DEVD - FMK). Significant caspase activation was observed in NMNAT1 KO cells, which was inhibited by the caspase inhibitor. During necrotic cell death, membrane permeability is enhanced, leading to increased lactate dehydrogenase (LDH) release. This can be measured from supernatants. In our study, we observed significantly higher LDH release in NMNAT1 KO cells treated with cisplatin than in wild-type cells. The necroptosis inhibitor necrostatin 1 (NEC1) inhibited LDH release, suggesting necroptotic cell death.

IV.2.4 Effect of cisplatin treatment on NAD^+ and ATP levels in NMNAT1 KO cells

Significantly lower basal NAD^+ levels were measured in NMNAT1 KO cells, which were further reduced by cisplatin treatment. A dramatic change in ATP levels was also observed in NMNAT1 KO cells after cisplatin treatment, whereas no change was observed in wild-type cells. Both the caspase inhibitor and the necroptosis inhibitor significantly inhibited ATP loss, suggesting that the decrease in ATP levels is partly due to impaired energy production and increased ATP consumption required for the completion of active cell death.

IV.2.5. Examination of metabolic pathways

To characterize the energy-producing pathways of cells, the oxygen consumption rate (OCR) was used to measure cellular respiration and the extracellular acidification rate (ECAR) was used to determine the rate of glycolysis. No significant difference in basal respiration was observed between the two cell lines. The mitochondrial stress test also showed similar values in both cell lines. However, cisplatin treatment completely abolished mitochondrial respiratory reserve capacity in NMNAT1 KO cells. Basal glycolytic activity was found to be significantly higher in the NMNAT1 KO cell line. No significant differences were observed between the two cell lines in glycolytic stress assays without treatment or with cisplatin treatment. This finding suggests that cisplatin has no effect on glycolytic activity independent of NMNAT1 status in most cases. Cellular metabolism is characterized by the ratio of the activity of the two main

energy-producing processes, the OCR/ ECAR ratio. Following cisplatin treatment, the OCR/ ECAR ratio decreased in NMNAT1 KO cells, indicating that the cells rely on glycolysis rather than respiratory energy production for their metabolism.

IV.2.6. Role of PARylation in the increased sensitivity of NMNAT1 KO cells

PARP activity was monitored by detecting the amount of PAR polymer produced. We found that cisplatin led to poly(ADP-ribose) (PAR) formation in WT cells, whereas this was not detectable in NMNAT1 KO cells. In addition, cisplatin treatment only slightly reduced colony-forming activity in wild-type cells, whereas a significant reduction was observed in NMNAT1 KO cells. Combined treatment with cisplatin and PARP inhibitor (olaparib) resulted in significantly lower proliferation in wild-type, cisplatin-treated cells, but no further reduction was detected in NMNAT1 KO cells. These data suggest that cisplatin-induced DNA damage is unable to activate PARP1 in NMNAT1 KO cells, and the lack of a substrate for PARP1 limits the ability of cells to efficiently repair DNA damage.

IV.3 Identification of drug targets that cause enhanced cell death in NMNAT-1 KO cells

IV.3.1 High throughput screening assay

High Throughput Screening (HTS) was performed in parallel on wild type and NMNAT1 knockout cells using Tecan Freedom EVO robot with a compound library containing 775 FDA (Food and Drug Administration) approved drugs. For screening, wild-type (WT) and NMNAT-1 knockout (KO) osteosarcoma cell lines were treated uniformly with the compounds of the molecular library at a final concentration of 10 μM. A 24-h treatment was applied and subsequently cytotoxicity was determined in both cell lines using Calcein AM assay. Compounds were considered to be effective when evaluated with at least 25% higher toxicity in the NMNAT1 KO cell line compared to wild-type cells. Nine compounds fulfilled the criteria: bortezomib, actinomycin-D, digoxin, teniposide and five anthracyclines (idarubicin, daunorubicin, doxorubicin, mitoxantrone, epirubicin).

