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Molecular characterization of mechanisms involved in the antitumor activities of NAD biosynthesis inhibitors

Matsumoto Saki

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UNIL | Université de Lausanne
Faculté de biologie et de médecine

Central Laboratory of Hematology, Department of Laboratory Medicine and Pathology, Lausanne University Hospital (CHUV)

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Thèse de doctorat ès sciences de la vie (PhD)

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par

Saki Matsumoto

Ingénieure de l'Institut des Sciences et Industries du Vivant et de l'Environnement (AgroParisTech)
Grade de Master

Jury

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Molecular characterization of mechanisms involved in the antitumor activities of NAD biosynthesis inhibitor

Lausanne, le 22 septembre 2023

pour le Doyen
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Prof. Romano Regazzi

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ABSTRACT

Cancer research has made remarkable progress in the last decades and the development of new therapeutics contributed to the improvement of cancer treatment. However, many cancers remain uncurable today. In order to develop effective therapeutic strategies, it is crucial to identify specific vulnerabilities of cancer cells, their mechanisms of adaptation to therapy-induced stresses, and the mechanisms of therapy-resistance acquisition.

Nicotinamide phosphoribosyltransferase (NAMPT) inhibitors have been developed and extensively studied as promising anticancer agents. NAMPT inhibitors display selective cytotoxicity toward cancer cells by depleting cellular nicotinamide adenine dinucleotide (NAD), an essential molecule in cell functions and survival. However, the efficacy of NAMPT inhibitors is limited in clinical settings. Further investigations are needed to determine the molecular mechanisms involved in the antitumor effect of NAMPT inhibitors, as well as factors that modulate tumor response to NAMPT inhibitors.

In this study, we aimed to characterize the mechanisms by which leukemia and lymphoma cells gain resistance to NAMPT inhibitor APO866 through three distinct projects.

First, to investigate the molecular mechanisms contributing to the development of resistance to APO866, we generated acute myeloid leukemia (AML) models of *in vivo*-acquired resistance, using human AML cell line ML2 xenografts in mice. We performed whole transcriptomic analyses of resistant ML2 by RNA sequencing, combined with functional studies, to identify the alterations associated with resistance to APO866. We demonstrated that acquired resistance is conferred by a profound transcriptomic reprogramming that induces the activation of cellular pro-survival signaling as well as adaptive metabolic changes.

In the second project, to examine the possible involvement of gut microbiota in human NAD metabolome and in tumor response to NAMPT inhibitors, we performed studies using bacteria-infected cell cultures, as well as ML2 xenograft models in microbiota-depleted mice. Overall, we showed that gut microbiota-dependent conversion of NAD-related metabolites contributes to NAD homeostasis in distant tumor cells. This counteracts the effect of NAMPT inhibitors and therefore decreases their efficacy as antitumoral agent.

Finally, we identified a novel precursor of NAD biosynthesis that can be exploited by leukemia cells treated with NAMPT inhibitors to suppress its anticancer toxicity.

In summary, this work provides novel evidence that the efficacy of NAMPT inhibitors in hematological cancers can be affected by gut microbiota or by the presence of a previously unidentified NAD biosynthesis precursor. Furthermore, we characterized models of acquired resistance to APO866, generated *in vivo*. This is also the first study to report transcriptomic analyses that investigate the gene expressions associated with the NAMPT inhibitor treatment and with resistance to NAMPT inhibitors. Overall, the use of combinatory approaches with NAMPT inhibitors appear to be promising to overcome their limited efficacy in the clinics and the resistance acquisition. These findings will contribute to the development of therapeutic strategies to treat cancers with NAMPT inhibitors.

Keywords: NAD, NAMPT, APO866, drug resistance, leukemia, lymphoma

RÉSUMÉ

La recherche sur le cancer a évolué de manière remarquable durant les dernières décennies. Le développement de nouvelles thérapies innovantes a permis l'amélioration du traitement des cancers. Toutefois, de nombreux cancers restent incurables aujourd'hui. Afin de mettre au point des stratégies thérapeutiques plus efficaces, il est crucial d'identifier les vulnérabilités des cellules cancéreuses qui peuvent servir de nouvelles cibles thérapeutiques, et de déterminer les mécanismes par lesquels les cancers s'adaptent aux stress liés aux agents thérapeutiques, afin de les exploiter pour contrecarrer le développement de résistance aux thérapies.

Les inhibiteurs de nicotinamide phosphoribosyltransférase (NAMPT) ont été développés et étudiés intensivement en tant que composés anticancéreux prometteurs. Les inhibiteurs de NAMPT ont démontré des effets cytotoxiques contre les cellules cancéreuses de manière spécifique, via la déplétion de nicotinamide adénine dinucléotide (NAD), un métabolite essentiel aux fonctionnements et à la survie cellulaires. Toutefois, l'efficacité des inhibiteurs de NAMPT est limitée chez les patients. Des efforts supplémentaires sont nécessaires afin de déterminer les mécanismes moléculaires impliqués dans les effets antitumoraux de ces inhibiteurs, ainsi que des facteurs qui impactent la réponse des cellules tumorales aux inhibiteurs.

L'objectif de cette étude était de caractériser les mécanismes par lesquels les cellules leucémiques et de lymphome acquièrent la résistance à l'inhibiteur de NAMPT APO866, en conduisant trois projets distincts.

En premier lieu, dans le but d'étudier les mécanismes moléculaires contribuant au développement de résistance à APO866, nous avons généré des modèles de leucémie myéloïde aiguë (LMA) de résistance acquise *in vivo* en utilisant des xénogreffes de la lignée cellulaire de LMA humaine ML2 dans des souris. Nous avons réalisé des analyses des transcriptomes entiers des cellules ML2 résistantes par RNA-sequencing, combinées avec des études fonctionnelles, afin d'identifier les altérations liées à la résistance à APO866. Nous avons démontré que la résistance acquise est conférée par une profonde réorganisation transcriptomique qui active plusieurs voies de signalisations pro-survie ainsi que des adaptations métaboliques.

Afin d'examiner l'implication du microbiote intestinal dans la régulation du métabolome du NAD et dans la réponse tumorale aux inhibiteurs de NAMPT, nous avons réalisé des études en utilisant des cultures de cellules infectées par des bactéries, et des modèles de xénogreffes de cellules ML2 dans des souris dont le microbiote intestinal a été déplété. Nous avons montré que des conversions des métabolites précurseurs du NAD se produisent au sein du microbiote intestinal lorsqu'il est présent et que ces métabolites contribuent à la maintenance de NAD dans les tumeurs distantes, permettant ainsi de contrer l'efficacité antitumorale des inhibiteurs de NAMPT.

Enfin, nous avons identifié un nouveau précurseur de synthèse de NAD, qui peut être exploité par les cellules leucémiques traitées aux inhibiteurs de NAMPT, supprimant leurs effets cytotoxiques.

En résumé, cette étude fournit de nouvelles preuves que l'efficacité des inhibiteurs de NAMPT dans les cancers hématologiques peut être affectée par le microbiote intestinal ou par la présence d'un précurseur de NAD non identifié auparavant. Nous avons caractérisé des modèles de résistance envers APO866, générés *in vivo*. Cette étude est également la première à rapporter des données transcriptomiques entières qui ont permis de rechercher les expressions de gènes associées au traitement avec l'inhibiteur de NAMPT et à la résistance aux inhibiteurs. En somme, des approches combinatoires avec les inhibiteurs de NAMPT semblent être prometteuses pour surmonter leur efficacité limitée en clinique et l'acquisition de résistance. Ces découvertes contribueront au

développement de stratégies thérapeutiques efficaces pour traiter les cancers avec des inhibiteurs de NAMPT.

Mots-clés : NAD, inhibiteurs de NAMPT, résistance aux thérapies, cancers hématologiques

RÉSUMÉ (GRAND PUBLIC)

Thèse de doctorat

« Caractérisation moléculaire des mécanismes impliqués dans les effets antitumoraux des inhibiteurs de la biosynthèse du NAD »

Saki Matsumoto

Le cancer est une maladie qui se caractérise par un dérèglement et une prolifération incontrôlée des cellules de notre organisme. Selon les statistiques, une personne sur cinq dans le monde se verra diagnostiqué un cancer au cours de sa vie. Grâce aux progrès de la recherche durant les dernières décennies, de nombreux cancers peuvent aujourd'hui être traités efficacement, et même être guéris pour certains. Toutefois, le taux de survie est faible pour d'autres cancers qui restent incurables. Les principaux obstacles sont les effets secondaires indésirables des traitements, la résistance aux traitements et les rechutes. Afin de mettre au point de nouvelles solutions thérapeutiques, les efforts de recherche portent sur la découverte de nouvelles cibles thérapeutiques, la détermination des facteurs prédictifs des réponses au traitement, et l'identification des mécanismes de résistance.

Les inhibiteurs de nicotinamide phosphoribosyltransférase (NAMPT) ont été développés et étudiés intensivement pour leurs effets antitumoraux prometteurs. Le NAMPT est une enzyme qui existe dans les cellules humaines et qui permet la production de nicotinamide adénine dinucléotide (NAD), une molécule essentielle au bon fonctionnement des cellules. Les cellules cancéreuses étant particulièrement dépendantes du NAD pour leur survie, le ciblage et la déplétion du NAD avec les inhibiteurs de NAMPT engendrent la mort des cellules cancéreuses. Plusieurs travaux ont démontré l'efficacité de ces inhibiteurs dans des modèles de cancers hématologiques et solides. Toutefois, leur efficacité est limitée chez les patients dans les études cliniques. Par conséquent, des efforts sont nécessaires afin de mieux comprendre les mécanismes impliqués dans les effets antitumoraux de ces inhibiteurs, ainsi que pour déterminer les facteurs diminuant la réponse des cellules cancéreuses au traitement.

L'objectif de cette thèse a été de déterminer les mécanismes par lesquels les cellules leucémiques et de lymphome acquièrent la résistance aux inhibiteurs de NAMPT. Dans un premier projet, nous avons réalisé une analyse se basant sur la technologie de séquençage à haut débit et démontré que des altérations des expressions de certains gènes rendent les cellules leucémiques capables de s'échapper à l'induction de la mort cellulaire par l'inhibiteur. Dans un deuxième projet, nous avons montré que le microbiote intestinal affecte l'efficacité thérapeutique des inhibiteurs de NAMPT dans les tumeurs distantes. Enfin, nous avons identifié une nouvelle molécule qui peut servir à la biosynthèse de NAD dans les cellules cancéreuses de manière indépendante de NAMPT, ce qui peut donc impacter négativement l'efficacité thérapeutique des inhibiteurs de NAMPT.

En résumé, cette étude a permis de décrire les mécanismes par lesquels les cellules cancéreuses résistent aux inhibiteurs de NAMPT. Une réorganisation des expressions de gènes permet aux cellules cancéreuses d'acquérir une résistance à la mort cellulaire. Elles profitent également des apports de métabolites depuis leur environnement pour effacer les effets antitumoraux des inhibiteurs de NAMPT. Ces découvertes contribuent au développement de stratégies thérapeutiques efficaces pour traiter les cancers avec des inhibiteurs de NAMPT.

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LIST OF ABBREVIATIONS

Akt	Protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
DEG	Differentially expressed gene
DGE	Differential gene expression
ECAR	Extracellular acidification rate
EMT	Epithelial-mesenchymal transition
ETC	Electron transport chain
FC	Fold change
GSEA	Gene set enrichment analysis
KO	knock-out
MAPK	Mitogen activated protein kinase
MDR	Multi-drug resistance
MFI	Mean Fluorescence Intensity
mTOR	Mechanistic target of rapamycin
NA	Nicotinic acid
NAAD	Nicotinic acid adenine dinucleotide
NAMN	Nicotinic acid mononucleotide
NAPRT	Nicotinic acid phosphoribosyltransferase
NAR	Nicotinic acid riboside
NAD	Nicotinamide adenine dinucleotide
NADK	Nicotinamide adenine dinucleotide kinase
NADS	Nicotinamide adenine dinucleotide synthase
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
NFkB	Nuclear factor kappa B
NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide adenilyltransferase
NR	Nicotinamide riboside
NRK	Nicotinamide riboside kinase
NSCLC	Non-small cell lung cancer
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PARP	Poly-(ADP-ribose)-polymerase
PI3K	Phosphoinositide 3-kinase

PPP	Pentose phosphate pathway
QAPRT	Quinolinic acid phosphoribosyltransferase
ROS	Reactive Oxygen Species
rpm	Rotations per minute
RT	Room temperature
SIRT	Sirtuin
TCA	Tri-carboxylic acid
TME	Tumor microenvironment
WT	Wild type

CHAPTER 1. INTRODUCTION

1 CANCERS

1.1 HEMATOLOGICAL CANCERS

Cancers are a leading cause of death worldwide. Nearly 19.3 million new cases and 10 million deaths were counted in 2020 according to the International Agency for Research on Cancer (IARC) report [1]. The statistics show that one in five people develop cancer during their lifetime. Despite extensive research on cancer biology and development of new therapeutics, many cancers remain uncurable today.

Hematological cancers comprise all malignancies that affect cells from blood, bone marrow and lymphoid organs. Owing in part to the relatively easy tumor samplings, hematological cancers have been the pioneer field in cancer research discoveries and therapeutic developments [2]. They are referred to as leukemia, lymphoma and myeloma depending on the type of cell affected.

Leukemias are a heterogenous group of hematological malignancies characterized by an intensive proliferation of immature blood cells. They are divided into different subtypes according to cell maturity (acute or chronic) and cell type (lymphoblastic or myeloid). Acute myeloid leukemia (AML) is characterized by an uncontrolled proliferation of immature myeloid progenitor cells. It is the most common type of acute leukemia in adults and remains a disease associated with poor prognosis. Standard treatment consists of cytarabine and anthracycline (e.g., daunorubicin) induction regimen [3]. Although the standard induction therapy is highly effective in killing leukemic cells and a high rate of complete remission is observed, most patients undergo relapse. In addition, elderly patients or patients with comorbidities do not tolerate intensive chemotherapy treatment. Among several new therapeutic agents, the development and introduction of Venetoclax, a potent BH3 mimetic, was shown to be a promising treatment for newly diagnosed, relapsed and refractory patients [4]. However, the response is limited in duration and the development of resistance is frequently observed. Today, the 5-year survival with adult AML is very poor (27 %). More effective therapies with lower toxicities are needed for the treatment of AML.

Acute lymphoblastic leukemia (ALL) is a diverse group of malignancies that affect immature lymphoid cells in the bone marrow, peripheral blood and extramedullary sites. While advances have

led to a remarkable improvement in pediatric ALL outcomes over the last 50 years, with a five-year overall survival rate approaching 90 %, ALL remains a disease in adults associated with poor prognosis [5], [6]. In adult ALL patients, long-term disease-free survival rate decreases with age, reaching nearly 25 % in those of more than 50 years-old [7]. Furthermore, relapsed and refractory cases remain a challenge in the disease cure. In the last decade, genetically engineered chimeric antigen receptor expressing T cells (CAR-T cells) have been largely studied and represent a promising treatment for ALL treatment, although several challenges including cytokine release syndrome or neurotoxicity remain to be addressed.

Lymphomas originate from lymphocytes and can develop in the lymph nodes, spleen, bone marrow, blood and other organs. Lymphomas are divided into two categories: Hodgkin lymphomas and non-Hodgkin lymphomas. Hodgkin lymphoma is characterized by the presence of specific large lymphoma cells called Reed-Sternberg cells in the lymph nodes. Non-Hodgkin lymphomas encompass all other lymphomas, which can affect either B cells or T cells. Among then, Burkitt lymphoma is a rare and aggressive type of B cell lymphoma. Owing to advances in treatment, the prognosis of patients diagnosed with lymphoma has improved in the past decades and the survival rates are relatively high. The mainstays of treatment consist of chemotherapy, radiotherapy and targeted therapies, and recently, stem cell transplantation and CAR-T cell therapies have also been approved for some types of lymphomas. However, severe side effects, recurrent disease and resistance development remain the main challenges of lymphoma treatment.

Extensive research for understanding the biology of cancers has greatly contributed to the improvement of cancer treatment in the last decades. Further investigation is needed to develop more effective therapies with higher specificity and less side effects, and powerfulness toward relapsed and refractory tumors.

1.2 HALLMARKS OF CANCERS

Cancers are characterized by the development of abnormal cells that proliferate uncontrollably. The continuous growth and division of cancer cells despite the limited resources are enabled by the reprogramming of cellular metabolic pathways. In addition, cancer cells display rearranged cellular signaling pathways that allow them to overcome cellular stresses occurring from aberrant proliferation, such as DNA damage and oxidative stress. These features of cancer cells that promote their growth and survival have been described as the hallmarks of cancer [8] (**Figure 1**), which are detailed in the following paragraphs.

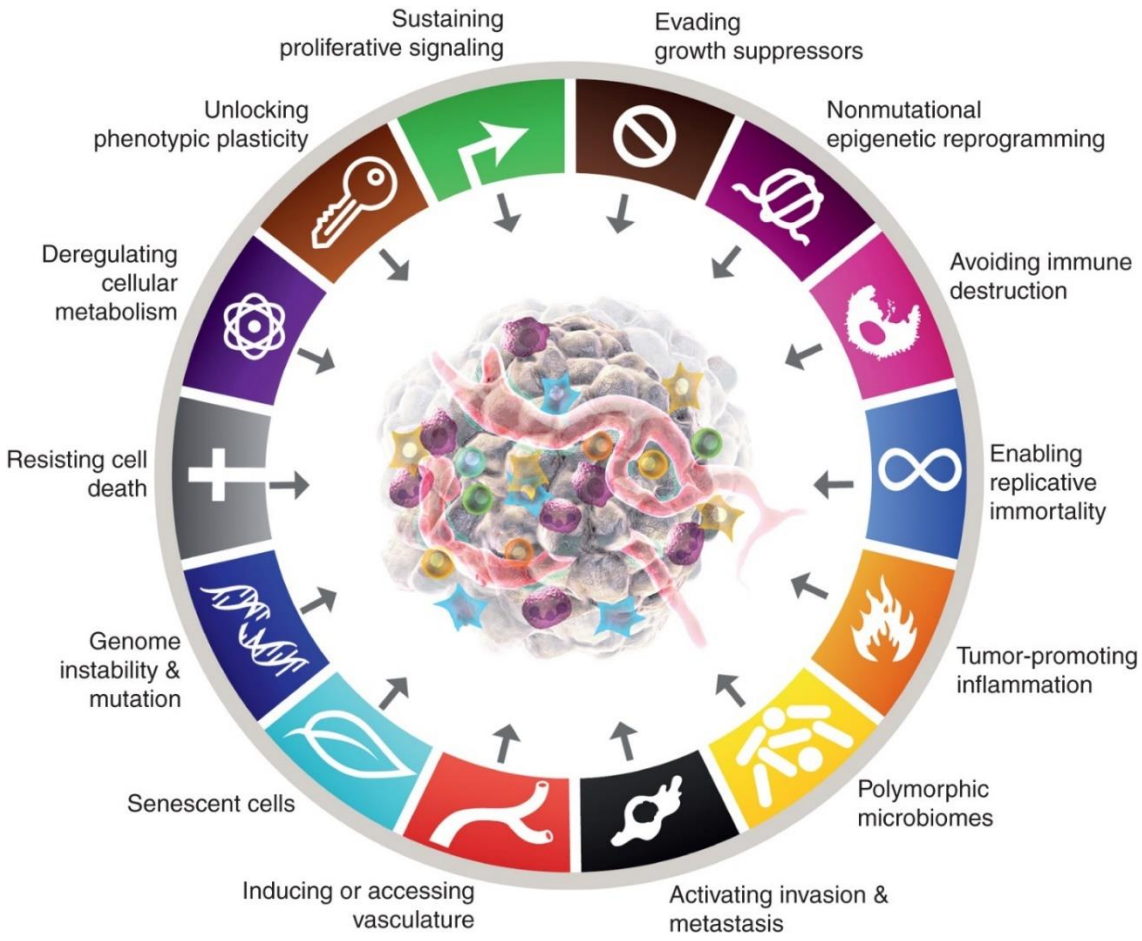

Figure 1 Hallmarks of Cancers (from Hanahan 2022)[9]

The first hallmarks of cancers are their abilities to sustain proliferative signals and to evade growth suppression. In healthy cells, external growth signals are needed for the cell proliferation, so that cells grow only when sufficient resources are present in the environment. Moreover, different signals control cell divisions to assure cell homeostasis. Contrastingly, in cancer cells, oncogenic alterations lead to continuous generation of proliferative signals, which make them independent from external stimuli. For example, overexpression of growth-stimulating factors such as c-Myc [10], or expression of constitutively active mutant of growth-promoting protein Ras [11] contribute to this hallmark. Besides, cancer cells are able to evade the anti-proliferative signals, via the inactivation or the loss of expression of genes encoding so-called growth suppressor proteins such as P53 or PTEN. Notably, p53 gene mutation or deletion is observed in more than 50 % of human tumors [12], [13]. Continuous cell division is also supported by replicative immortality, via the upregulation of telomerases, to control the lengths of the telomeres in their DNA.

Uncontrolled proliferation under limited resources may cause depletion of nutrients and decrease of oxygen level. Cancers exhibit deregulated cellular energetics that confer them the ability to grow at high speed in unfavorable conditions. Notably, many cancers show a reliance on an

enhanced aerobic glycolysis and a reduced oxidative phosphorylation, a phenomenon known as the Warburg effect [14]. This altered metabolism allow faster production of energy and building blocks for cellular components to support rapid cell proliferation.

In high stress conditions, normal cells undergo programmed cell death called apoptosis. Cancer cells acquire the ability to evade from apoptosis via different mechanisms. For instance, anti-apoptotic members of B cell lymphoma-2 (Bcl-2) family proteins, which are the primary regulators of apoptosis, can be highly expressed in cancer cells [15], [16]. Conversely, pro-apoptotic signaling can be attenuated by inactivation of p53 mutation for example [12].

The immune system plays an important role in the control of tumorigenesis and tumor progression. Increasing evidence demonstrate that cancer cells have different strategies to impede with the antitumoral effects of the immune system. The mechanisms include escaping the immune surveillance by interfering with immune checkpoints such as PD-1/PD-L1 or CTLA4 [17], modulating the recruitment and the functions of immune cells through pro-inflammatory cytokines production and nutrient uptake, and making a pro-tumoral environment. This hallmark is of major importance as it demonstrates the ability of cancers not only to adapt to environmental stresses but to construct a favourable environment for their growth.

While these hallmarks of cancers underline the complexity and the high adaptability of cancers that make them difficult to treat, on the other hand these alterations make cancer cells more dependent on specific processes or proteins. Thus, they consist of potential therapeutic targets that can be exploited for cancer treatment. Recent research has led to the development of new targeted therapies including metabolism-targeting drugs, anti-apoptotic drugs, and immunotherapies. Understanding the biology of cancers and identifying their vulnerabilities are key in the development of effective treatments.

In this context, nicotinamide adenine dinucleotide (NAD) has emerged as a suitable target for anticancer therapies.

2 NAD METABOLISM

2.1 FUNCTIONS

Nicotinamide adenine dinucleotide (NAD) is an essential metabolite in cell life (**Figure 2**). NAD alternates between the reduced NADH and oxidized NAD^+ forms and acts as a redox carrier for more than 300 redox reactions in the cells, involved in the energy metabolism and the cellular redox signaling. In addition, NAD serves as a co-substrate for numerous enzymes such as protein deacetylases sirtuins (SIRTs), poly-(ADP-ribose)-polymerases (PARPs) and ADP-ribose synthases

CD38, CD157 and SARM1. By participating in their reactions, NAD functions as an important second messenger regulating multitude of cellular fundamental processes such as: gene transcription, DNA repair, stress signaling, mitochondrial function, energy metabolism, cell cycle progression, circadian rhythm, calcium signaling, immune function and inflammation etc. [18]. In the following paragraphs, we describe the functions of NAD to highlight the importance of NAD in cell homeostasis, proliferation and survival.

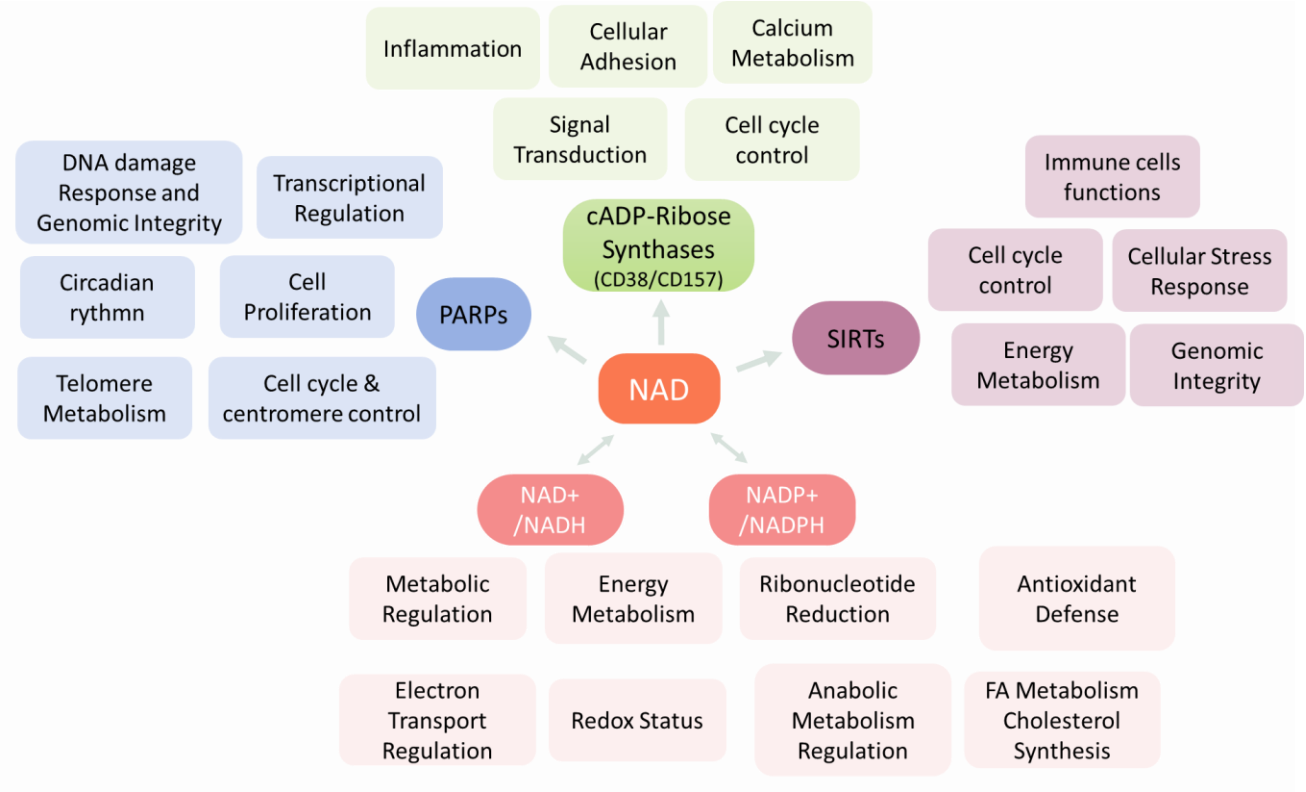

Figure 2 Central role of NAD in cellular functions. *NAD participates in cellular functions through the activities of NAD-consuming enzymes PARPs, SIRTs and cADP-Ribose synthases and as co-factor of NAD(P)H-dependent enzymes. Abbreviations: cADP, cyclic adenosine diphosphate. FA, fatty acid. PARP, poly-(ADP-ribose) polymerase. SIRT, sirtuins.*

Genomic Stability and gene expression

NAD coordinates genomic stability and regulates gene expression as well as RNA processing, mainly via the activities of NAD-dependent enzymes. For instance, NAD-dependent deacetylase SIRT1 deacetylates multiple transcription coactivators and histones, thereby remodelling the epigenome [19]. The decrease in NAD level leads to lower SIRT activity and therefore aberrant gene transcription due to histone hyperacetylation. NAD-dependent enzymes PARPs, by mediating the PARylation of topoisomerases, histones, DNA polymerases and DNA ligases, assure the genome stability and regulate the genomic and transcriptomic biology [20]. Notably, PARPs play a central role

in the DNA damage response. Moreover, NAD itself can serve as a nucleotide analogue and be used for RNA capping and DNA ligation in response to stresses [21], [22].

Energy metabolism

NAD plays a major role in the energy metabolism. NAD serves as co-factor for enzymes involved in different metabolic pathways including glycolysis, oxidative phosphorylation (OXPHOS), pentose phosphate pathway (PPP), serine one-carbon metabolism, nucleotide base synthesis, fatty acid and lipid synthesis [23]. In the glycolytic pathway, NAD is used by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the sixth step and by lactate dehydrogenase (LDHA) at the end of the process. The end-product of glycolysis, pyruvate, is metabolized to acetyl-coA through a NAD-dependent conversion and enter the tri-carboxylic acid (TCA) cycle. In the TCA cycle, NAD^+ is reduced to NADH in multiple steps involving isocitrate dehydrogenase (IDH), oxoglutarate dehydrogenase (OGDH) and malate dehydrogenase (MDH). Finally, NADH produced in the TCA cycle is oxidized to NAD^+ by NADH dehydrogenase (or the mitochondrial respiratory complex I) in the electron transport chain (ETC) and contributes to the generation of ATP. Therefore, the maintenance of a sufficient level of NAD is crucial for ATP production, which in turn is required for diverse cellular processes.

NAD is also implicated in the regulation of metabolic functions through the activities of NAD-consuming enzymes. For example, nuclear SIRT1 controls the oxidative metabolism in response to low nutrients signals, while mitochondrial SIRT3 regulates fatty acid oxidation, TCA cycle and urea cycle by deacetylating numerous enzymes in these pathways [19].

Maintenance of cellular redox balance

Active cells constantly produce reactive oxygen species (ROS) and antioxidants, and the impairment of the redox balance leads to an oxidative stress. ROS can be generated in metabolic reactions occurring in mitochondria, or by a range of cytosolic enzymes (NADPH oxidase (NOX), xanthine oxidase (XO), lysyl oxidase (LOX), or cytochrome P450 (CYPs)) and all of them require NAD(P)H as a redox co-factor. On the other hand, NADPH serves as the reductive power for ROS-detoxifying enzymes such as glutathione reductase (GR) or glutathione peroxidase (GPX) and thioredoxin reductase (TxR). Therefore, NAD(P)H is a major regulator of cellular redox balance. Moreover, through the activities of NAD-dependent enzymes, NAD is involved in the expression or activities of enzymes in the antioxidant system. For instance, SIRT3 is an essential modulator of oxidative stress that modulates both ROS generation and detoxification [18].

Immune functions

A growing number of studies demonstrated the implication of NAD metabolism in the regulation of immune cell biology. Notably, enhanced glycolysis supported by NAD is important in

the activation of inflammatory macrophages. Sirtuins are crucial regulators of innate and adaptive immune cell metabolism and functions [24], [25]. For instance, HIF1α, a direct target of SIRT1, is a key protein linking metabolism and immunity, which regulates the metabolism, differentiation, and activation of immune cells through induction of cytokines production [26]. Hence, NAD metabolism is closely linked to the immune functions.

Altogether, NAD plays an essential role in the execution and the regulation of fundamental processes in cell life. Consistently, the intracellular NAD pool needs to be constantly replenished and maintained at homeostasis to ensure cell growth.

2.2 NAD BIOSYNTHESIS AND HOMEOSTASIS

2.2.1 NAD biosynthesis

In the last decades multiple studies contributed to depicting the NAD biosynthetic pathways in mammalian cells. Consistent with the central role of NAD in cell life, several distinct pathways allow the biosynthesis of NAD from different precursors. In mammalian cells, NAD is synthetized through three main pathways (**Figure 3**) [27]. First, in the *de novo* pathway, dietary amino acid tryptophan is degraded and converted to NAD through a series of enzymatic steps involving notably quinolinic acid phosphoribosyltransferase (QAPRT) activity. Secondly, cells produce NAD via the Preiss-Handler pathway, in which nicotinic acid (NA) and nicotinic acid riboside (NaR) are transformed to nicotinic acid mononucleotide (NAMN) by the enzymatic activities of nicotinic acid phosphoribosyltransferase (NAPRT) and nicotinamide riboside kinase (NRK), respectively. NAMN is further converted by nicotinamide mononucleotide adenylyltransferase (NMNAT) to nicotinic acid adenine dinucleotide (NAAD), which can be amidated into NAD by NAD synthase (NADS) activity. Finally, the salvage pathway generates NAD from nicotinamide (Nam) and nicotinamide riboside (NR), via the activities of nicotinamide phosphoribosyl-transferase (NAMPT) and NRK respectively to produce nicotinamide nucleotide (NMN). Finally, NMN is converted by NMNAT to generate NAD. The salvage pathway governed by NAMPT is of major importance, as it enables the recycling of Nam, the end-product of NAD degradation by NAD-consuming enzymes such as PARPs or sirtuins, and does not rely on NAD precursors from external sources. It contributes greatly to the maintenance of intracellular NAD.

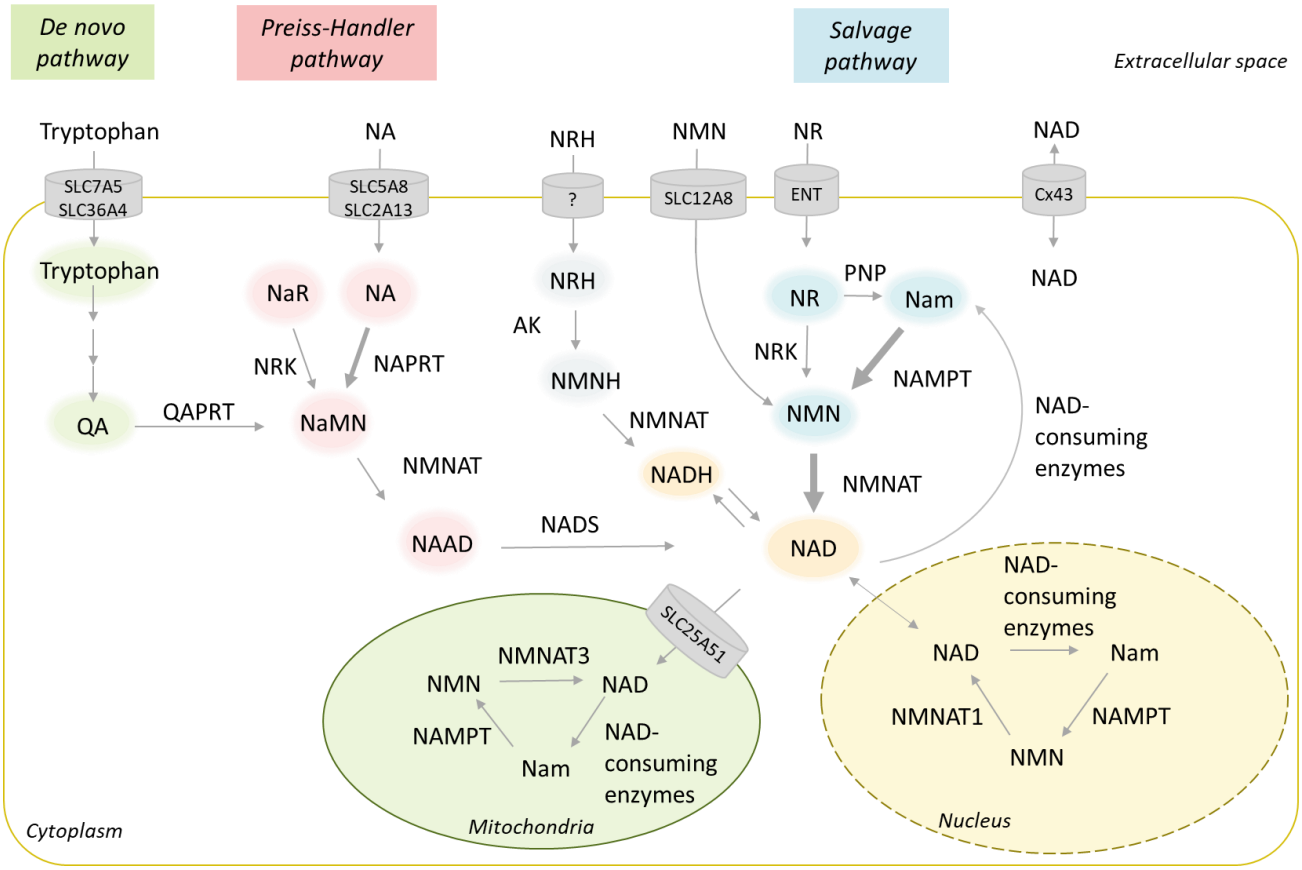

Figure 3 NAD biosynthesis in mammalian cells. *Abbreviations: AK, adenosine kinase. Cx43, connexin 43. NA, nicotinic acid. NAAD, nicotinic acid adenine dinucleotide. NAMN, nicotinic acid mononucleotide. NAPRT, nicotinic acid phosphoribosyltransferase. NAR, nicotinic acid riboside. NAD, nicotinamide adenine dinucleotide. NADK, NAD kinase. NADS, NAD synthase. NAM, nicotinamide. NAMPT, nicotinamide phosphoribosyltransferase. NMN, nicotinamide mononucleotide. NMNAT, NMN adenilyltransferase. NR, nicotinamide riboside. NRH, dihydronicotinamide riboside. NRK, nicotinamide riboside kinase. PNP, purine nucleoside phosphorylase. QA, quinolinic acid. QAPRT, QA phosphoribosyltransferase.*

Recently, NRH and NARH have been shown to be additional precursors for NAD biosynthesis [28], [29]. Furthermore, recent reports demonstrated that purine nucleoside phosphorylase (PNP) plays an important role in the metabolism of NR, by mediating the cleavage of NR to Nam [30]. Some studies also demonstrated the existence of interconversions between NAD precursors in stress conditions. For instance, bone marrow stromal cell antigen 1 (BST1) was shown to mediate the conversions between NA and NR, leading to a switch between the *Preiss-Handler* and the salvage pathways [31]. These discoveries suggest the complexity of NAD biosynthetic pathways and the potential existence of yet undescribed precursors or interconversions in the NAD metabolome.

2.2.2 Regulation of NAD homeostasis

As mentioned above, the maintenance of NAD is important for cell homeostasis. The intracellular level of NAD is tightly regulated to be maintained at a sustained level. Several enzymes, transcription factors or micro RNAs (miRNAs) have been described to regulate the NAD biosynthetic enzymes as well as the activities of NAD consuming enzymes. For instance, several enzymes and

transcription factors including SIRT, c-Myc, or FoxO1, regulate the expression of NAMPT, which plays an important role in the regulation of cellular NAD level [32].

It is also important to emphasize that NAD homeostasis depends not only on NAD synthesis and consumption, but on its subcellular compartmentalization. NAD level in each of nuclear, cytosolic and mitochondrial compartments must be maintained at their respective suitable levels. Notably, mitochondrial pool of NAD is the largest, accounting for up to 70 % of cellular NAD in some tissues [33]. In addition, not only the total NAD(H) level but also the ratio of NAD^+/NADH, as well as their phosphorylated forms $NADP^+$/NADPH, need to be maintained at definite levels in each compartment. While cytosolic and nuclear NAD^+/NADH ratios are at similar ranges (700:1), mitochondrial NAD^+/NADH ratio is maintained distinctly at a much lower level (8:1) [34]. The total amount and the redox balance of NAD(H) in subcellular compartments are maintained via the differential distribution of NAD biosynthetic enzymes, the activities of dehydrogenases or the malate-aspartate shuttle, and in the mitochondria, via the redox reactions in the TCA cycle and electron transport chain as well as active NAD import [35], [36].

These processes altogether tightly regulate cellular NAD metabolism to protect cellular homeostasis.

2.3 NAD AND CANCERS

Given the essential role of NAD in the cellular fundamental processes, deregulation of NAD metabolism is associated with various pathologies such as neurodegeneration, infection, fatty liver disease, kidney injury, cardiac diseases, muscular diseases, and cancers [37]. An increase of NAD level in the tissues is reported to be associated with high inflammation to promote the development of diseases [37]. On the other hand, age-associated decline in NAD levels is a common feature observed in age-associated diseases [31]. In the field of anti-aging, administrations of NAD intermediates such as NMN or NR as NAD-boosting agents have been extensively investigated, and growing evidence indicate their effectiveness in competing age-associated dysfunctions and promoting health conditions [38].

In the field of cancer, a deregulation of NAD homeostasis is also reported in various tumor types. High expressions of NAD biosynthetic enzymes have been reported in different cancers and associated with disease progression [39].

Indeed, NAD is an essential molecule for cancer metabolism. As mentioned above, rapid proliferation, altered metabolism shifted to aerobic glycolysis and rapid energy production are the major traits of cancer cells. Cancer cells are therefore highly dependent on sustained level of NAD to support their metabolism.

Moreover, NAD is known to be implicated in tumorigenesis through the regulation of various tumor suppressors and oncogenes. Notably, via the activities of NAD-dependent enzymes PARPs and SIRTs, NAD controls the expression or the activities of tumor suppressors such as p53, superoxide dismutase 2 (SOD2) or Bcl-2 family protein Bax, and of oncogenes including c-Myc, Akt, or isocitrate dehydrogenase (IDH) [40].