IV.3.2. Validation of hit compounds

Confirmation of our findings was performed on both wild type and NMNAT1-KO U2OS cells. The cytotoxicity of the compounds identified in the screening was assayed 24 h after treatment using a Calcein-AM viability assay with a wide range of concentrations (1.22 nM to 40,000 nM), including 10 μM concentration used in the screening. In 8 out of nine compounds, the sensitizing effect of the NMNAT1 KO phenotype was confirmed. The effect of all identified anthracyclines was verified. Actinomycin D, bortezomib and teniposide also

caused a significant increase in cytotoxicity of the KO cell line compared to WT cells. Also in the case of digoxin, concentration-dependent cytotoxicity was observed in both cell lines, but toxicity in the KO cell line was not significantly higher in this case compared to wild type cells. The most striking difference was observed for actinomycin D, as the absence of NMNAT1 from a concentration of ~40 nM induced a significant sensitizing effect. Actinomycin D (Act D) is a polypeptide antibiotic isolated from the genus Streptomyces. Actinomycin D intercalates with a DNA guanine and cytosine-rich region, thereby inhibiting RNA polymerases, thereby inhibiting transcription. As the molecular basis of its tumor killing mechanism is only partially understood, there is currently no literature data on interactions with NMNAT1 enzyme. Therefore, actinomycin D was chosen for further studies.

V.4. Investigation of the role of NMNAT1 in actinomycin D-treated osteosarcoma cells

IV.4.1 Characterization of actinomycin D-induced cell death

We investigated how the viability of actinomycin D-treated cells changes with specific inhibition of apoptosis or necroptosis. Actinomycin D treatment (40 nM) was applied to wild-type and NMNAT1 KO cells, either alone or in combination with a caspase-3 inhibitor (DEVD-fmk) or a necroptosis inhibitor (Nec1). Our study showed that DEVD-fmk can significantly increase the viability of wild-type and NMNAT1 KO cells treated with actinomycin D, whereas Nec1 pretreatment did not cause any change in viability compared to those treated with actinomycin D alone. Further investigation of apoptotic cell death was performed by high content analysis using a kinetic assay in the presence of the caspase-3 inhibitor DEVD-fmk. Actinomycin D treatment caused a significant increase in caspase-3 activity in both cell lines, which was effectively inhibited by the caspase-3 inhibitor. In the NMNAT1 KO cell line, caspase-3 activation was already detectable and the proportion of caspase-positive cells was more than twice the proportion of caspase-3 positivity measured in wild-type cells. The possible presence of necrotic cell death was assessed by measuring lactate dehydrogenase (LDH) levels from supernatants, 24 hours after actinomycin D treatment. No significant LDH release was detected in WT cells. While significant LDH release was measured in the KO cell line. However, the LDH release from KO cells could be considered as secondary necrosis, since DEVD-fmk caspase-3 inhibitor was able to block it completely.

IV.4.2. Examination of cellular NAD^+ and ATP levels after Actinomycin D treatment

NMNAT enzymes are involved in the synthesis of NAD^+ through two pathways (salvage pathway and Preiss-Handler pathway). Thus, the absence of the enzyme and the significantly higher cytotoxicity observed in the NMNAT1 KO cell line following actinomycin

D treatment, lead us to conclude that possible changes in NAD^+ and ATP content of KO cells are worth investigating. As shown previously, basal NAD^+ levels were significantly lower in NMNAT1 KO cells than in wild-type U2OS cells. Actinomycin D caused a significant decrease in NAD^+ levels in both cell lines. NAD^+ levels in the KO cell line were approximately 1/5 of those in the treated wild-type samples. Cellular ATP is a universal metabolite required for almost all energy-dependent processes. Since ATP production is dependent on NAD^+, we then examined whether cellular ATP levels also change after actinomycin D treatment. Our previous results suggest that basal ATP levels are not significantly different in WT and KO cells. Actinomycin D treatment caused a significant decrease in ATP levels in wild-type cells. However, NMNAT-1 KO cells also showed significantly decreased ATP levels compared to wild-type treated samples.

IV.4.3. Actinomycin D induces DNA damage and PARP activation

No significant DNA damage was detected in wild-type U2OS cells. However, high levels of DNA damage were observed in KO cells 6 hours after ActD treatment. Increased DNA damage may indicate impaired DNA repair. The DNA damage activates the NAD^+-dependent enzyme PARP1, which, based on literature data, may interact with NMNAT1 at PARP1-dependent promoters. Therefore, we investigated how DNA damage alters the amount of poly-ADP-ribose (PAR) polymer PARylation was detected by western blot using anti-PAR antibody (10H). Increased PARP activation was detected in wild-type cells 6 h after ActD treatment, whereas PARP activation was significantly reduced in KO cells, probably due to low NAD^+ content.