Recent studies also suggest the role of NAD in modulating the immune cells function in the tumor environment. For example, NAD regulates PD-L1 induction in tumor cells through STAT1-dependent interferon gamma (IFN-γ) signaling pathway [41]. Sirtuins play important roles in the differentiation and function of CD8 T cells, and in the function of tumor infiltrating lymphocytes (TILs) [24]. In addition, CD38 induces an immunosuppressive environment by promoting the production of immune-suppressive myeloid-derived suppressor cells (MDSCs) and regulatory T cells [42]. Thus, CD38 mediates the immune modulation of the tumor microenvironment (TME) and plays a major role in tumorigenesis, tumor progression and metastasis in both hematological malignancies and solid tumors. Consistently, CD38 overexpression is associated with several malignancies, and associated with poor prognosis in chronic lymphocytic leukemia (CLL) [43]. Furthermore, NAMPT itself was also shown to act as a chemokine and affect cellular signaling related with immune functions [44]. These NAD-dependent modulations of immune cells reduce their antitumoral activities and promote tumor growth.

In summary, cancer cells are highly dependent on NAD and NAD-dependent functions. Therefore, inducing the depletion of NAD in cancers by using specific small molecule inhibitors of NAD biosynthesis has emerged as a potential therapeutic approach for cancer treatment.

2.4 THE KEY ROLE OF NAMPT IN CANCERS

The increased activities of NAD-consuming enzymes in cancer cells cause rapid NAD degradation. Therefore, cancer cells are highly dependent on the rapid turnover of degraded NAD by the salvage pathway for maintaining the intracellular NAD level. NAMPT, as the rate-limiting enzyme of the salvage pathway, plays an important role in the rapid NAD replenishment in cancer cells. Accordingly, elevated expression of NAMPT has been observed in a broad range of solid and hematological cancers, including colorectal, gastric, ovarian, prostate, breast cancers, melanoma, gliomas, sarcomas and lymphomas [45], [46]. In general, a direct correlation between NAMPT expression and cancer aggressiveness is observed. NAMPT overexpression has been clinically associated with cancer progression, metastasis, drug resistance and poor prognosis [45], [47]–[49].

NAMPT also exists in an extracellular form commonly called eNAMPT, also known as pre-B cell colony-enhancing factor (PBEF) or visfatin. Whether eNAMPT possesses enzymatic activity is still debated, but the enzymatic activity seems to be limited in any case by the scarce availabilities of its substrates ATP and phosphoribosyl pyrophosphate (PPRP). On the other hand, eNAMPT possesses

several well-established non-enzymatic functions in the regulation of cellular activities. eNAMPT acts as a cytokine in multiple signaling pathways including PI3K-Akt, mTOR, Erk1/2, p38 MAPK and NFκB [50]. By regulating these pathways, eNAMPT plays a significant role in the regulation of cancer metabolic reprogramming and promotes proliferation, stemness, and epithelial–mesenchymal transition (EMT) [51]. Hence, eNAMPT plays a key role in cancers. Consistently, increased amount of secreted eNAMPT has been associated with cancers [44].

Moreover, NAMPT has been shown to directly affect the functions of different immune cells in context-dependent manner. For instance, eNAMPT promotes polarization of macrophages to pro-tumoral M2 macrophages and secretion of immune suppressive (IL-10) and tumor promoting cytokines (IL-6, IL-8) [49]. Hence, NAMPT contributes to the cancer hallmark of building tumor-promoting immune environment.

Given the central role of NAMPT in the NAD biosynthesis and in the regulation of multiple processes in cancer biology, impairing the NAD metabolism in cancers by targeting NAMPT has emerged as a promising therapeutic approach for cancer treatment.

3 NAD-TARGETING ANTICANCER THERAPIES

3.1 NAMPT INHIBITORS

NAMPT emerged as an attractive target for anticancer treatment given its multifaced functions in cancer pathogenesis and biology. To date, several NAMPT inhibitors have been reported and developed, as reviewed in the literature [46], [52].

APO866 (also known as FK866, (E)-Daporinad, and WK175) is the first described chemical NAMPT inhibitor [53]. APO866 has been identified in a screening of compounds with anticancer activities [54] and later demonstrated to be an inhibitor of NAMPT [53]. Until today APO866 is the most extensively studied NAMPT inhibitor in preclinical models and exhibit robust anticancer killing effects in several cancer types including hematological and solid malignancies. It presents a potency within nanomolar ranges, with an inhibitor constant K_i of 0.4 nM in cell-free assay, and an IC_{50} for cellular toxicity of approximately 1 nM [53].

OT-82 was identified as an anticancer agent in a drug screening [55]. OT-82 was shown to inhibit NAMPT and induce NAD depletion and subsequent cancer cell death [55].

CHS-828 (GMX1778) was first developed as an anticancer agent and later suggested to inhibit NAMPT by Olesen *et al.* in 2007 [56]. GMX1777 is a prodrug of CHS-828 with enhanced chemical properties in terms of pharmacokinetics.

Many other NAMPT inhibitors are reported in the literature and include GNE-617 and GNE618 (Genentech), A-1293201 and A-1326133 (Abbvie) etc. In addition, several dual NAMPT inhibitors or combinatory approaches with NAMPT inhibitors are also reported and studied in both preclinical and clinical settings. For example, KPT-9274 is a dual inhibitor of NAMPT and p21-activated kinase 4 (PAK4) [57], while STF-31 is a dual inhibitor of NAMPT and glucose transporter 1 (GLUT1) [58]. Dual inhibitors of NAMPT and histone deacetylase (HDAC) are also studied as promising anticancer agents [59], [60]. The potentiation of NAMPT inhibitor by a combinational approach with other agents is also extensively investigated.

3.2 ANTICANCER EFFECTS OF NAMPT INHIBITORS

NAMPT inhibitors including APO866 have proven strong anti-proliferative and cytotoxic effects in several cancer types in both *in vitro* and *in vivo* studies. *In vitro*, NAMPT inhibitors were shown to induce a rapid depletion of intracellular NAD, with subsequent ATP depletion and cell death [52]. In contrast, they showed limited toxicity toward healthy cells. *In vivo* efficacies of several NAMPT inhibitors have been reported in different tumor xenograft models of human malignancies including AML, glioblastoma, non-small cell lung cancer (NSCLC), ovarian, prostate, pancreatic, and colorectal cancers [52]. For instance, in mouse xenograft model of Burkitt lymphoma, intraperitoneal injections of APO866 inhibited tumor growth within 7 days, resulting in a significant prolongation of animal overall survival [61]. In the same study, APO866 was also shown to effectively inhibit tumor initiation. Similar observations have been reported with administration of GMX1777 by intravenous infusion in multiple myeloma (MM), small cell lung cancer and colon carcinoma xenograft models [62], [63], or with oral administration of GNE-617 and GNE-618 in colon, pancreatic and prostate cancers [64].

The promising observations of NAMPT inhibitors in preclinical models led to their assessment in clinical trials (**Table 1**). To date, GMX-1778, OT-82 andKPT-9274 were tested in phase I trials for safety in patients, and APO866 in phase II, in a wide range of solid and hematological cancers. APO866 has been evaluated as a single-agent treatment of advanced melanoma, cutaneous T-cell lymphoma, and refractory B-cell CLL (NCT00432107, NCT00431912, NCT00435084). Although APO866 demonstrated good tolerability in patients, it failed to show objective tumor response due to dose-limiting toxicities such as thrombocytopenia. Other NAMPT inhibitors that have been tested in clinical settings such as CHS-828 could not show objective response either so far (NCT00003979). Dual inhibitor KPT-9274 is currently evaluated in phase I trials enrolling patients with solid tumors, non-Hodgkin lymphoma and relapsed and refractory AML (NCT02702492, NCT04281420).

The lack of antitumor benefits in the clinical trials suggests that efforts to optimize the use of NAMPT inhibitors in the clinics are necessary. To achieve this, it is important to have a better understanding of their precise mechanism of action, as well as any possible mechanism of resistance.

Table 1 NAMPT inhibitors in clinical studies

Phase	Drug	Type	Condition	Treatment	ClinicalTrials.gov Identifier	Name of the Study	Results
II	FK866	NAMPT inhibitor	Melanoma	Single agent	NCT00432107	Study to Assess APO866 for the Treatment of Advanced Melanoma	Completed
II	FK866	NAMPT inhibitor	Cutaneous T-cell Lymphoma	Single agent	NCT00431912	Study of APO866 for the Treatment of Cutaneous T-cell Lymphoma	Completed
I/II	FK866	NAMPT inhibitor	B-cell Chronic Lymphocytic Leukemia	Single agent	NCT00435084	Study to Assess the Safety and Tolerability of APO866 for the Treatment of Refractory B-CLL	Completed
I	**GMX1778 (CHS-828)**	Oral NAMPT inhibitor	Solid tumors	Single agent	NCT00003979	CHS 828 in Treating Patients with Solid Tumors	Withdrawn
I	**OT-82**	Oral NAMPT inhibitor	Relapsed or refractory lymphoma	Dose escalation and expansion	NCT03921879	Safety and Efficacy of OT-82 in Participants with Relapsed or Refractory Lymphoma	Unknown (No results posted)
I	**KPT-9274**	oral dual inhibitor of PAK4 and NAMPT	Solid tumors, non-Hodgkin's lymphoma	Single agent or co-administered with Niacin or Nivolumab	NCT02702492	PAK4 and NAMPT in Patients with Solid Malignancies or Non-Hodgkin's Lymphoma	Terminated
I	**KPT-9274**	oral dual inhibitor of PAK4 and NAMPT	Solid tumors, non-Hodgkin's lymphoma	Single agent or co-administered with Niacin	NCT04281420	Study of Evaluating Dual Inhibitor of PAK4 and NAMPT ATG-019 in Advanced Solid Tumors or Non-Hodgkin's Lymphoma	Recruiting
I	**KPT-9274**	oral dual inhibitor of PAK4 and NAMPT	Acute Myeloid Leukemia	Single agent	NCT04914845	KPT-9274 in Patients with Relapsed and Refractory Acute Myeloid Leukemia	Recruiting

3.3 Cellular Pathways Related to the Anticancer Effects of NAMPT Inhibitors

3.3.1 NAMPT inhibition affects cellular metabolism and signaling

Disturbance of cellular NAD homeostasis by NAMPT inhibitors has shown anti-proliferative and cytotoxic effects in cancers. To date, several studies contributed to dissecting the mechanisms through which NAD depletion induces these effects. They have demonstrated that several metabolic and signaling pathways are affected by NAMPT inhibition and lead to cell death induction.

In the following paragraphs, some major metabolic and signaling pathways associated with the antitumor effects of NAMPT inhibitors are described, with an emphasis on emerging strategies to potentiate NAMPT inhibitors.

3.3.2 Apoptosis

NAMPT inhibitors were shown to trigger a rapid NAD depletion, which induces cell death through apoptosis. Apoptosis is a type of programmed cell death that is energy-dependent and characterized by DNA fragmentation and morphological changes. It prevents damaged cells from consuming essential nutrients and transmitting toxic molecules to surrounding cells. It is therefore an important process in the control of tissue homeostasis. Apoptosis is triggered by external and intracellular stimuli and involves different signaling cascades, through two major pathways. In the intrinsic pathway, cellular stress signals such as DNA damage or hypoxia activate pro-apoptotic Bcl-2 family proteins, which induce mitochondrial membrane permeabilization. Consequently,

mitochondrial apoptotic factors such as cytochrome c and apoptosis-inducing factor (AIF) are released in the cytoplasm and trigger the activation cascade of caspases. Caspases are the main effectors of apoptosis that cleave numerous substrates in the cells, eventually causing cell shrinkage and cell death. In the extrinsic pathway, binding of ligands to death receptors triggers the caspase activation cascade which also results in cell death execution.

In several reports, APO866 was demonstrated to induce caspase activation and apoptotic cell death [52] in cell lines of solid tumors [65] and hematological malignancies [53], [61], as well as in primary cells [66]. Consistently, the use of apoptosis activator tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in combination with APO866 in human leukemia has shown a synergistic antitumor effect [67]. In the same line, Bruton's tyrosine kinase (BTK) inhibitors, other apoptosis inducing agents, efficiently lead to a synergistic antitumor effect on Waldenstrom Macroglobulinemia Cells [68].

3.3.3 Autophagy

Autophagy is referred to as type II programmed cell death. It is a self-degrative catabolic process that eliminates the damaged organelles or proteins and allows the recycling of cellular components and the maintenance of cellular homeostasis. A major regulator of autophagy is the mTOR signaling pathway.

Cell death induction by NAMPT inhibitors has been reported to involve an activation of autophagy. Indeed, autophagy activation was observed in multiple myeloma [69], [70], in neuroblastoma [71], and in hematological cancers [61].

Moreover, a crosstalk between autophagy and apoptosis has also been reported. Indeed, both apoptosis and autophagy were observed and shown to be required in the cell death induction in hematological cancer cell lines [72], as well as in primary cells of adult T-cell leukemia/lymphoma (ATL) [73].

3.3.4 DNA damage and oxidative stress

Any form of programmed cell death is triggered upon increase of cellular stresses. Both DNA damage stress and oxidative stress were shown to mediate APO866-induced cell death.

Oxidative stress is caused by an imbalance between ROS generation and antioxidant system capacity. Elevation of ROS levels is a critical step in the anticancer effect of NAMPT inhibitors, as demonstrated by an increase in cytosolic and mitochondrial ROS upon treatment [65], [74], [75]. Notably, NAMPT inhibition was shown to impair the expression of FOXO3a transcription factor, which is an activator of oxidative stress response [76]. Excessive ROS induced by NAMPT inhibitors was associated with impairment of mitochondrial membrane potential, causing OXPHOS dysfunction and subsequent depletion of ATP [74]. The use of ROS scavengers such as catalase or tocopherol could

reverse these effects and prevent cell death [74], [77], indicating a crucial role of oxidative stress in NAMPT inhibitors-induced cell death. In the same line, NAMPT inhibitor sensitized cancer cells to hydrogen peroxide H_2O_2 [78] or β-lapachone, a ROS generating agent [79], [80].

NAD depletion was also shown to induce DNA damage, mainly due to the increase of DNA-damaging ROS, and downregulation of PARP activities. The immediate response to DNA damage is the activation of DNA repair process and cell cycle arret, however if stresses persist, apoptotic cell death is triggered. DNA damage seems to contribute to NAMPT inhibitor cytotoxicity, as suggested by studies showing that DNA damage agents such as 5-fluorouracil [81], cisplatin [82] or cytarabine [66] increased the sensitivity of cancer cells to NAMPT inhibition. Moreover, inhibition of DNA damage repair proteins HDAC potentiated NAMPT inhibition in AML [83].

3.3.5 Energy metabolism

In line with the central role of NAD in cellular energetics, NAMPT inhibition was shown to impair several metabolic pathways. Tan *et al.* showed that NAMPT inhibition with FK866 attenuated glycolysis at glyceraldehyde-3-phosphate dehydrogenase step, which consequently reduced the pentose phosphate pathway, serine biosynthesis and TCA cycle activities [84]. The deregulation of metabolic pathways directly leads to cellular ATP depletion. Accumulation of upstream glycolytic metabolites also induced the decrease of metabolites associated with nucleotide, lipid and amino acid synthesis in NSCLC [85]. Thus, NAMPT inhibition causes global metabolic perturbations resulting ultimately to cell death. Recently, APO866 was shown to increase pancreatic cancer cells sensitivity to metformin, a mitochondrial ETC inhibitor [86], supporting the importance of the crosstalk between NAD level and metabolism in the anticancer effect of NAMPT inhibition.

3.3.6 Cellular stress signal transduction pathways

Multiple cellular signaling pathways were shown to mediate the activation of cell death upon NAMPT inhibition.

In several cancer models, NAMPT inhibition was observed to inhibit mTOR activation, which induce autophagic cell death [87]. In hepatocarcinoma cells, NAMPT inhibition by FK866 activated AMP-activated protein kinase (AMPK) and therefore blocked mTOR activation and its downstream targets p70S6 kinase and 4EBP1 [88]. Similarly, APO866 induced AMPK activation and eIF2a phosphorylation and activation in leukemia cells, leading to a translational arrest [89]. In multiple myeloma cells APO866 activated mTOR via the inhibition of Erk1/2, leading to an autophagic cell death [90]. In these studies, the use of mTOR inhibitors as co-treatment enhanced the antitumor effects of NAMPT inhibition.

In the same line, MAPK pathways are involved in the cytotoxicity of NAMPT inhibitors. As mentioned above, NAMPT inhibition was observed to inactivate Erk1/2 in multiple myeloma [90],

and NSCLC together with Akt, and MEK1/2 attenuation [91]. On the other hand, JNK pathway was shown to be activated by NAMPT inhibition [77].

Overall, NAMPT inhibition causes a dramatic change in a wide range of cellular signaling and processes. Targeting these pathways are promising strategies to potentiate the therapeutic effect of NAMPT inhibitors. Further investigation of the precise mechanism of action will be beneficial for optimizing the use of NAMPT inhibitors for cancer treatment.

4 RESISTANCE

4.1 COMMON MECHANISMS OF DRUG RESISTANCE IN CANCERS

Resistance to therapies remains the major cause of relapse and death in hematological malignancies. It is the main obstacle for achieving cures in patients.

Two broad categories of resistance are distinguished, the primary (or innate) resistance and the acquired resistance. The primary resistance corresponds to the pre-existence in the organism, before the treatment, of innate factors that render the therapy ineffective. On the other hand, acquired resistance is developed during the treatment in tumors that were initially sensitive. Various mechanisms of acquired resistance have been reported in the literature [92]. One of the common mechanisms is the modification of the drug target. Mutagenesis in the drug target gene or downregulation of drug target expression levels are frequently observed and minimize the deleterious effect of the drug-target interaction. Increase of drug efflux, reduction of drug uptake, or drug sequestration are also common mechanisms that reduce intracellular drug presence. They often lead to the development of multidrug resistance. Besides, modification of the drug metabolism (inactivation or ineffective activation) can reduce drug activity. Other pro-survival adaptive changes include enhancement of DNA repair, inhibition of apoptosis, and epithelial-mesenchymal transition (EMT). Importantly, a mechanism of resistance that is frequently observed in targeted therapies is the activation of alternate compensatory pathways with redundant or similar regulatory functions, that allows bypassing the inhibited pathway and results in the maintenance of cellular processes.

Understanding how resistance acquisition occurs is important to enable the prediction and the prevention of resistance development, as well as the reversal of resistant phenotype. Increased knowledge on molecular resistance mechanisms helped improving the efficacy of cancer therapies, for instance by combinatory or dual targeting approaches. However, due to high cancer heterogeneity, resistance mechanism can be drug-, cancer type-, and even patient-dependent. Drug resistance is still a major problem in cancer treatment.

4.2 PREVIOUSLY DESCRIBED MECHANISMS OF RESISTANCE TOWARDS NAMPT INHIBITORS

In our preclinical mouse models, we observe that although APO866 effectively blocks tumor progression, tumor relapses can occur at long-term observations (data not shown). And these relapsed tumors are likely to be less sensitive to treatments. These observations suggest the possibility that cancer cells are able to gain resistance against NAMPT inhibitors. Several studies have reported the acquisition of resistance to NAMPT inhibitors in *in vitro* cancer models and described the mechanism of resistance (**Table 2**).

Table 2 Mechanisms of resistance to NAMPT inhibitors reported in in vitro studies

	Cancer types	NAMPT inhibitor	*model type*
• Point mutations in NAMPT gene			
Watson *et al.* (2009)	HCT-116 colon cancer cells	CHS-828	*in vitro*
Olesen *et al.* (2010)	OC-NYH NSC lung cancer cells	CHS-828	*in vitro*
Wang *et al.* (2014)	RD rhabdomyosarcoma cells MiaPaCa-2 pancreatic cancer cells NCI-H460 NSC lung cancer cells	GNE-618	*in vitro*
• Multi-drug resistance (Implication of ABC transporter gene)			
Ogino *et al.* (2018)	HCT-116 colon cancer cells	FK866	*in vitro*
• Up-regulation of *de novo* pathway			
Guo *et al.* (2017)	HT1080 Fibrosarcoma cells	GMX1778	*in vitro*
Thongon *et al.* (2018)	CCRF-CEM T cell ALL cells	FK866	*in vitro*
• Metabolic adaptations			
Thongon *et al.* (2018)	MDA MB231 breast cancer cells	FK866	*in vitro*

Most commonly, genetic mutations have been reported to be the major cause of resistance acquisition in models of colorectal cancer, non-small cell lung cancer rhabdomyosarcoma and pancreatic cancer [63], [93], [94]. In these models, point mutations in NAMPT gene modified the binding site of the protein with NAMPT inhibitor CHS-828 and reduced the affinity of the inhibitor to the enzyme.

In another model of colon cancer, resistance to NAMPT inhibitor FK866 was shown to come from the development of multi-drug resistance (MDR), with an upregulation of ABC transporters, which allow drug excretion to the extracellular space to reduce its toxicity [95].

Interestingly, non-mutational resistance mechanisms were also described in the studies by Guo *et al.* (2017) [96] and Thongon *et al.* (2018) [97], in which fibrosarcoma cells and T-ALL cells showed an upregulation of the *de novo* pathway as an alternative route for NAD biosynthesis to bypass the blockade of NAMPT-dependent pathway. Some metabolic adaptations implicating an upregulation of lactate dehydrogenase A (LDHA) and of glycolysis were also described by Thongon *et al* [97].

However, the development of resistance in *in vivo* settings and in relapsing tumors have not been described yet.

5 AIMS OF THE STUDY

NAMPT inhibitors have proven robust anticancer killing effect in different cancer types *in vitro* and in animal models. However, the therapeutic effect of NAMPT inhibitors is still limited in patients. Identification of the precise molecular mechanism of action of NAMPT inhibitors as well as the factors positively or negatively impacting tumor response to the inhibitors is needed for developing strategies to optimize the efficacy of NAMPT inhibitors.

The main focus of the research in our group is the characterization of the anticancer effect of NAMPT inhibitors in hematological cancer models and the investigation of strategies to potentiate their therapeutic efficacy. The aim of this thesis is to identify possible mechanisms by which hematological cancers can become insensitive to APO866 treatment, in leukemia and lymphoma models.

In the first project, I aimed to characterize possible mechanisms of resistance toward NAMPT inhibitors (APO866). To this end, we generated a model of resistance acquired in *in vivo* settings, using human AML cell line ML2 cells xenografts in mice, in order to reproduce the situation of relapsing leukemia in patients. I investigated the whole transcriptomes of resistant cancer cells by RNA sequencing to identify the alterations of gene expression associated with resistance to APO866, and further characterized their phenotypes by functional analyses.

In the second project, I aimed to investigate the factors that can affect the efficacy of APO866 *in vivo*. I focused on the fact that bacterial cells possess NAD biosynthetic enzymes that are absent in mammalian cells and hypothesized that gut microbiota may contribute to modulating the levels of NAD-related metabolites in tumors. I investigated whether these supplies of NAD-related metabolites from the gut microbiota may affect the efficacy of NAMPT inhibitors against leukemia using xenograft mouse model of human AML.

In the third project, I aimed to broaden our understanding of the global picture of NAD-related metabolome. I aimed to evaluate the role of nicotinaldehyde in mammalian NAD metabolome and its possible implication in the cancer response to NAMPT inhibitors.

CHAPTER 2: RESULTS

PROJECT 1

The following chapter presents the project results in a publication-ready format.

Summary of the results

Drug resistance is an important issue in achieving effective anticancer treatments. To date, the mechanisms of resistance to NAMPT inhibitors have not been extensively explored. Besides, only *in vitro* models of resistance have been described so far, whilst *in vivo* settings may generate distinct resistance mechanisms given the possible interactions with the tumor environment. Identifying the possible mechanisms on which cancer cells may rely to escape the antitumor effect of APO866 is crucial for designing rational strategies to optimize its antitumor therapeutic efficacy.

In this project, we aimed to decipher the molecular mechanisms that contribute to the development of resistance to APO866. We generated *in vivo*-acquired APO866-resistant human acute myeloid leukemia ML2 cells in xenograft mouse models. We analyzed the whole transcriptomes of the resistant ML2 by RNA sequencing in comparison with sensitive ML2, as well as in drug-treated or untreated conditions. Transcriptomic analyses revealed profound and constitutive modifications in the transcriptomic programs of resistant ML2 compared to the parental ML2. Notably, we observed upregulations of interleukin-mediated signaling and several anti-apoptotic signaling pathways. We demonstrated that activated PI3K/Akt and ERK pathways, increased activity of transcription factor NFκB and anti-apoptotic protein Mcl-1 contributed to APO866 resistance. Accordingly, specific pharmacological inhibition of these factors sensitized resistant cells to APO866. Of note, downregulation of translational and proteasomal complexes were shown to play a key role in the resistance phenotype. Besides, a shift to glycolysis rather than mitochondrial respiration was shown to support energy production under NAD depletion-related stresses. Overall, we identified a mechanism of acquired resistance to NAMPT inhibitor APO866 in AML, which relied on the activation of pro-survival cellular signaling and metabolic adaptations. These findings suggest that combinatory approaches are promising for enhancing the therapeutic efficacy of APO866.

Personal Contributions

I designed the study, I analysed the whole transcriptomic data, I designed and performed the *in vitro and in vivo* experiments. I wrote the manuscript under the supervision of Dr. Aimable Nahinama, PD and Prof. Michel Duchosal.

In vivo-acquired resistance to NAMPT inhibitor APO866 in AML is conferred by transcriptomic and metabolic alterations

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Abstract

Inhibitors of nicotinamide phosphoribosyltransferase (NAMPT) are promising anticancer compounds that display selective cytotoxic effects by depleting cellular nicotinamide adenine dinucleotide (NAD) in tumors.

In this study, we aimed to characterize the mechanism of resistance to NAMPT inhibitor APO866 in acute myeloid leukemia (AML). We developed APO866-resistant ML2 models *in vivo*, using mouse xenografts. To identify the gene expression alterations associated with APO866 resistance, we analyzed the whole transcriptomes of resistant ML2 by RNA sequencing in comparison with parental APO866-sensitive ML2, as well as in drug-treated or untreated conditions. These analyses revealed profound and constitutive modifications of transcriptomic programs in the resistant ML2 compared to parental ML2. Notably, we identified upregulations of interleukin-mediated signaling and several anti-apoptotic signaling pathways. We found that activated PI3K/Akt and ERK pathways, increased activity of NFκB transcription factor and anti-apoptotic protein Mcl-1 contributed to APO866 resistance. In addition, downregulation of translational and proteasomal complexes were shown to have a key role in the resistance phenotype. Besides, a shift to glycolysis rather than mitochondrial respiration was shown to support energy production under stresses related to NAD depletion.

Overall, we identified a mechanism of acquired resistance to NAMPT inhibitor APO866 in AML, which relied on the activation of pro-survival cellular signaling and metabolic adaptations. These findings allow to set up novel combinatory approaches to enhance the therapeutic efficacy of APO866.

Key words*: NAD, NAMPT inhibitor, AML, drug resistance, whole transcriptomic analysis.*

1 INTRODUCTION

Resistance to therapeutic agents is a major issue facing current cancer research and treatment. Drug resistance is classified into two broad categories, the intrinsic (or primary) resistance or acquired (or secondary) resistance. Intrinsic resistance corresponds to the pre-existence of resistance mechanisms prior to the treatment. Acquired resistance occurs when resistance mechanisms are developed in the tumors during the treatment. Resistance acquisition is frequently associated with tumor relapse and metastasis. The molecular mechanisms responsible for drug resistance are multiple. Some common mechanisms include alteration of drug targets, expression or activation of drug efflux pumps, reduced susceptibility to apoptosis, and activation of compensation mechanisms [1]. In addition, increasing evidence demonstrate that not only tumor-intrinsic factors but also the tumor microenvironment contributes greatly to resistance development [2].

Acute myeloid leukemia (AML) is a heterogenous group of malignancies characterized by uncontrolled proliferation of immature myeloid cells in the bone marrow and peripheral blood. Despite the standard induction therapy displays a high rate of complete remission, overall survival is poor mainly due to relapsed and drug-tolerant leukemic cells [3]. Innovative new targeted therapies such as venetoclax have been approved or are currently under clinical development [4]. However, they do not eradicate therapeutic resistance in these patients. Indeed, recent studies have shown the development of acquired resistance to these molecularly targeted drugs in AML [5]. Elucidating the molecular basis of drug resistance and understanding how resistance can be prevented, targeted and treated becomes increasingly important to improve cancer therapies.

Nicotinamide phosphoribosyltransferase (NAMPT) is a key enzyme for nicotinamide adenine dinucleotide (NAD) biosynthesis, which plays an essential role in cancer biology, through its NAD biosynthetic function but also through other well-established functions [6]. NAMPT overexpression is observed in many cancer types and high expression is associated with tumorigenesis, tumor progression, and poor prognosis [7], [8]. Therefore, NAMPT has emerged as potential anticancer therapeutic target and several specific inhibitors have been developed so far [9]. These include APO866 (known also as FK866), CHS-828, OT-82 and others. NAMPT inhibitors have proven anticancer toxicity in *in vitro* and preclinical models, of multiple cancer types including solid tumors and hematological malignancies [10]. However, in clinical trials, although good tolerability was observed no objective tumor response could be obtained [9]. Several strategies to increase their efficacy are currently investigated, including antibody-conjugated delivery or combinatory approaches with other

anticancer agents [11]. To achieve the development of more effective therapies, a better understanding of the mechanism of the anticancer cytotoxic effect of NAMPT inhibition is crucial.

The anticancer activity of NAMPT inhibitors is primary due to the induction of NAD depletion. In mammalian cells, NAD is synthesized through several pathways from different precursors [12]. NAMPT mediates the major NAD biosynthesis pathway called the salvage pathway, in which it catalyzes the reaction of nicotinamide (Nam) to NMN. NMN can also be produced from nicotinamide riboside (NR) through the activity of NR kinase (NRK). NMN is then transformed to NAD by NMN adenylyl-transferase (NMNAT). An alternative route is the *Preiss-Handler* pathway, in which nicotinic acid (NA) and nicotinic acid riboside (NaR) are converted to NA mononucleotide (NaMN) and further converted to NA adenine dinucleotide (NaAD) by NMNAT. NAD is then produced from NaAD by the activity of NAD synthase (NADS). Finally, the *de novo* pathway synthesizes NAD from dietary tryptophan through multiple reaction steps, including notably quinolinic acid phosphoribosyl-transferase (QAPRT) activity. Among the different pathways, the salvage pathway mediated by NAMPT is thought to be of major importance in cancer cells [12]. Indeed, NAMPT mediates the recycling of the end-product of NAD degradation by NAD-consuming enzymes such as sirtuins or poly-(ADP-ribose) polymerases (PARPs) [13]. As cancer cells rely on rapid NAD turnover to sustain their altered metabolism and rapid proliferation, they are highly dependent on the activity of NAMPT. In different cancer models, NAMPT inhibitors such as APO866 were shown to induce rapid depletion of intracellular NAD pool, which subsequently causes cellular stresses, ATP depletion and cell death [10]. Both apoptotic and autophagic processes are involved in the cell death induction by NAMPT inhibitors [14], [15]. Mechanistically, the oxidative stress as demonstrated by an increase of reactive oxygen species (ROS) levels, mitochondrial dysfunction, ATP depletion and activation of several cellular signaling pathways are involved, including mechanistic target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and mitogen-activated protein kinases (MAPK) pathways [16]–[21].

To date, numerous studies contributed to describing the mechanism of action of NAMPT inhibitors in cancer cells. On the other hand, several studies have reported and described the mechanisms of resistance acquisition in *in vitro* models [22]–[28]. Genetic mutations are the most observed mechanism of resistance against NAMPT inhibitors and were shown to modify the structure of the binding site of the enzyme, reducing its affinity with NAMPT inhibitor CHS-828, in colon cancer, lung cancer, pancreatic cancer and rhabdomyosarcoma [22]–[24]. In a model of colon cancer, an upregulation of ABC transporters expression conferred unspecific resistance to APO866 with the development of multi-drug resistance (MDR) [27]. Mechanisms that do not rely on genomic alteration were described in models of GMX1778-

resistant fibrosarcoma and FK866-resistant T-ALL cells [25], [28]. In these cells, the *de novo* pathway was upregulated as an alternative route for NAD biosynthesis. Furthermore, metabolic adaptations including an upregulation of lactate dehydrogenase A (LDHA) and of glycolysis were observed in FK866-resistant breast cancer cells [28]. These latter mechanisms provide interesting hints for conceiving strategies to reverse resistance to NAMPT inhibitors by specifically targeting the developed compensation pathways as new vulnerabilities of resistant cells. However, the development of resistance in *in vivo* models and in relapsing tumors have not yet been described.

In this study, we investigated the molecular mechanisms of acquired resistance to NAMPT inhibitors in AML. With this aim, we developed *in vivo*-acquired APO866-resistant human AML ML2 models using mouse xenografts. We analyzed the whole transcriptomes of the resistant cells in comparison with the parental APO866-sensitive cells, as well as in drug-treated compared to untreated conditions. We found a profound and constitutive modification of the transcriptomic programs in the resistant ML2, characterized by an activation of several pro-survival signaling pathways including PI3K/Akt, ERK and NFκB pathways. Drug treatment further activated these cellular signaling pathways in resistant ML2 and induced an attenuation of translational and proteasomal complexes. These alterations collectively conferred resistance to APO866-induced apoptosis, as this was reversed by using specific chemical inhibitors. Besides, a metabolic adaptation was shown to support energy production under stresses related to NAD decrease. Overall, we identified a mechanism of acquired resistance to NAMPT inhibitor APO866 in AML, which relied on a broad reorganization of cellular signaling pathways, protein homeostasis and metabolism. These findings provide a strong rationale for combinatory approaches to enhance the therapeutic efficacy of APO866.

2 MATERIALS AND METHODS

2.1 Cell lines and culture conditions

Acute myeloid leukemia cell line ML2 cells were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). Cells were cultured in RPMI medium (Invotrogen AG, 61870-01) supplemented with 1 % penicillin/streptomycin (PS, Amimed, 4-01F00-H) and 10 % heat inactivated fetal calf serum (FCS, Amimed, 2-01F30-I) at 37 °C in a humidified atmosphere.

2.2 Chemicals

NAMPT inhibitor APO866 was provided by TopoTarget (Switzerland) and CHS-828 was purchased from Cayman chemical (200484-11-3). Ruxolitinib (HY-50856), copanlisib (HY-15346), ZSTK474 (HY-50847), DHMEQ (HY-14645), S63845 (HY-100741), ravoxertinib (HY-15947), venetoclax (HY-15531), verapamil (HY-A0064) were purchased from MedChemExpress. Cycloheximide (#2112) and bortezomib (#2204) were purchased from Cell Signaling Technology.

2.3 Generation of APO866-resistant ML2 in mouse xenograft models

Severe combined immune deficiency (SCID) mice (Iffa Credo, L'Arbresle, France) were housed in micro-isolator cages in a specific pathogen-free room within the animal facilities of the University Hospital of Lausanne. Mice were allowed to acclimatize to their new environment for 1 week prior to use. Animal experiments were conducted according to the institutional regulations after approval of the animal ethics committee of the University of Lausanne. ML2 cells were expanded, washed with PBS and suspended in warm PBS. 10 x 10^6 ML2 cells were injected subcutaneously in the right flank of seven mice. Once tumor reached a volume of 100 mm^3, mice were treated with APO866 (15 mg/kg body weight) intraperitoneal injection in 200 µL 0.9% saline, twice a day for 4 days per week, until tumor volume is reduced to undetectable level. Mice were monitored for tumor re-apparition for several weeks without treatment and when tumors relapsed and reached again 100 mm^3, mice were treated with APO866 in the same regimen. These treatment/observation cycles were repeated accordingly to tumor growth, and when tumors ultimately became tolerant to the drug and reached 1000 mm^3, they were extracted from mice and suspended *in vitro*. All animals were monitored daily for signs of illness and terminated immediately if tumor size reached a volume of 1000 mm^3.

2.4 Flow cytometry for evaluation of cell death, ROS, mitochondrial membrane, cell surface and intracellular antigen expression and caspase activity

To evaluate the effect of different drugs on cell death, cells were incubated in presence of the drugs or DMSO as control for required time (96 h if not specified otherwise). They were harvested and stained with annexin V (eBioscience BMS306FI/300) and 7-aminoactinomycin D (7AAD, Immunotech, A07704) as described by the manufacturer and analyzed by flow cytometry. Early apoptotic cells were identified as annexin$^+$ 7AAD$^-$ and dead cells (late apoptotic or necrotic) cells as 7AAD$^+$. Specific cell death induced by different treatments was defined as =[(D–CT)/(100–CT)]*100 where D corresponds to the percentage of cell death in drug-treated sample and CT to percentage of cell death in control DMSO-treated sample. Live

cell number was counted with CountBright™ Plus Absolute Counting Beads (Invitrogen, C36995).

Mitochondrial membrane potential was evaluated with Tetramethylrhodamine methyl ester dye (TMRM, Invitrogen, T668), by incubating washed cell suspension in PBS containing 100 nM TMRM for 30 min at 37 °C and analyzing the fluorescence by flow cytometry. Intracellular levels of ROS, namely mitochondrial and cytosolic superoxide anion radicals $O_{2^{\cdot}}^{-}$ and total H_2O_2, were assessed using specific fluorescent probes, Mitosox (Molecular Probes, M36008), DHE (Marker Gene Technologies, MGT-M1241-M010) and carboxy-H2DCFDA (Molecular Probes, C-400) respectively. Briefly, harvested cells were washed with PBS (or DPBS for carboxy-H2DCFDA) and resuspended in PBS containing 2.5 µM of Mitosox, 10 µM of DHE or 20 µM of H2DCFDA, for 30 min at 37 °C and the fluorescence level was assessed by flow cytometry.

CD markers expressions were assessed with CD86-(MHCD8601, LifeTechnologies Europe BV), CD89-(SC-19680, Santa Cruz Biotechnology), CD23-PE (IM3609U, Beckman Coulter) antibodies. IgG1-PE antibody (A07796, Beckman Coulter) was used as control. Phospho-Erk1/2 and TNFα levels were assessed by treating the cells with anti-p-erk1/2 (12-9109-42, Life Technologies) and anti-TNFα (MA5-44111, Life Technologies) PE-conjugated antibodies after cell permeabilization with Foxp3 fixation/permeabilization kit (00-5521-00, Thermofisher).

Caspase 3 activation was assessed using Caspases 3/7 Glo assay kit (Promega) following manufacturer's instructions.

2.5 Determination of intracellular total NAD, NAD(H) and NADP(H) and ATP content by biochemical assay

Total amount of intracellular NAD(H) was quantified using the NAD cycling assay (as described previously [29]). NAD-related metabolites were quantified by LC-MS/MS as described previously [30]. NAD^+ and NADH contents were measured with NAD^+/NADH assay (AB65348, Abcam) following manufacturer's instructions. NADP+ and NADPH contents were measured with NADP/NADPH-Glo™ assay (G9081, Promega) according to manufacturer's instructions. Total ATP was measured using the ATP determination Kit (Life Technologies, A22066) according to manufacturer's instructions.

2.6 Transcriptomic analysis by RNA sequencing

For whole transcriptomic analysis, cells were treated with APO866 (10 nM) for 96 h and harvested at 40h and at 96h of incubation. After a PBS wash and centrifugation, the cell pellets were snap frozen and stored at -80 °C. The frozen pellets were shipped on dry ice to Genewiz

(Germany) for RNA extraction, library preparation, sequencing reactions and initial bioinformatics analyses. Briefly, upon RNA extraction, initial PolyA selection-based mRNA enrichment, RNA samples were quantified using a Qubit 4.0 Fluorometer (ThermoFisher Scientific) and RNA integrity was checked with RNA Kit on an Agilent 5300 Fragment Analyzer (Agilent Technologies, Palo Alto, CA, USA). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were first enriched with Oligo(dT) beads and fragmented for 15 minutes at 94 °C. First strand and second strand cDNAs were subsequently synthesized. cDNA fragments were end-repaired and adenylated at 3'ends. Universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by limited-cycle PCR. The sequencing libraries were validated using Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA) and quantified with the Qubit 4.0 Fluorometer (ThermoFisher Scientific). The sequencing libraries were multiplexed and loaded on the flow cell of the Illumina HiSeq 6000 instrument according to the manufacturer's instructions. The samples were sequenced using a 2×150 Pair-End (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software. Raw sequence data (.bcl files) generated from Illumina HiSeq were converted into fastq files and de-multiplexed using Illumina bcl2fastq program version 2.20. One mismatch was allowed for index sequence identification. Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Homo sapiens GRCh38 reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. Unique gene hit counts were calculated with the feature counts from the Subread package v.1.5.2. DESeq was used for comparison of gene expression levels between the different sample groups. P values and log2 fold changes (FC) were calculated using the Wald test. Genes with an adjusted P value (Padj) of <0.05 were referred to as DEGs.

Pathway enrichment analysis was performed by analysis of the respective DEGs with the Reactome pathway Knowledgebase [31]. GeneSCF v.1.1-p2 software was used for gene ontology analysis. The goa_human GO list was used to cluster the set of genes based on their biological processes and determine their statistical significance (adjusted p-value <0.05). Gene set enrichment analysis (GSEA) was performed using GSEA software v.4 (Broad Institute, Cambridge) [32], [33] and visualized with Cytoscape software [34].