V.4.4. Analysis of acetylation of the p53 protein

Low NAD^+ levels are known to inhibit the activity of nuclear NAD^+-dependent enzymes such as PARP1 or SIRT1. Since SIRT1 is an NAD^+-dependent deacetylase enzyme, a decrease in its activity is expected to cause an increase in the acetylation of its target proteins (e.g. p53 protein). Acetylation of p53 protein on the Lys382 side chain was detected by western blot. In untreated samples, no acetylation was detected. However, after 20 h of ActD treatment, increased acetylation was detected in both WT and KO cells, showing significantly higher levels in KO cells.

IV.4.5. investigation of the induction of p53-dependent pro-apoptotic genes

Elevated acetylation of p53 may induce increased expression of p53-dependent pro-apoptotic genes, such as NOXA or BAX. mRNA expression of these genes was examined by qPCR 15 h after ActD treatment. NOXA gene expression was increased in both cell lines, but reached significantly higher levels in NMNAT1 KO cells. In contrast, BAX gene expression showed a significant increase only in the NMNAT1 KO cell line upon actinomycin D treatment. NOXA or BAX protein expression levels were detected by western blot. Both NOXA and BAX protein levels showed significantly higher induction in NMNAT1 KO cells than in WT cells.

IV.4.6. NMNAT1 deficiency inhibits cell proliferation

IV.4.6.1 Cell proliferation and cell cycle assay

Cell proliferation in our study model was determined by HCA. For this study, digital phase-contrast images were taken before and four days after ActD treatment, and current cell counts were determined using an image analysis software (Harmony). During the four-day period, the number of untreated WT and KO cells increased approximately sevenfold compared to the plated cell count, and there was no significant difference between the two cell lines. ActD treatment slowed down cell proliferation in both WT and KO cells, but KO cells showed a dramatic decrease in proliferation compared to their wild-type counterparts as a result of treatment. The proportion of cell populations at different cell cycle phases were determined by flow cytometry. Without treatment, no significant difference was found between the two cell lines, but after ActD treatment, the NMNAT1 KO cell line showed a significant decrease in the proportion of S and G2/M phase populations. Thus, a higher proportion of cells in the KO cell line were in G1 phase than in the WT line. No significant change was detected in WT cells after treatment.

IV.4.6.2. The effect of SIRT1 enzyme inhibition on cell proliferation

In addition to the induction of pro-apoptotic genes, increased acetylation of p53 also affects cell proliferation through the induction of the p21 gene. ActD induced p21 mRNA and protein expression in NMNAT1 KO cells, in contrast, no significant changes were observed in WT cells.

IV.4.6.3 The effect of PARP1 enzyme inhibition on cell proliferation

Since published data suggest that inhibition of PARP1 slows down cell proliferation, we examined the effect of ActD treatment-induced PARP activation on cell proliferation. Pretreatment with PARP inhibitors (Olaparib and PJ34) reduced proliferation in both lines.

When ActD was used as a combined treatment with PARP inhibitors, we found that cell proliferation was significantly reduced in wild-type cells, but no significant change was observed in NMNAT1 KO cells compared to samples treated with actinomycin D alone. These data suggest that the reduced basal NAD levels in NMNAT1 KO cells do not interfere with the basal activity of PARP (so it can be inhibited by a specific inhibitor). However, in the case of ActD treatment, the current available NAD pool is not sufficient for PARP1 activation in NMNAT1 KO cells in response to DNA damage due to inhibited cellular NAD^+ synthesis. This limits the DNA damage repair of the cells, which also contributes to the reduced proliferative capacity of the cells.

IV.4.7. The effect of NMNAT1 KO phenotype and actionomycin D treatment on RNA content

It has been previously reported that both the NMNAT1 enzyme and ActD affect RNA synthesis. To investigate RNA homeostasis, our experiments measured the amount of total RNA and the expression of 45S and 18S ribosomal RNAs. We found that NMNAT1 KO cells have three times higher total RNA levels compared to wild-type cells. ActD treatment significantly decreased total RNA levels in NMNAT1 KO cells, while it had no effect on WT cells. The absence of NMNAT1 increased the expression of the pro-form of 18S ribosomal RNA (45S). 45S RNA expression was completely blocked by ActD treatment in both cell lines. Interestingly, there was no detectable difference in the amount of mature 18S rRNA between the two lines. ActD treatment increased the level of mature 18S rRNA in wild-type cells, but did not cause a significant change in NMNAT1 KO cells.