2.7 Immunoblotting

Proteins samples were separated by SDS-PAGE on a 10 % polyacrylamide gel on blotted on PVDF membrane. Rabbit anti-actin (#4967) antibody was purchased from Cell Signaling and used as a protein loading control. After incubation with primary antibodies, polyclonal goat anti-mouse IgG conjugated with IRDye 680 (LI-COR, B70920-02) or goat anti-rabbit IgG

conjugated with IRDye 800 (LI-COR, 926-32210) were applied. Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR).

2.8 Metabolic profiling by Seahorse assay

Metabolic profiling of leukemia cells was performed using a seahorse XF analyzer (Agilent). Mitochondrial respiration was assessed using a Seahorse XF Cell Mito Stress test Kit following manufacturer's instructions. Cell-Tak (CLS354240, Corning) was used to prepare adherent monolayer cultures of non-adherent ML2 cells.

2.9 Statistical analyses

Unless specified otherwise, data are expressed as mean ± SD of at least three independent replicates. Statistical analyses were performed using GraphPad Prism 8.0 Software (GraphPad Software, US). Unpaired or paired t tests were applied for evaluating differences between groups. Statistical significance was established for p value<0.05.

3 RESULTS

3.1 APO866 Resistant ML2 models are generated *in vivo* in mouse xenografts

First, APO866-resistant ML2 cells were generated in mouse xenograft models. Human AML cell line ML2 cells were injected subcutaneously in seven SCID mice. Tumors that grew and reached a volume over 100 mm^3 were treated with intraperitoneal injections of APO866 (15 mg/kg). While APO866 first efficiently eradicated the tumors, after approximately eight weeks of monitoring without drug treatment tumors re-appeared (**Figure 1A**). Relapsed tumors were treated again with APO866 when they were over 100 mm^3 of volume and treatment was interrupted when tumor volumes decreased below 100 mm^3 or were undetectable. These cycles were repeated until the tumors became drug-tolerant and grew regardless of drug treatment. Tumors that reached 1000 mm^3 were extracted and the derived cells were suspended *in vitro*. Eventually, we harvested ML2 cell populations from two tumor xenografts that were called resistant ML2 A (ML2 RA) and B (ML2 RB) and were cultured in classical RPMI culture medium. A sub-population of ML2 RB cells was cultured continuously in RPMI medium containing 10 nM of APO866 and called resistant ML2 C (ML2 RC).

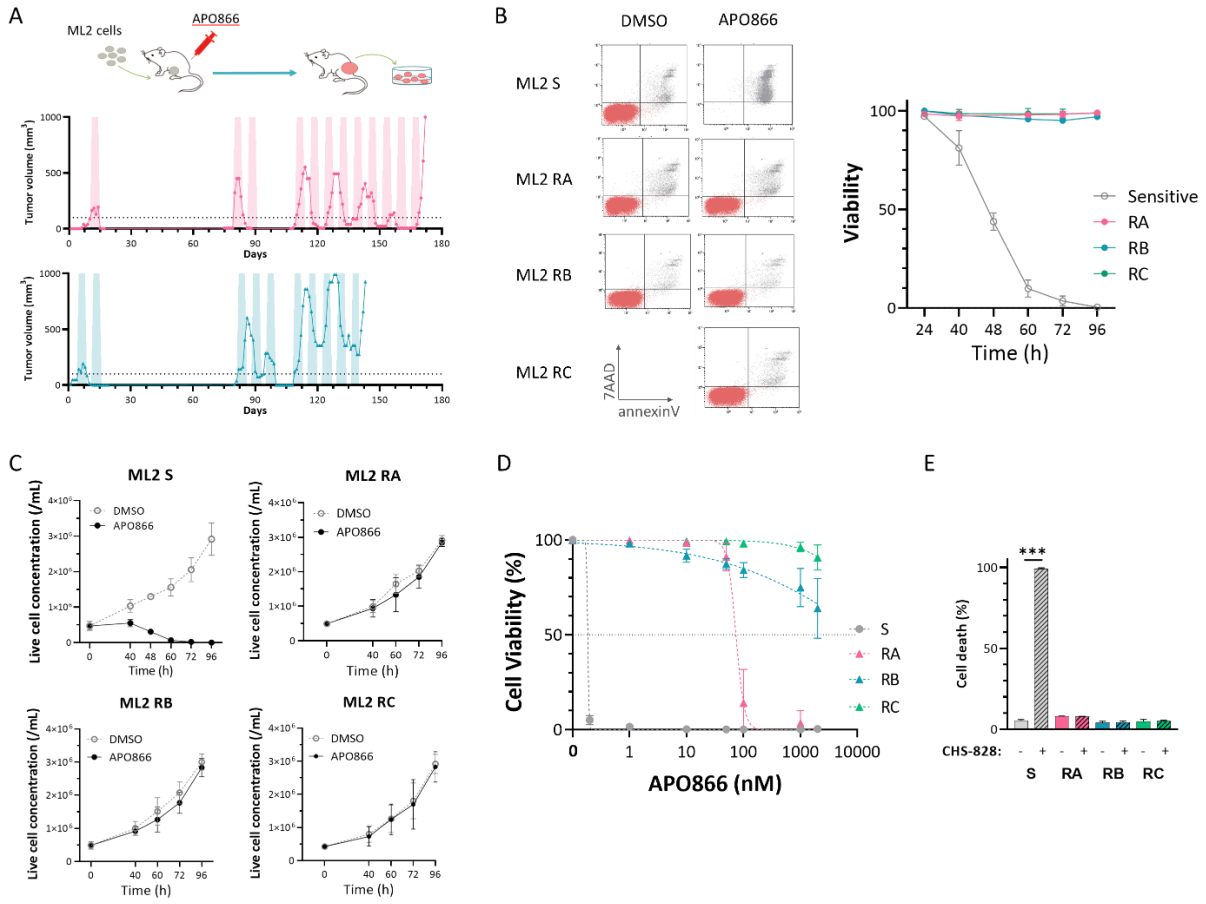


Figure 1: Generation of resistant ML2 in mouse xenograft models. A) Evolution of tumor volumes in two ML2 xenografts. Filled areas indicate the time periods where tumors were under APO866 treatment. **B)** Cell death of parental ML2 (ML2 S) and of APO866-tolerant ML2 incubated in presence of 10 nM APO866 or DMSO (negative control) for 96h. Left panel: representative flow cytometry analyses of ML2 cells stained with annexin V (x axis) and 7AAD (y axis) dyes. Right panel: Time-dependent cell viability in presence of APO866 (10nM). **C)** Evolution of cell number of parental and resistant ML2 clones in presence of APO866 (10nM) or DMSO. **D)** APO866 dose-dependent cell viability after 96 h of incubation. **E)** Cell death upon treatment with NAMPT inhibitor CHS-828 (1 nM)(+) or DMSO (-). *n>3, error bars=SD. ***P<0.001*.

First, we assessed the sensitivity of the harvested ML2 cells toward APO866 *in vitro* by incubating them in presence of APO866 (10nM) and evaluating their viability by flow cytometry. While APO866 killed 100 % of parental ML2 (ML2 S) at 96 h, ML2 RA, RB and RC survived to APO866 treatment showing only 7.0 %, 1.7 % and 3.8 % respectively of APO866-induced cell death at 96 h (**Figure 1B**). Resistant ML2 were able to proliferate in a comparable manner as the parental ML2 in absence of the drug, as assessed by the increase of live cell number (**Figure 1C**). In addition, the proliferation was unaffected by drug presence (APO866 10nM). We evaluated the IC_{50} (dose that kills 50 % of cells after 96 h treatment) of APO866 in resistant ML2 with an APO866 dose-dependent assay. We found IC_{50} of 78 nM for ML2 RA, and over 1000 nM (IC_{50} unreached) for ML2 RB and RC, which were at least more than 100 times higher compared to the IC_{50} in sensitive ML2 (0.08 nM) (**Figure 1D**). Notably, ML2 RC was completely insensitive to APO866 at the highest tested dose of 2000 nM, with only <10 %

of cell death. Therefore *in vivo*-derived ML2 have acquired resistance to APO866. While ML2 RA and RB maintain their resistance without drug pressure, ML2 RC displays the most powerful resistance toward APO866. In addition, we tested the sensitivity of APO866-resistant ML2 to another NAMPT inhibitor CHS-828. Resistant ML2 exhibited cross resistance to CHS-828 (**Figure 1E**). Hence, the resistance is not compound-specific but common to other NAMPT inhibitors. Altogether, we successfully generated ML2 clones with *in vivo*-acquired resistance to NAMPT inhibitors.

3.2 Resistant AML ML2 cells respond to NAD depletion induced by APO866 and suppress its anticancer cytotoxic effects

We further characterized the phenotypes of resistant ML2 cells. As a frequently observed mechanism of resistance is the modification of the drug target, we first assessed the expression level of NAMPT in resistant ML2. We evaluated the intracellular NAMPT protein levels by western blot, however no significant difference between parental and resistant clones was observed (**Figure 2A**). This observation suggests that resistant ML2 cells have acquired a resistant phenotype to NAMPT inhibitors that is independent from an alteration of NAMPT expression level. To assess the implication of multi drug resistance, we tested the effect of drug efflux pumps inhibitor verapamil and found no effect on the response to APO866 (Supplementary Figure S1). Therefore, resistant ML2 do not rely on an altered target expression nor MDR.

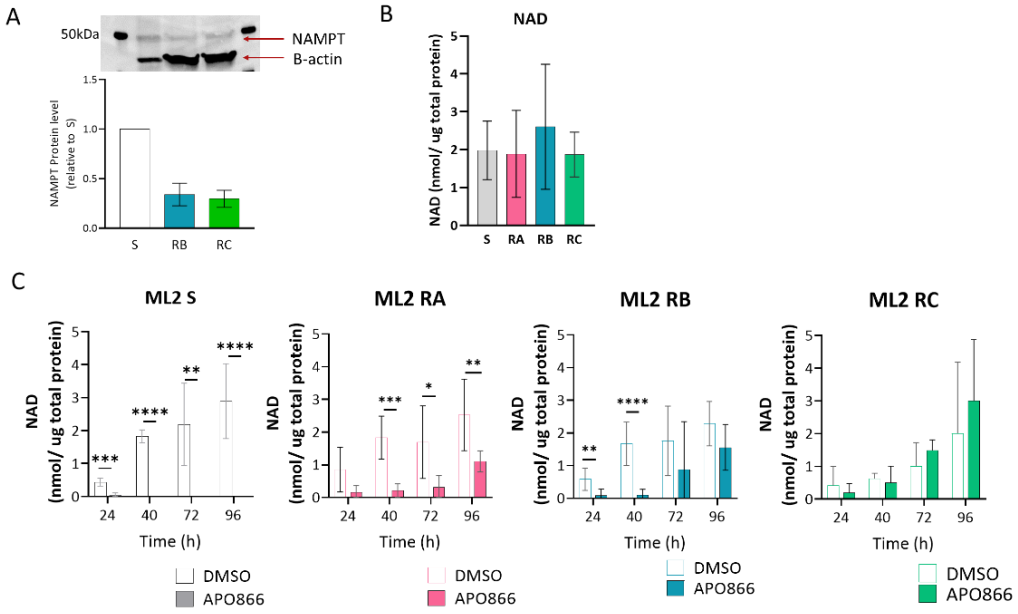


Figure 2: NAMPT expression and Intracellular NAD levels in resistant ML2. A) NAMPT protein levels assessed by western blot in APO866-sensitive (S) and resistant ML2 (RB, RC) cells. Representative western blot image and quantification of NAMPT protein levels normalized by actin levels. **B)** Intracellular NAD level in resistant and sensitive ML2 clones, normalized by total protein concentration. **C)** Time course evolution of intracellular NAD level in resistant and parental clones during incubation with DMSO or APO866 (10nM). *n>3, error bars=SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.*

The primary effect of NAMPT inhibitors in cancer cells is the depletion of intracellular NAD, which triggers further downstream cytotoxic events [10]. We quantified the intracellular NAD level in resistant and sensitive ML2. We found that the basal levels of total NAD(H) were comparable in resistant and sensitive ML2 clones (**Figure 2B**). We next evaluated the level of NAD upon treatment with APO866 (10 nM) in a time course manner. Interestingly, APO866 induced a significant decrease of NAD level in resistant ML2 RA and RB by 24 h of treatment, at approximately 15 to 19 % of the basal levels (**Figure 2C**). This demonstrates that APO866 is active in resistant ML2 cells, although the decrease of NAD level is less important than in sensitive ML2. Remarkably, resistant ML2 RC maintained its NAD level independently from APO866 treatment. Strikingly, in resistant ML2 RA and RB, from 72 h of treatment, NAD levels showed an increase and were restored at around 44 to 60 % at 96 h. These variations of NAD levels were confirmed with a quantification by LC-MS/MS in ML2 RB (Supplementary Figure S2). Overall, these observations demonstrate that resistant ML2 are able to modulate intracellular NAD level in response to APO866-induced stress and replenish its NAD pool to avoid long-term depletion in presence of NAMPT inhibitors.

The mechanism of cell death induction by APO866 has been well described in previous studies [10]. Specifically, NAD depletion upon APO866 treatment induces ROS increase, mitochondrial membrane depolarization and ATP depletion. We questioned whether these events are prevented in resistant ML2. We evaluated the intracellular levels of ROS including intracellular hydrogen peroxide H_2O_2 and mitochondrial and cytosolic superoxide $O_2^{\cdot -}$. We did not observe any significant changes of ROS levels except a modest increase of cytosolic $O_2^{\cdot -}$ upon APO866 treatment in resistant ML2 (**Figure 3A-C**). In the same line, resistant ML2 cells did not show any significant APO866-induced mitochondrial membrane depolarization (**Figure 3D**), ATP depletion (**Figure 3E**), nor caspase 3 activation (**Figure 3F**). Therefore, by counteracting NAD depletion, resistant ML2 cells are able to attenuate the induction of oxidative stress and to suppress mitochondrial dysfunction by APO866, thus preventing subsequent cell death.

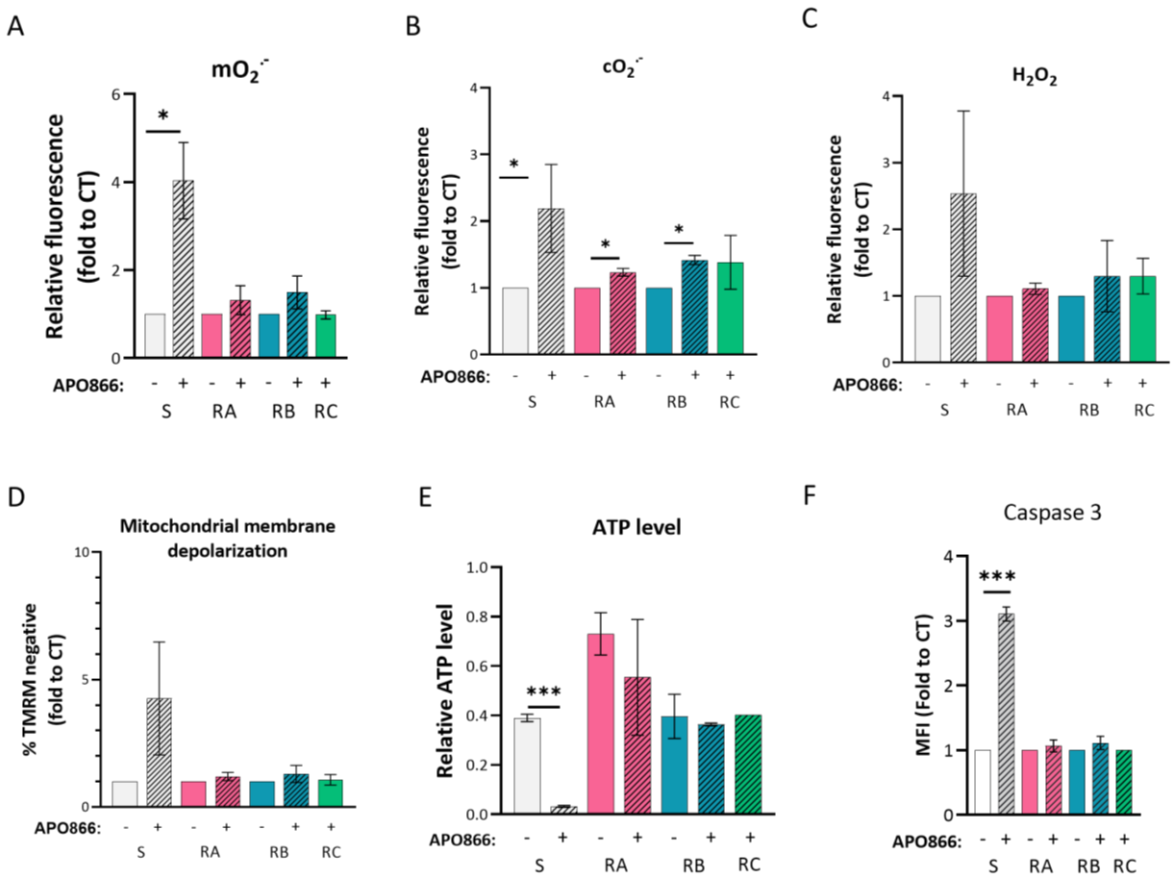
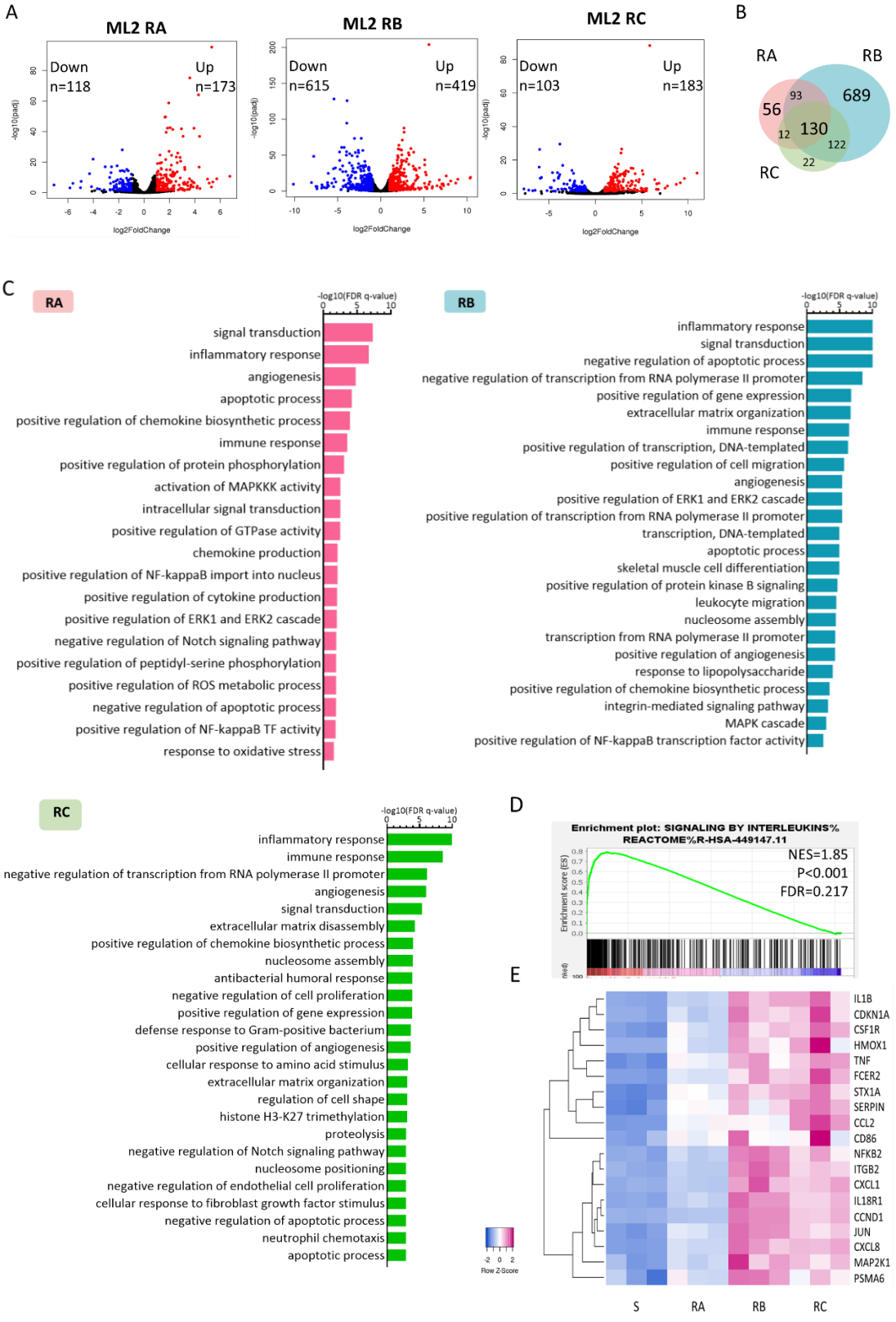


Figure 3: Resistant ML2 cells suppress APO866-dependent oxidative stress, mitochondrial dysfunction, ATP decrease and apoptosis activation. Intracellular levels of **A)** mitochondrial $O_2^{\cdot-}$, **B)** cytosolic $O_2^{\cdot-}$, **C)** cellular H_2O_2 and **D)** mitochondrial membrane depolarization measured by flow cytometry, in sensitive (S) and resistant ML2 (RA, RB and RC) at 48 h of incubation with DMSO (-) or APO866 (10 nM) (+). Presented values are normalized to level observed in DMSO-treated condition for each ML2 clone. **E)** Intracellular ATP levels in ML2 cells incubated with DMSO or APO866 (10nM) for 48h determined by luminescence assay. Represented values are normalized to total protein levels. **F)** Active caspase 3 levels, normalized to mean fluorescence intensity (MFI) measured in each ML2 clone treated with DMSO (control). *n>3, error bars=SD. *P<0.05, ***P<0.001.*

3.3 Gene expression profiles of Resistant ML2 cells present a profound and constitutive alteration with activated pro-survival signaling pathways

To further gain insights into the mechanism of resistance, we performed a whole transcriptomic analysis by RNA sequencing of parental and resistant ML2. We performed a differential gene expression (DGE) analysis of each resistant clone compared to the sensitive clone. We identified 291, 1034, and 286 DEG respectively in ML2 RA, RB and RC (with thresholds of Benjamin-Hochberg adjusted p-value <0.05, absolute log2 fold change (FC) >1) (**Figure 4A**). Among them, 130 genes were common to all three resistant clones (**Figure 4B**). Gene ontology (GO) analysis of the DEG in each resistant clone revealed an enrichment of biological processes involved in signal transduction, inflammatory response and pathways

related to apoptosis, which were mostly redundant between the resistant clones albeit to different extends (**Figure 4C**). Overall, we found that resistant ML2 present transcriptomic profiles with activated cellular signaling that seemingly make them predisposed to cellular stresses and promote cell survival.



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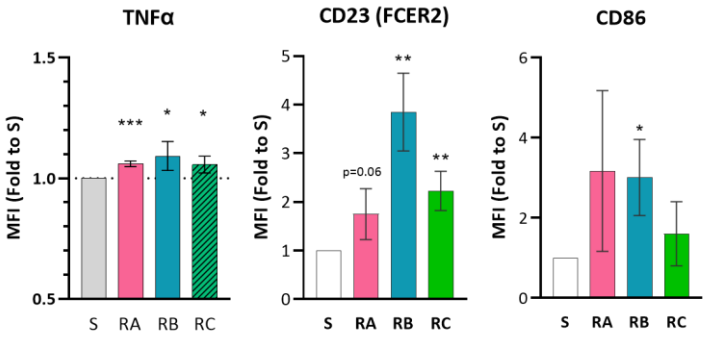

Figure 4: Resistant ML2 cells present transcriptional reprogramming compared to parental ML2. A) Volcano plots of differentially expressed genes (DEGs) in resistant RA, RB and RC in a comparison to sensitive clone. **B)** Venn Diagram showing shared and unique DEGs among resistant clones. **C)** Top enriched GO biological processes determined by functional analyses based on significant DEGs in RA, RB and RC. **D)** GSEA enrichment plot of Signaling by interleukins Reactome pathway (in RA compared to S) and **E)** heatmap of common and significant core-enriched genes in Signaling by Interleukins parthway of all resistant clones. Each column represents a biological replicate of each ML2 clone. **F)** TNFα, CD23 and CD86 levels measured by flow cytometry using fluorescent probe-conjugated specific antibodies. Represented values are mean fluorescence intensity (MFI) normalized by MFI of control anti-IgG antibody. *n=3, error bars=SD. *P<0.05, **P<0.01, ***P<0.001.*

We further investigated more in-depth by performing a gene set enrichment analysis (GSEA) to identify upregulated or downregulated pathways. We found positively enriched pathways in all resistant ML2 clones, which mainly consisted of cellular signaling and regulations of cellular processes (Supplementary Figure S3A-C). Downregulated pathways were only observed in ML2 RC, which corresponded to a significant attenuation of ribosomal and of proteasomal system (Supplementary Figure S3D). Top ranked upregulated pathways in all resistant ML2 were the Signaling by interleukins and the IL-4 and IL-13 signaling (**Figure 4D**). The activation of these pathways was associated with increased expressions of intracellular signaling mediators, cell surface receptors and inflammatory cytokines including TNFα, IL-8 and IL-1β (**Figure 4E**). To confirm the expression changes observed in transcriptomic data, we quantified the amounts of the most significantly upregulated cytokines and cell surface receptors using specific antibodies and flow cytometry. We found elevated protein levels of TNFα, CD23 and CD86 in resistant ML2 compared to the sensitive clone, confirming the observation in the gene expression data (**Figure 4F**).

Collectively, these data demonstrate that the acquisition of resistance is associated with a profound modification of the transcriptomic programs, characterized by an activation of inflammatory response and cellular signaling pathways. In the most powerful resistant clone ML2 RC, modulation of protein homeostasis with downregulation of ribosomal and proteasomal complexes seems to contribute to drug resistance.

3.4 Resistant ML2 cells modulate gene expressions of stress signaling pathways upon APO866 treatment

Next, we examined the transcriptional changes that are induced in resistant ML2 cells upon APO866 treatment. We determined the whole transcriptomes of resistant ML2 RA and RB at 40 h of incubation in presence of APO866 (10 nM) or DMSO and performed a DGE analysis and a GSEA.

With the initial significance thresholds (adjusted p-value <0.05 and absolute log2(FC) >1), only four genes in ML2 RA and one gene in ML2 RB were identified as differentially expressed (Supplementary Figure S4A-B). Extending the analysis with a threshold of log2(FC) >0, we identified 1371 DEG in APO866-treated ML2 RA (Supplementary Figure S4A). No other significant DEG were detected in RB, suggesting that its transcriptional program is weakly affected by APO866-induced stress (Supplementary Figure S4B). Next, we performed a functional analysis with GSEA to identify the functional changes upon APO866 treatment. We observed activations of a large number of cellular signaling pathways. Most of them were those enriched in the basal gene expression profiles compared to sensitive ML2 and consisted of signaling by interleukins, NFκB signaling and MAPK cascade, transcription factor AP-1 network, etc. (**Figure 5A, 5B**). Interestingly, we observed a downregulation of ribosomal and proteasomal proteins-encoding genes (**Figure 5C, 5D**). Considering that these expressions were constitutively downregulated in the most powerful resistant ML2 RC, which is maintained permanently under drug pressure, the translation and proteasomal degradation processes seem to be key factors involved in resistance to APO866. Moreover, mitochondrial respiration appeared to be downregulated upon APO866 exposure (**Figure 5C, 5D**). Overall, APO866 induces in resistant ML2 the activation of stress signaling, the attenuation of translational and proteasomal complexes, and of mitochondrial respiration.

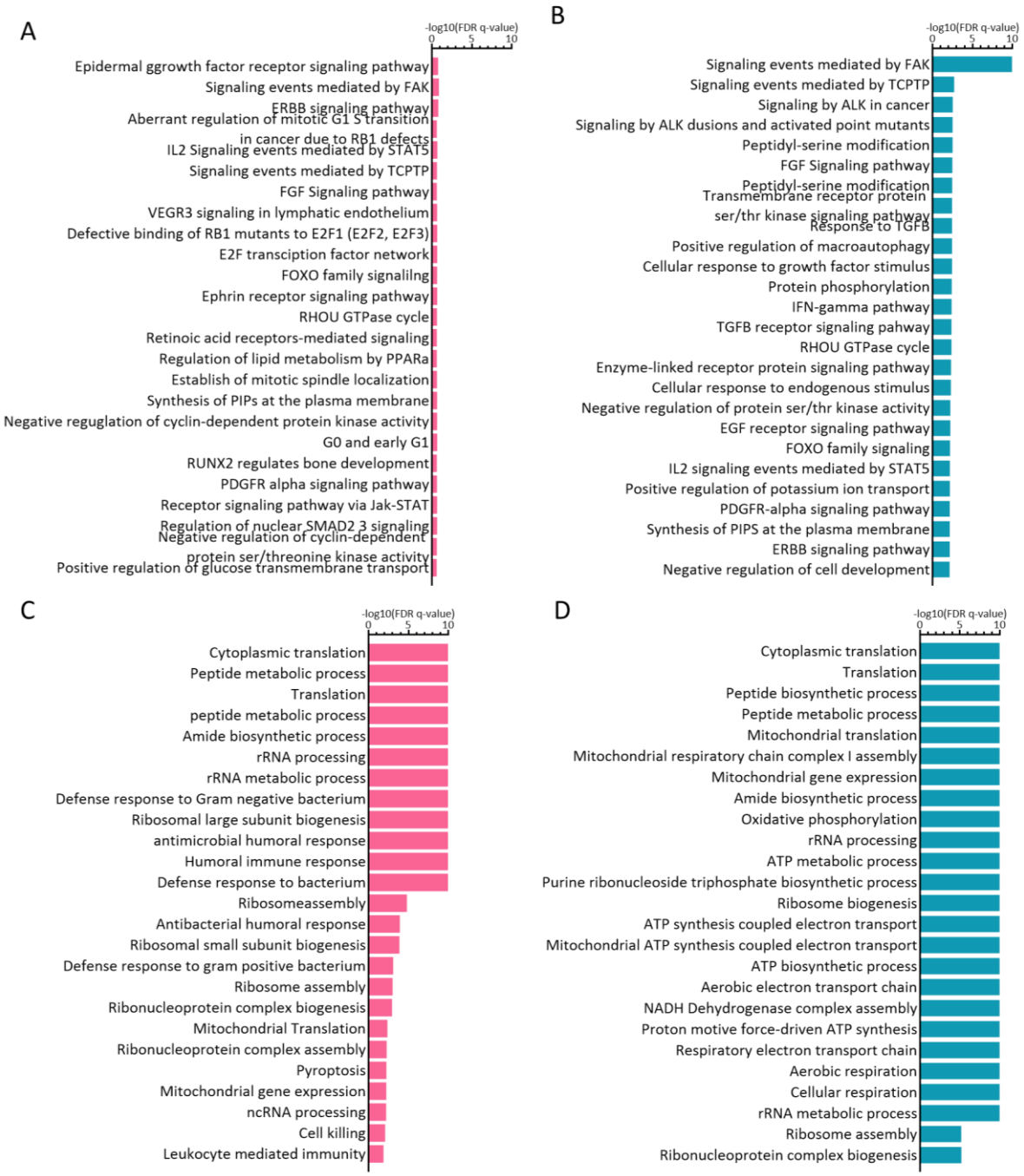

Figure 5: APO866 induces transcriptional changes in resistant ML2 cells. Whole transcriptomes of resistant ML2 RA and RB were determined at 40 h of treatment with APO866 (10 nM) or DMSO as control, and enriched pathways were detected by GSEA. Upregulated pathways in **A)** RA and **B)** RB, and downregulated pathways in **C)** RA and **D)** RB.

We questioned whether these alterations induced by APO866 correspond to signatures of drug cytotoxicity or rather to ML2 cell response as a protective mechanism. Therefore, we examined the transcriptional changes induced by APO866 in the parental ML2. By whole transcriptomic determination and DGE analysis of APO866 (10 nM) vs DMSO- treated ML2 at 40h of treatment, we identified 2710 DEG (1226 up, 1484 down) (**Figure 6A**). This shows the enormous impact of APO866 on the global gene expressions in AML cells. The functional

enrichment analysis showed enrichment of pathways related to apoptosis, regulation of transcription, cell division and cellular signal transduction (**Figure 6B**). We identified many processes that are previously reported to be affected by NAMPT inhibitors, including cell cycle arrest (cytostatic effect), apoptosis or MAPK cascades, corroborating the previous studies [20], [35]. By comparison with the alterations in resistant ML2, we found several pathways that were altered by APO866 in both resistant and sensitive ML2 (including TGFβ signaling, MAPK cascades), suggesting that they may consist of major pathways involved in the response to APO866. In contrast, attenuated translational and proteasomal complexes seem to be specific features of resistant ML2.

Overall, our results revealed a unique transcriptional reprograming that is induced in resistant ML2 upon exposure to APO866.

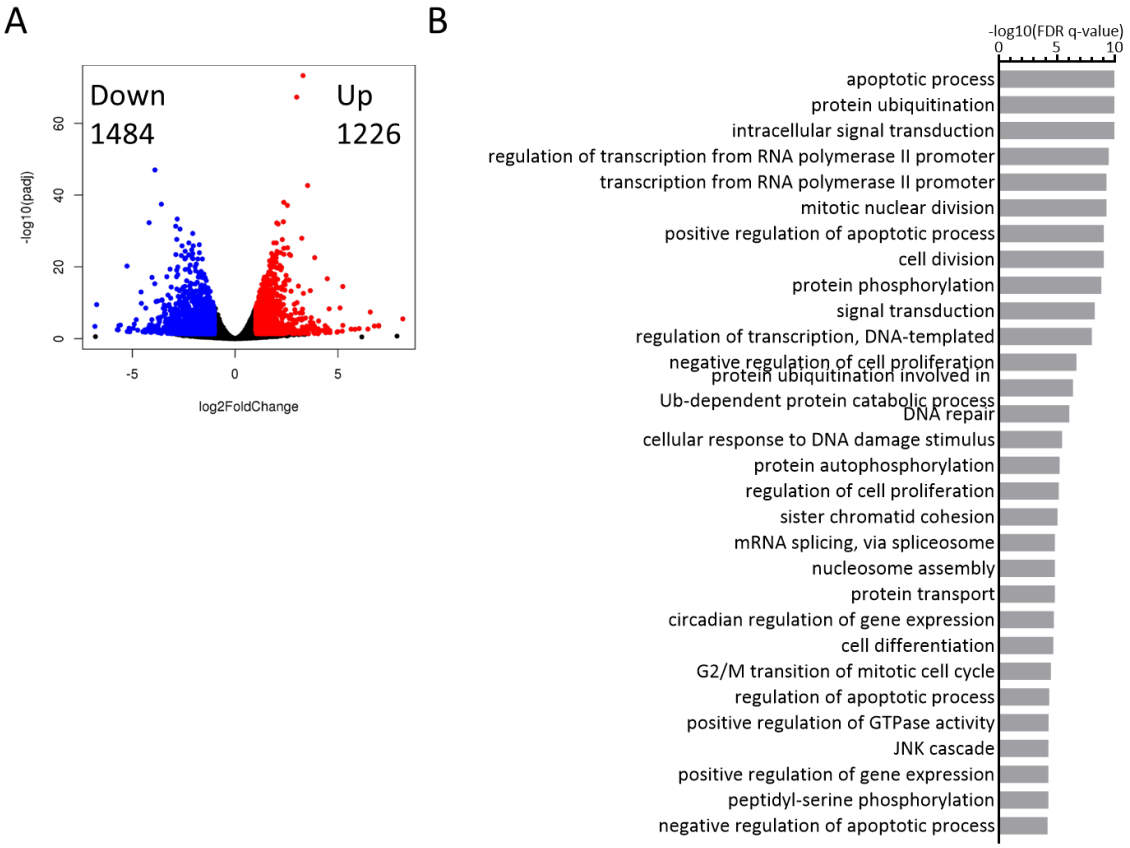

Figure 6: APO866 treatment dramatically alters the global gene expressions in parental ML2 cells. Whole transcriptome of parental (or sensitive) ML2 S at 40h of treatment with APO866 (10 nM) or DMSO (as control) was determined by RNA sequencing and enriched pathways were detected by GSEA. **A)** DEG in APO866-treated ML2 S cells compared to control. **B)** Top-ranked pathways in GO enrichment analysis of DEG showing enrichment induced by APO866.

3.5 Activated signaling PI3K, Erk and NFκB pathways enable resistant ML2 to block apoptosis.

Next, we aimed to explore the biological relevance of the previous observations. The transcriptomic analyses of resistant ML2 showed enrichment of several cellular signaling pathways that are involved in the regulation of cell survival in both constitutive and drug-responsive manners. We investigated whether the activation of these pathways is implicated in the resistance to APO866.

In all resistant ML2 cells, we have observed a significant activation of signaling by interleukins, which appeared to be IL-4 and IL-13-mediated signaling (**Figure 7A**). IL-4 and IL-13 signaling are mediated mainly by JAK-STAT, and PI3K/Akt, Ras/Erk signaling [36]. We assessed the levels of phosphorylated forms of Erk and of Akt by flow cytometry and by western blot. We observed increased levels of phosphorylated proteins in resistant ML2, indicating an activation of this signaling pathways (**Figure 7B**). Next, we assessed their possible contribution to the response to APO866 treatment using specific inhibitors as single agent or in combination with APO866. We found that resistant ML2 RA and RB were more sensitive to PI3K inhibition (with ZSTK474 and copanlisib) alone compared to sensitive ML2 (**Figure 7C** and supplementary figure S5A). Co-treatment with APO866 (at IC_{30} of each ML2 clones) enhanced its cell-killing effect in resistant clones (**Figure 7C**). Therefore, active PI3K signaling in resistant ML2 has a pro-survival function against APO866. Interestingly, in sensitive ML2 PI3K inhibition reduced APO866 cytotoxicity. This observation indicates that PI3K signaling is involved in the anticancer effect of APO866 and has differential effect depending on cell context. In addition, in the most powerful resistant ML2 RC, PI3K inhibition was able to partially reverse resistance to high dose of APO866 (500 nM) (supplementary figure S5A).

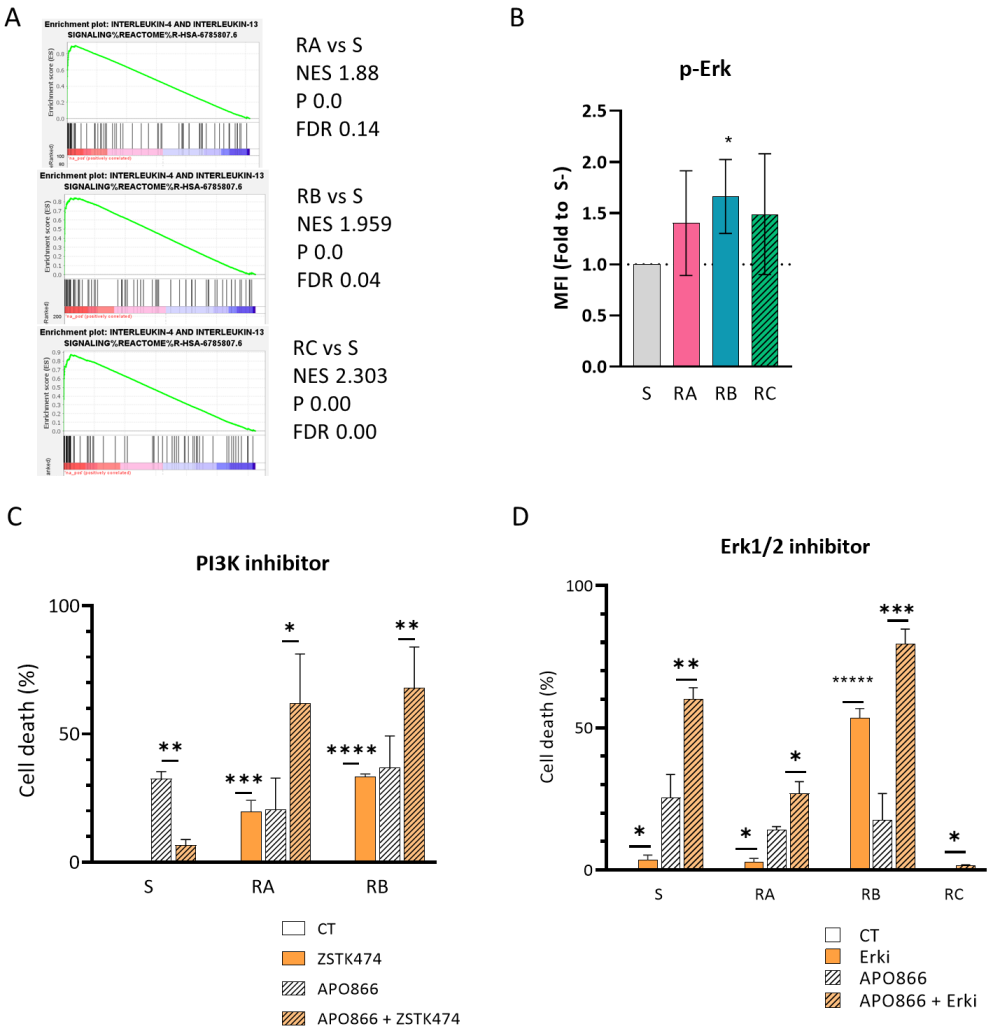

Figure 7: Activated PI3K and Erk pathways allow resistant ML2 to block apoptosis. A) GSEA plots of IL-4 and IL-13 Signaling showing enrichment in the resistant clones compared to the sensitive clone, with their corresponding normalized enrichment score (NES), p-value and FDR q-values. **B)** p-Erk levels assessed by flow cytometry. Represented values are MFI normalized by MFI anti-IgG1-PE antibody. **C)** Cell death upon 96h treatment with PI3K inhibitor ZSTK474 (1 µM) as single agent or in combination with APO866 (at IC_{30} of each clone). **D)** Cell death upon ERK1/2 inhibition with Ravoxertinib (2 µM) as single agent or in combination with APO866 (at IC_{20-30} of each clone). Presented data in **C)** and **D)** are specific cell death induced by each treatment (as defined in the materials and methods). *n>3, error bars=SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, *****P<0.00001.*

We assessed the effect of the pharmacological inhibition of Erk1/2 with a specific inhibitor Ravoxertinib (GDC-0994). In comparison to parental ML2, resistant ML2 RB showed a significantly higher sensitivity to Erk1/2 inhibition (**Figure 7D**). In combination with APO866, Erk1/2 inhibition presented synergistic (in RA) or additive (in RB) effects on APO866-induced cell death. Accordingly, active Erk1/2 signaling contributes to cell survival and resistance against APO866. Resistant ML2 RC viability was not affected by Erk1/2 inhibition alone or in co-treatment with APO866.