V. CONCLUSIONS

NMNAT1 knockout osteosarcoma cells are highly sensitive to cisplatin treatment

- An NMNAT1 KO cell line was successfully generated from U2OS osteosarcoma cells.
- NMNAT1 KO cells had decreased NAD^+ levels and clonogenic activity.
- Cisplatin induced both necrotic and apoptotic cell death pathways.
- Decreased DNA repair was observed in the NMNAT1 KO cells after treatment.
- Cisplatin treatment resulted in decreased NAD^+ and ATP content in the NMNAT1 KO cell line.
- DNA-damage dependent PARylation was not induced in NMNAT1 KO cells.
- NMNAT1 KO tumor cells are more sensitive to cisplatin treatment.

NMNAT1 is a survival factor in the actinomycin D-induced osteosarcoma cell line

- We identified 8 compounds that caused at least 25% greater cytotoxicity in the NMNAT1 KO cell line than in wild-type U2OS cells from an FDA-approved compound library with High-throughput screening (HTS).
- Actinomycin D induced enhanced cytotoxicity of the NMNAT1 KO osteosarcoma cell line compared to wild-type osteosarcoma cells.
- Actinomycin D induces cell death by triggering apoptosis in U2OS cells.
- Increased acetylation of p53 protein and increased expression of the pro-apoptotic genes, BAX and NOXA were observed in the NMNAT1 KO cell line after treatment.
- Actinomycin D treatment resulted in more severe DNA damage in NMNAT1 KO cells.
- Actinomycin D caused inhibition of proliferation and cell cycle in the NMNAT1 KO cell line
- Inhibition of rRNA synthesis is caused by actinomycin D treatment.

VI. SUMMARY

Chemotherapy is not effective enough for a significant proportion of cancers and the side effects could be extremely harmful for the patients. That's why it is of high importance to increase the sensitivity of tumor cells. NMNAT enzymes are involved in the synthesis of NAD^+ and may therefore influence a number of biochemical processes. However, we have limited information about their role in tumor cells. Therefore, an NMNAT1 gene-deficient (knock-out) osteosarcoma cell line was generated. During the characterization of the cell line, we detected decreased NAD^+ levels and proliferation of the NMNAT1 KO cell line. We compared the effect of cisplatin on wild-type and NMNAT1 KO osteosarcoma cells. Cisplatin induced necrotic and apoptotic cell death pathways. We observed reduced DNA repair in NMNAT1 KO cells. NAD^+ - and ATP content was further decreased on the KO cells, while, a highly blocked PARylation process could be detected. Our results show that NMNAT1 KO tumor cells are more sensitive to cisplatin treatment.

In the second part of our work, a high-throughput screening assay was performed, to identify drug candidates that also cause increased cell death in the absence of NMNAT1. The effects of one hit compound was further characterized in wild-type and NMNAT1 KO osteosarcoma cells. This compound was actinomycin D, which caused apoptotic cell death in the osteosarcoma cells. Actinomycin D caused enhanced cytotoxicity and DNA damage in the NMNAT1 KO cell line. Increased acetylation of p53 protein, and increased expression of the pro-apoptotic genes BAX and NOXA were observed after the treatment. Inhibition of proliferation, cell cycle and rRNA synthesis were also observed in the NMNAT1 KO osteosarcoma cell line.

In conclusion, our findings suggest that inhibition of NMNAT1 expression or activity, may be an effective target for tumor therapy. Furtherly, in the case of currently used cytostatic agents, it is expected that the time of the treatment and/or dose could be reduced in a therapy, combined with the inhibition of NMNAT1, to achieve greater efficacy, which may contribute to better overall patient outcome and reduction of side effects.

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List of publications related to the dissertation

1. **Kiss, A.**, Csíkos, C., Regdon, Z., Polgár, Z., Virág, L., Hegedűs, C.: NMNAT1 Is a Survival Factor in Actinomycin D-Induced Osteosarcoma Cell Death.
   Int. J. Mol. Sci. 22 (16), 1-17, 2021.
   DOI: http://dx.doi.org/10.3390/ijms22168869
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   Cancers (Basel). 12 (5), 1-27, 2020.
   IF: 6.639

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List of other publications

3. Guti, E., Regdon, Z., Sturniolo, I., **Kiss, A.**, Kovács, K., Demény, M. Á., Szöőr, Á., Vereb, G., Szöllősi, J., Hegedűs, C., Polgár, Z., Virág, L.: The multitargeted receptor tyrosine kinase inhibitor sunitinib induces resistance of HER2 positive breast cancer cells to trastuzumab-mediated ADCC.
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