Pan-JAK inhibitor Ruxolitinib had only a minor effect on the response to APO866 (Supplementary Figure S5B), suggesting a minor role of JAK activation in the resistance to APO866. Hence, the activation of signaling by interleukins as appeared in the transcriptomic analyses seems to be mediated by PI3K/Akt and Erk pathways, not necessary activated by IL-4 or IL-13 and their receptors but regulated by other factors.

The MEK/Erk signaling pathway plays a pivotal role in cancer growth and survival [37], [38]. There are also many cross-talks between MEK/Erk and PI3K/Akt pathways [39].

The transcriptomic study showed an activation of NFκB signaling, which is one of the downstream pathways of PI3K/Akt and MEK-Erk pathways (**Figure 8A**). NFκB is a transcriptional factor that promotes cell survival by regulating the expression of various target genes, whose products inhibit the apoptosis machinery [40]. We questioned whether NFκB is involved in the protective signaling. The inhibition of NFκB with a specific inhibitor DHMEQ synergistically enhanced sensitivity of resistant ML2 RA and RB to APO866 (IC_{30}) (**Figure 8B**). Therefore, NFκB mediates resistance to APO866 in resistant ML2 RA and RB. In addition, resistant ML2 RC was found to be sensitive to NFκB inhibition (**Figure 8B**).

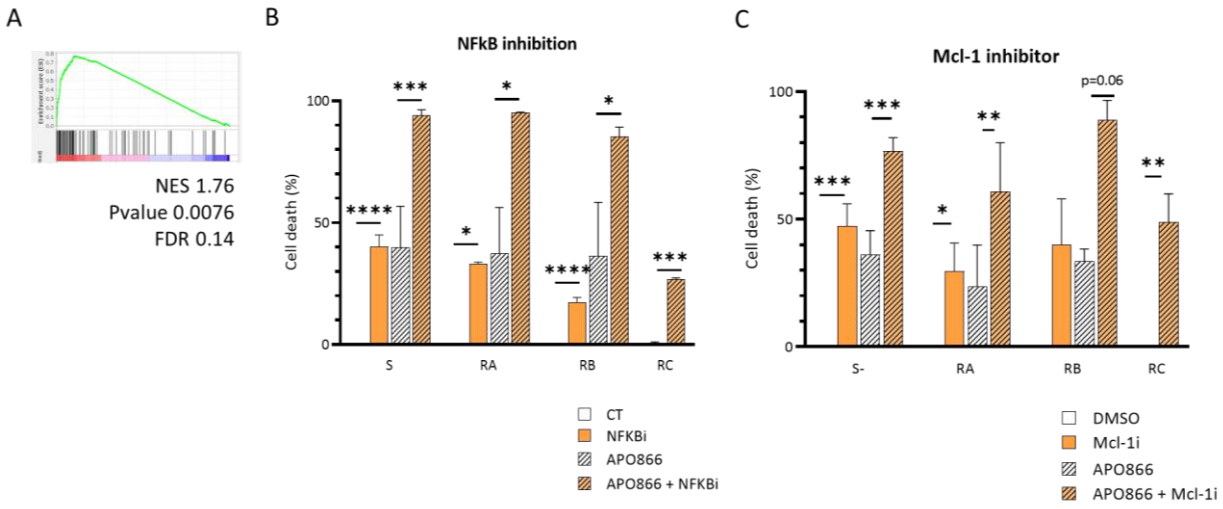

Figure 8: Resistant ML2 block apoptosis by activation of NFκB pathway and by Mcl-1 activity. A) GSEA plot of NFκB signaling pathway enriched in the resistant clone. **B)** Specific cell death of ML2 cells treated with NFκB inhibitor DHMEQ at 20 µM (or DMSO control) as single agent or in combination with APO866 (IC30 in each clone) for 96h. **C)** Specific ell death of ML2 cells treated with Mcl-1 inhibitor S63845 at 100 nM (or DMSO control) as single agent or in combination with APO866 (IC_{30} in each clone) for 96h. *n>3, error bars=SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.*

The induction of apoptosis is regulated by Bcl-2 family of proteins, which is composed of pro-apoptotic and anti-apoptotic proteins [41]. Mcl-1 is a Bcl-2 family member protein with anti-apoptotic function. Its expression is regulated by multiple pathways and factors including MEK/Erk, NFκB, JAK/STAT, ATF4 (ER stress) or HIFα [42]. In the transcriptomic analysis,

an induction of MCL1 gene was observed upon drug exposure in resistant ML2. To assess the potential implication of Mcl-1 in APO866 resistance, we evaluated the effect of a specific inhibitor S63845 as single agent or in co-treatment with APO866 on cell death. Inhibition of Mcl-1 significantly enhanced the sensitivity of resistant ML2 to APO866 (**Figure 8C**). Therefore, overexpression of Mcl-1 upon drug exposure strongly contributes to APO866 resistance in ML2 cells.

Altogether, resistant ML2 rely on activated pro-survival signaling pathways mediating PI3K/Akt, Erk and NFκB activities, and on Mcl-1 activation to evade APO866 cytotoxicity. The exact flow of the signaling cascade is still to be determined.

3.6 Resistant ML2 cells modulate their metabolism and control their NAD(P)(H) levels

The previous results demonstrated the ability of resistant ML2 to reprogram the cellular signaling and counteract cell death induction by APO866. On the other hand, given that NAD homeostasis is crucial in the cell survival, we may speculate that a metabolic adaptation is also required for resistant ML2 to overcome APO866 effect. Indeed, in the transcriptomic analysis a downregulation of oxidative phosphorylation (OXPHOS) genes expressions was observed in resistant ML2 upon drug exposure (previous section). To investigate the metabolic profiles of resistant ML2 and their changes upon APO866 treatment, we analyzed the mitochondrial respiration and glycolytic activities in resistant ML2 by Seahorse MitoStress assay (Agilent). We found that the basal oxygen consumption rates (OCR) were slightly lower in ML2 RA and RB compared to sensitive ML2 (**Figure 9A**). Upon APO866 treatment, a significant drop of OCR was induced in sensitive ML2. Contrastingly, only small and no significant decreases were observed in resistant ML2, showing that the mitochondrial respiration is less affected by APO866. Similar tendencies were observed in terms of spare respiratory capacity (SRC), with a lower fitness of mitochondrial respiration capacity in basal conditions but less effect of APO866 (**Figure 9B**). Accordingly, resistant ML2 proliferate with a reduced OXPHOS activity and they are less susceptible to APO866. Remarkably, we observed that ML2 RC have the same mitochondrial metabolic profiles as the sensitive ML2 although being in continuous presence of the inhibitor.

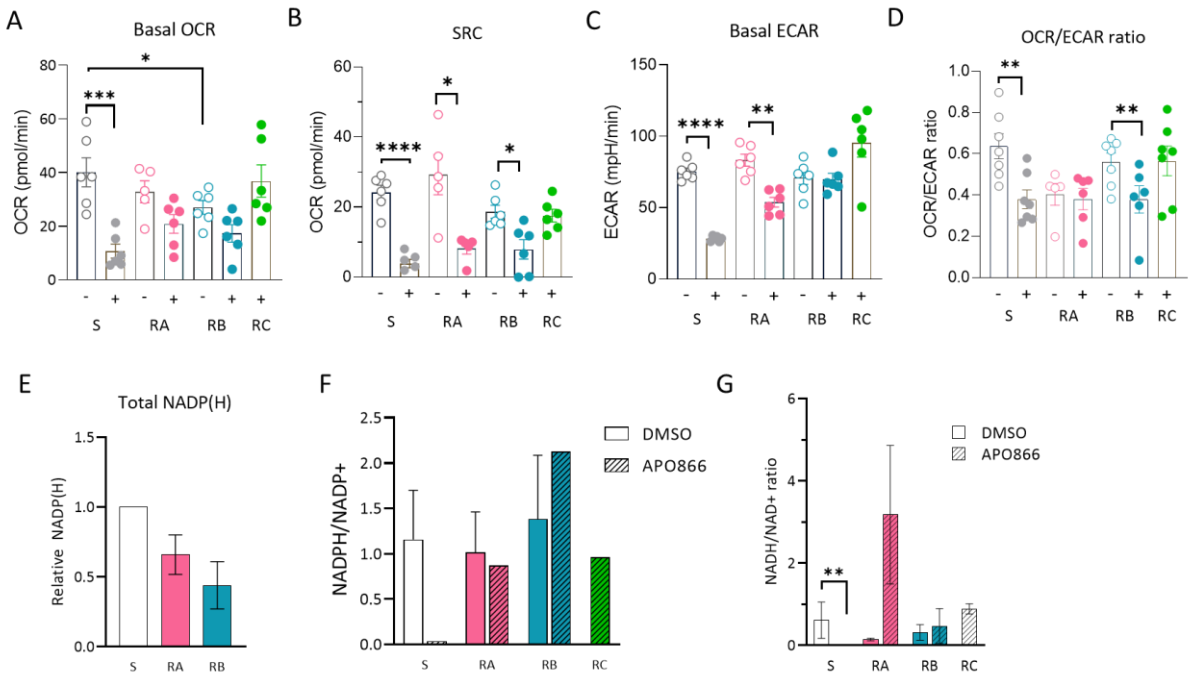

Figure 9: Metabolic activities and NAD(P)H levels of resistant ML2 cells are less affected by APO866. A-E) metabolic profiling with seahorse assay. **A)** basal OCR **B)** Spare respiratory capacity (SRC) **C)** basal ECAR **D)** OCR/ECAR ratio **E)** Total NADP(H) normalized to amount in Sensitive ML2 **F)** NADPH/NADP$^+$ ratio **G)** NADH/NAD$^+$ ratio. *n=>3, error bars=SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.*

In parallel, we measured the extracellular acidification rate (ECAR) to evaluate the glycolytic activity. We found an increase of ECAR upon drug treatment in ML2 RB, suggesting an activation of the glycolytic pathway (**Figure 9C-D**). Accordingly, there is a switch to glycolysis, which may consist of a compensatory mechanism to sustain ATP production in presence of NAMPT inhibitor.

While these metabolic pathways do not consume NAD, they both affect the NAD$^+$/NADH ratio in distinct manners. The preference of glycolysis upon OXPHOS in ML2 RB may contribute to the maintenance of the redox balance while producing sufficient ATP for proliferation. To test this hypothesis, we measured NADH and NAD$^+$, as well as NADPH and NADP$^+$ levels distinctively and evaluated the redox ratios. We found that resistant ML2 present a reduced pool of NADP(H) compared to sensitive ML2 but with a comparable NADPH/NADP+ ratio (**Figure 9E-F**). Upon treatment with APO866, the NADPH/NADP$^+$ redox balances were maintained in ML2 RA, and even increased in ML2 RB (**Figure 9F**). Similarly, NADH/NAD$^+$ ratio was also shown to be comparable to basal level in sensitive ML2 and maintained (ML2 RB) or increased (ML2 RA) in presence of APO866 (**Figure 9G**). Therefore, resistant ML2 RA and RB are able to maintain the redox ratios and promote the reducing power in presence of APO866. Given that glycolytic reactions promote NAD$^+$ to

NADH conversions at least in aerobic condition, while the mitochondrial respiration oxidizes NADH to NAD^+, it is plausible to interpret that the metabolic switch to glycolysis is favorable for increasing the redox balance of NAD(P)H/$NAD(P)^+$. We may imagine that the reducing power promotes ROS detoxifications.

In line with these observations, resistant ML2 were slightly more susceptible to rotenone (complex I inhibitor) compared to APO866-sensitive ML2, however, while rotenone had a synergistic effect in co-treatment with APO866 in sensitive ML2, no additive effect was observed in resistant ML2 (Supplementary Figure S6).

Overall, resistant ML2 RA and RB adaptively modulate their metabolic activities upon APO866 through transcriptomic modification and conserve NAD(P)H redox homeostasis.

4 DISCUSSIONS

In this study, we successfully developed *in vivo* models of human AML with acquired resistance to NAMPT inhibitor APO866 and we characterized them by whole transcriptomic analyses. APO866-resistant ML2 cells exhibited activation of cellular signaling pathways involved in stress response and control of cell survival. Notably, resistant ML2 cells showed activations of PI3K/Akt and Erk signaling pathways, and of NFκB transcription factor, which were implicated in preventing APO866-induced cell death. Furthermore, Mcl-1 played a major role in blocking APO866-induced apoptosis. The pharmacological inhibition of these factors effectively reversed the resistance to APO866, demonstrating the dependency of ML2 cells on these anti-apoptotic signaling pathways for acquired resistance. Moreover, we observed a downregulation of translational processes and of proteasomal complex, constitutively in the most powerful resistant ML2 RC and upon exposure to APO866 in resistant ML2 RA and RB. Inhibiting the translation process in parental ML2 conferred them resistance to APO866, suggesting that these processes consist of key regulators of ML2 cell response to APO866. Furthermore, a metabolic shift toward glycolysis supported the sustained energy production while maintaining NAD homeostasis, thereby promoting cell survival under stress conditions with reduced level of NAD. Overall, our study elucidated the mechanisms underlying resistance to NAMPT inhibitors in AML ML2 cells. Our findings suggest that the identified key pathways can serve as potential targets for overcoming resistance to NAMPT inhibition. Further investigations are required to determine how precisely these alterations are coordinated and contribute to resistance.

This study represents the first report of models of resistance to NAMPT inhibitors acquired in *in vivo* settings. The resistant ML2 cells were generated in two distinct mouse xenografts. Our analyses revealed substantial similarities in the transcriptomic profiles and the resistance phenotypes between the two ML2 clones. This observation suggests the central role of these common pathways in the antitumor activity of NAMPT inhibitors and in the cancer response to them. It should be further explored whether these features are specific to the ML2 cell line or to AML in general, or could be extended to other cancer types.

We demonstrated that activation of Erk pathway is associated with resistance toward APO866 in AML cells. This observation is consistent with a previous study which showed that NAMPT inhibition induced cell death in multiple myeloma by triggering a downregulation of Erk signaling and subsequent autophagic cell death [16]. Our results support these previous observations and underlines the importance of Erk pathway in the cancer response to APO866.

In contrast, we observed mixed effects regarding the implication of PI3K/Akt signaling in the response to APO866. In resistant ML2, we observed that inhibition of PI3K increased their sensitivity to APO866 treatment (at IC_{30}). Conversely, PI3K inhibition reduced the sensitivity of parental ML2 to APO866 treatment at low dose (0.5 nM, IC_{50}). Previous studies have reported that FK866 inhibits PI3K and induces autophagic cell death in multiple myeloma and leukemia cells [16], [19], suggesting a pro-survival role of PI3K in these cells. These contrasting observations suggest that PI3K activation may have bidirectional effects depending on the cellular contexts. Therefore, the magnitude of PI3K/Akt/mTOR activities and their coordination with other signaling pathways seem to be important in determining their effect on the cell fate.

We identified a major role of Mcl-1 in the protection of AML from APO866-induced apoptosis. Increased expression of Mcl-1 has been reported as a mechanism of resistance toward various anticancer therapies including radiotherapy and Bcl-2 inhibitor venetoclax [42]. Inhibitors of Mcl-1 are gaining interest to complement the effects of other apoptosis-inducing drugs or to overcome therapy resistance [43]. Our observations support the potential of Mcl-1 as a therapeutic target for treatment of AML with acquired resistance to apoptosis-inducing therapies including APO866.

Most of the signaling pathways that we found to be associated with APO866 resistance of ML2 cells are well described in the literature for their pro-tumoral functions. For instance, the activation of PI3K/Akt/mTOR pathway is frequently observed in AML as a consequence of genetic mutations such as the prevalent mutation of the growth factor receptor FLT3 [4], [44]. In addition, NRAS and KRAS mutations occurring in a subset of patients with AML (11 % and 5 % respectively) result in the constitutive activation of Ras/MEK/Erk pathway [45]. It has

been reported that a substantial proportion of AML patients of approximately 50 to 80 % exhibit constitutive PI3K/Akt/mTOR activation, which is associated with reduced survival [46], [47]. It is noteworthy to mention that ML2 cell line harbors a KRAS mutation causing a constitutive activation of RAS signaling and of downstream PI3K/Akt and Erk pathways [48]. Investigating whether increased activities of PI3K/Akt or Erk pathways deriving from genetic alterations may serve as predictive markers of resistance development toward NAMPT inhibitors would be of great interest.

We observed a downregulation of ribosomal and proteasomal complexes in resistant ML2 treated with APO866. However, the precise roles of these processes in the resistance to APO866 remained unexplored and is worth investigating further. Interestingly, we observed that inhibition of protein synthesis with cycloheximide blocks the cytotoxic effects of APO866 in drug sensitive ML2 cells, while enhancing resistance to APO866 in resistant ML2 RA and RB (Supplementary Figure S7A). In contrast, treatment with proteasome inhibitor bortezomib demonstrated an additive or synergistic effect on APO866-induced cell death in APO866-sensitive ML2 (Supplementary Figure S7B). Besides, resistant ML2 including ML2 RC were more sensitive to Bortezomib compared to the sensitive clone. Therefore, these processes are playing a key role in the biology of resistant ML2 and in the response of leukemia cells to NAMPT inhibitors. These findings are consistent with a previous report indicating that APO866 induces a translational arrest as a temporary defense mechanism in leukemia cells [19]. The translational arrest coupled with a reduction of proteasomal activity were likely to contribute to the downregulation of Mcl-1 level, thereby counteracting apoptosis induction by APO866. Altogether, these observations highlight the crucial role of the tight regulation of protein homeostasis in the response to APO866. In this regard, translational reprogramming is closely associated with cancer progression and therapy resistance [49]–[51]. It implies a reduction of global protein synthesis, to reduce energy consumption and adapt to the limited energy supplies, while promoting selective translation of mRNAs of proteins essential for adaptation and survival in a resources-limited TME [49]. Proteasomal degradation, on the other hand, is an essential regulator of various signal molecules and transcription factors [52]–[54]. It is worth exploring whether these alterations contribute to the regulation of some specific proteins in the context of response to NAMPT inhibition. For instance, our analyses showed transcriptional upregulations of HMOX1 (encoding heme oxygenase), Mcl-1, or NFκB, which are described to be tightly regulated by proteasomal activities [54]–[57]. These proteins represent potential candidates for further investigations.

Finally, our investigation revealed an association of the activation of inflammatory responses with acquired resistance. Increased levels of cytokines and inflammatory molecules such as TNFα or IL-8 are largely associated with tumorigenesis [58], [59]. Hence, it is worth

exploring whether these inflammatory factors play a role in the resistance phenotype and deciphering the underlying mechanism. In our xenograft models of SCID mice, while T and B cells are defective, NK cells, dendritic cells, macrophages and other myeloid immune cells are present in the tumor environment. Therefore, it would be interesting to assess the potential interaction of cancer cells with the host immune system. Notably, it is of particular interest to explore whether the interaction of tumor cells with immune cells contributes to the resistance acquisition and whether the pro-inflammatory phenotype of resistant ML2 cells enables immune escape. These will allow gaining insights into the interplay between inflammation and tumor response to NAMPT inhibitors.

Our whole transcriptomic analyses of APO866-sensitive and resistant ML2 cells have revealed significant alterations in various cellular processes. While our findings provide valuable insights on the tumor response to APO866, many aspects remain to be explored. For instance, it would be crucial to determine the precise signaling flow of the anti-apoptotic response in resistant ML2. Additionally, the transcriptomic changes induced by APO866 in sensitive ML2 warrant further investigation to elucidate the mechanisms underlying cell death induction by NAMPT inhibition. In conclusion, our data provide a valuable basis for understanding the molecular mechanisms of the antitumor effects of NAMPT inhibitors and the response of cancer cells to NAMPT inhibition.

5 CONCLUSION

This study reports the characterization of models of *in vivo*-acquired resistance to NAMPT inhibitors and provides whole transcriptomic data associated with the response of leukemia cells to NAMPT inhibition. We have successfully identified a mechanism of acquired resistance to NAMPT inhibitor APO866 in AML, in which pro-survival cellular signaling and metabolic adaptations played a crucial role. These findings suggest the potential of combinatory therapeutic approaches to enhance the efficacy of APO866 and to overcome drug resistance. The precise molecular mechanisms governing the response of cancer cells to NAMPT inhibitors remain to be determined in future studies. This study provides a valuable basis for further research to unravel the intricate effects of NAMPT inhibitors in cancer cells and for the development of potent strategies of more effective utilization of NAMPT inhibitors in the treatment of cancer.

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PROJECT 1 SUPPLEMENTARY DATA

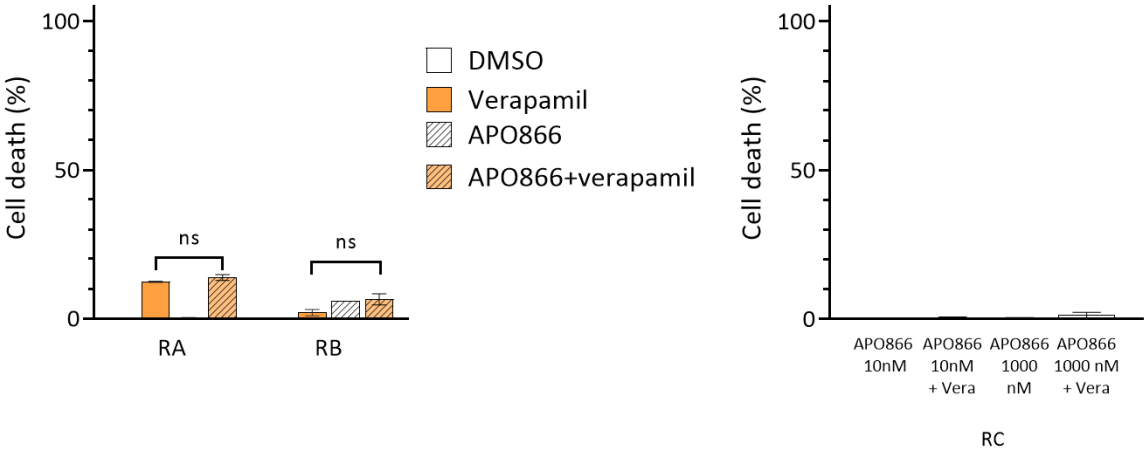

Figure S1: **Resistance to APO866 is independent of drug efflux pumps activity (MDR).** Specific cell death of ML2 incubated in presence verapamil (vera, 40 µM) as single agent or in co-treatment with APO866 (10 nM). *n=3, error bars=SD. ns=non-significant.*

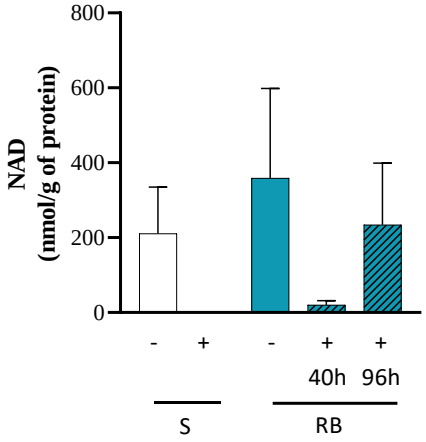

Figure S2: **NAD levels determined by LC-MS/MS in resistant and sensitive ML2**. Intracellular NAD levels determined by LC-MD/MD analysis, in ML2 cells treated with DMSO (-) or APO866 10 nM (+), for 96h. Presented data are measured at 96h, if not specified otherwise. *n=3, error bars=SD.*

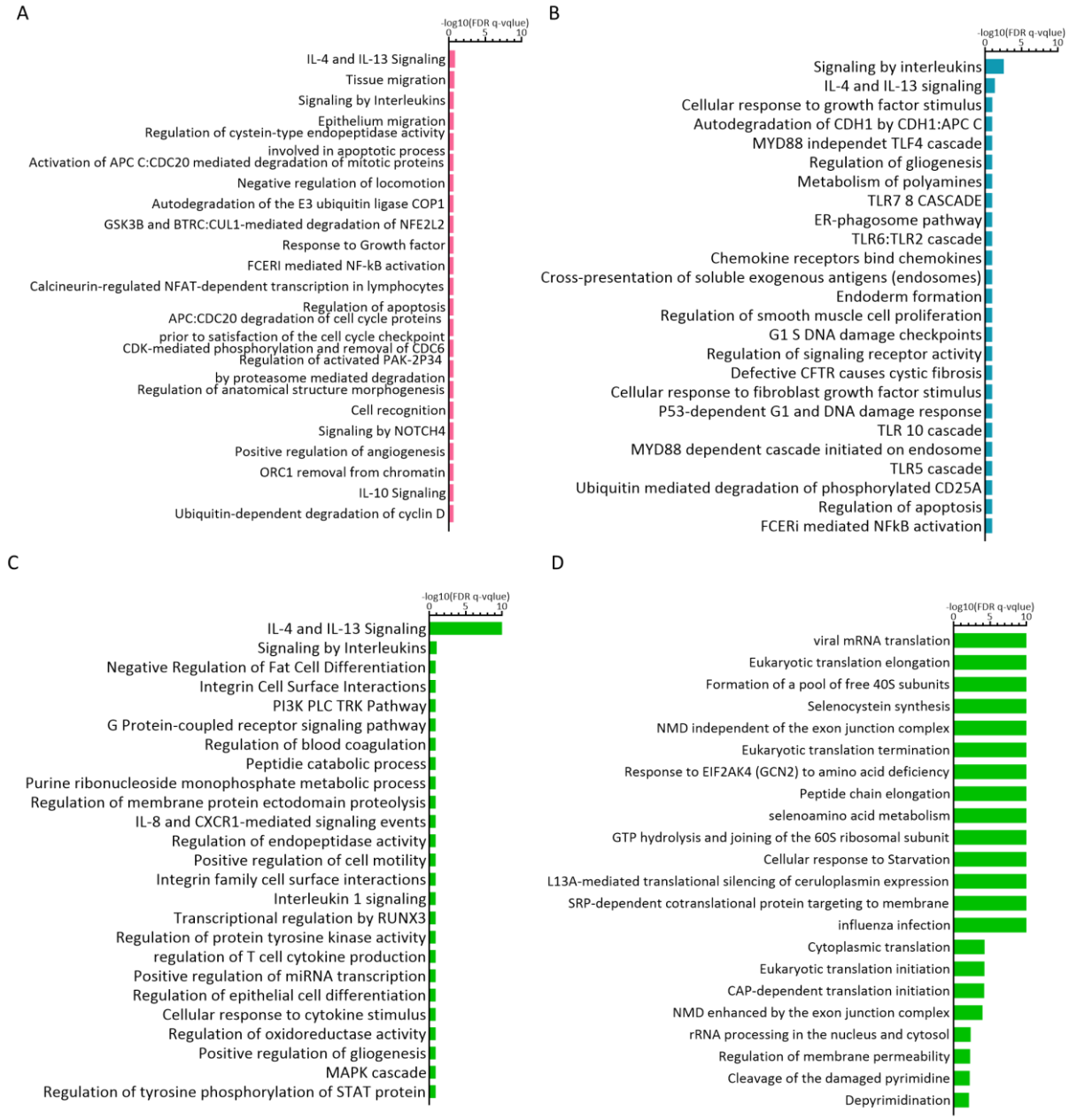

Figure S3: **GSEA of resistant ML2 cells**. Top-ranked pathways **A)** upregulated in RA (vs S), **B)** upregulated in RB (vs S), **C)** upregulated in RC (vs S) and **D)** downregulated in RC (vs S).

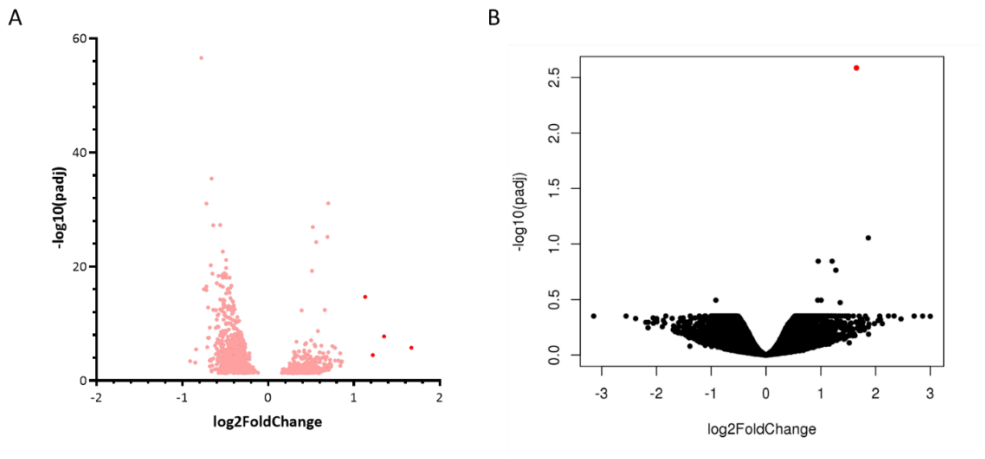

Figure S4: **Volcano plots of DEG in APO866-treated ML2**. DEGs in APO866-treated **A)** RA and **B)** RB compared to untreated condition. Red dots represent significant DEG with threshold log2(FC)>1 and pink dots represent DEG with log2(FC)>0. Black dots are non-significant DEG (p-value >0.05).

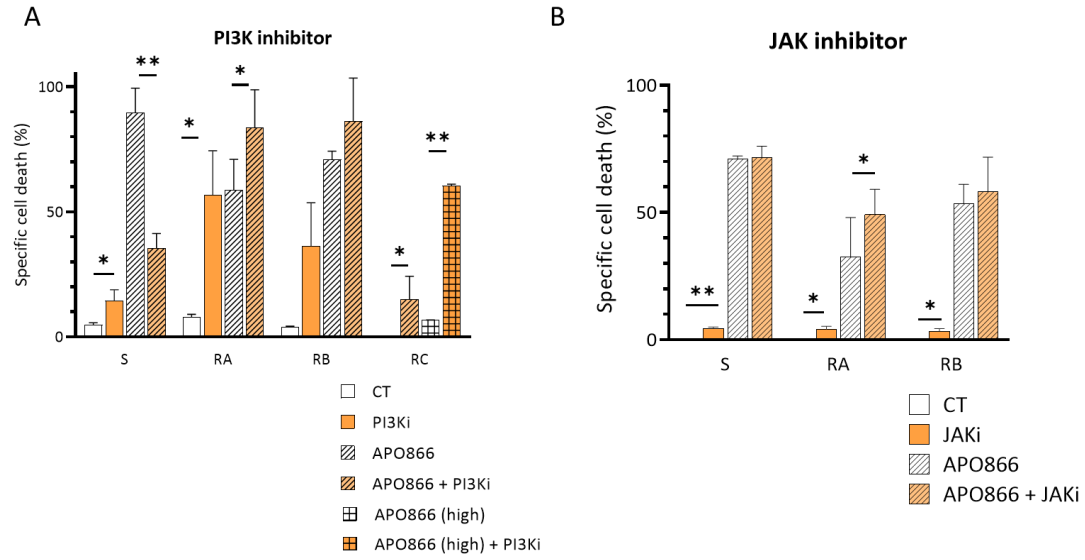

Figure S5: **Effects of pan-Jak and PI3K inhibition on APO866-induced cell death in ML2**. Specific cell death of ML2 incubated in presence of **A)** PI3K inhibitor copanlisib (1 µM) and **B)** pan-JAK inhibitor ruxolitinib (1 µM) as single agent or in co-treatment with APO866 (at IC_{20} to IC_{60}, or >1000 nM when indicated "high"). *n=3, error bars=SD. *p<0.05, **p<0.01.*

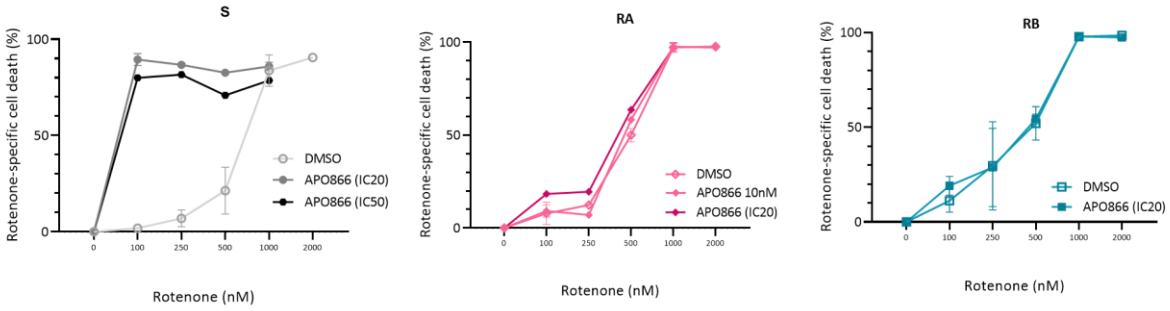

Figure S6: **Sensitivity of ML2 cells to mitochondrial ETC complex I inhibitor (rotenone).** Specific cell death of ML2 cells treated with a range of concentrations of rotenone (0 to 2000 nM), in presence or absence of APO866 (10nM, or respective IC_{20}).

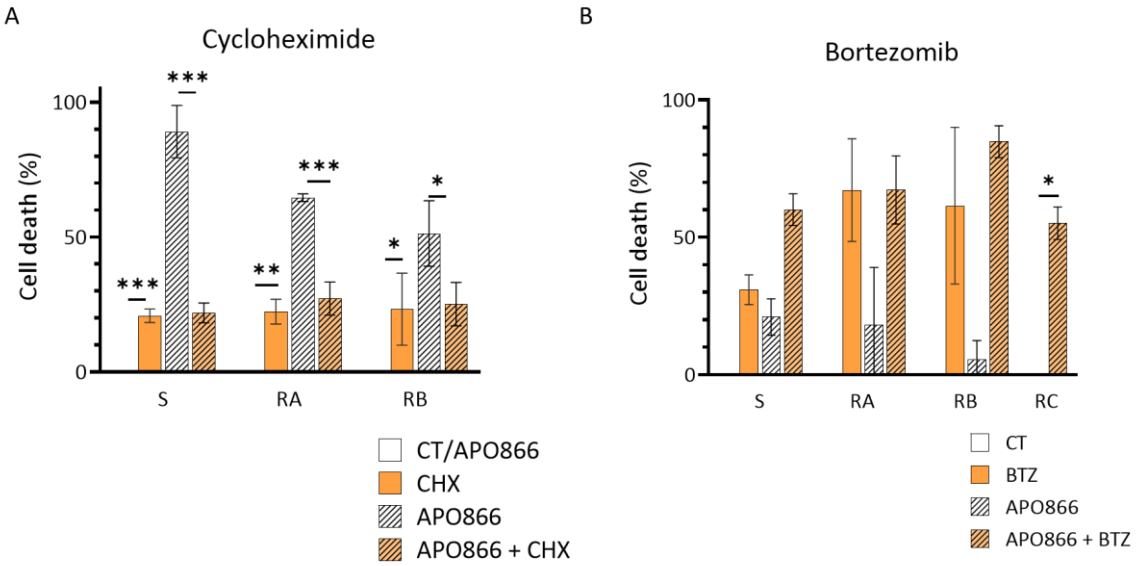

Figure S7: Effects of proteasome inhibitor and translation inhibitor on cell death of ML2 cells. Specific ell death of ML2 cells treated for 96 h with **A)** cycloheximide (500 nM) or with **B)** bortezomib (2 nM) as single agent or in combination with APO866. *n=3, error bars=SD. *p<0.05, **p<0.01, ***p<0.001.*

PROJECT 2

The following chapter is a reprint from the research article ElMokh *et al.*, published in *Cell Death & Disease* (2022).

ElMokh, O., Matsumoto, S., *et al.* Gut microbiota severely hampers the efficacy of NAD-lowering therapy in leukemia. *Cell Death Dis* **13**, 320 (2022).

https://doi.org/10.1038/s41419-022-04763-3

Summary of the results

Despite their striking antitumor activity demonstrated in *in vitro* studies, NAMPT inhibitors have failed to show antitumor effect in clinical trials. This suggests the need of better understanding the modulation of NAD metabolome in the organism. In this study, we show that gut microbiota contributes to counteracting APO866 antitumor effect by providing novel source of NAD precursor that can be exploited through an alternative pathway. Mechanistically, bacterial enzyme nicotinamidase contributes to the conversion of nicotinamide to nicotinic acid, providing an alternative NAPRT-dependent NAD biosynthesis pathway absent in mammalian cells. This allows tumor cells to avoid intracellular NAD depletion by NAMPT inhibitor. Of interest, we show that NAPRT inhibition or antibiotic treatment could restore the anticancer cytotoxicity of NAMPT inhibitor, demonstrating the promising efficacy of the dual targeting therapeutic strategy.

Personal Contributions

As a co-first author, I performed *in vitro* experiments, analysed the data, and wrote the manuscript, assisting Dr. Oussama ElMokh and with the guidance of Dr. Aimable Nahimana and Prof. Michel Duchosal.

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Gut microbiota severely hampers the efficacy of NAD-lowering therapy in leukemia

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Most cancer cells have high need for nicotinamide adenine dinucleotide (NAD^+) to sustain their survival. This led to the development of inhibitors of nicotinamide (NAM) phosphoribosyltransferase (NAMPT), the rate-limiting NAD^+ biosynthesis enzyme from NAM. Such inhibitors kill cancer cells in preclinical studies but failed in clinical ones. To identify parameters that could negatively affect the therapeutic efficacy of NAMPT inhibitors and propose therapeutic strategies to circumvent such failure, we performed metabolomics analyses in tumor environment and explored the effect of the interaction between microbiota and cancer cells. Here we show that tumor environment enriched in vitamin B3 (NAM) or nicotinic acid (NA) significantly lowers the anti-tumor efficacy of APO866, a prototypic NAMPT inhibitor. Additionally, bacteria (from the gut, or in the medium) can convert NAM into NA and thus fuel an alternative NAD synthesis pathway through NA. This leads to the rescue from NAD depletion, prevents reactive oxygen species production, preserves mitochondrial integrity, blunts ATP depletion, and protects cancer cells from death.

Our data in an in vivo preclinical model reveal that antibiotic therapy down-modulating gut microbiota can restore the anti-cancer efficacy of APO866. Alternatively, NAphosphoribosyltransferase inhibition may restore anti-cancer activity of NAMPT inhibitors in the presence of gut microbiota and of NAM in the diet.

Cell Death and Disease (2022)13:320 ; https://doi.org/10.1038/s41419-022-04763-3

INTRODUCTION

Compared to normal cells, most cancer cells have a high demand for nutrients and essential cofactors such as glucose, glutamine, and nicotinamide adenine dinucleotide (NAD^+), which sustain cancer cell proliferation and survival [1–3]. Tumor cells are expected to be more vulnerable to NAD^+ depletion than normal cells [4]. This notion led to the development of NAD^+ synthesis inhibitors for the clinical treatment of cancer [4–6].

Mammalian cells synthesize NAD^+ mainly through the salvage pathway utilizing nicotinamide (NAM) as a substrate, but also from other precursors that include tryptophan (via *de novo* pathway), nicotinic acid (NA, through the Preiss-Handler pathway), ribosylated NAM (NR) or NA (NAR), as well as the reduced form of NR (NRH) [7–14]. NAMPT is the rate-limiting enzyme that catalyzes the phosphoribosylation of NAM to produce nicotinamide mononucleotide (NMN). Despite the therapeutic efficacy of NAMPT inhibitors reported in several preclinical studies of solid and blood cancers [5, 15–21], the most promising agents (APO866 and GMX-1777) failed in clinical studies [22, 23], suggesting that alternative NAD^+ production routes may be active in humans. Shats et al. [24] recently reported that intestinal bacteria boost NAD^+ production in mammalian tissues through the activity of their enzyme, nicotinamidase (NMASE or PncA), which bypasses NAMPT inhibitors activity, and counteracts the anti-tumor effects of NAMPT inhibitor in a colon carcinoma cell line. Whether and how the microbiota may alter the anti-lymphoma/leukemia properties of NAMPT inhibitors has not been addressed.

In humans, the concentration of vitamin B3 (NA, NAM and related riboside derivatives) in the blood is very variable, with values ranging from 35 to 1487 nM and it is influenced by the type of diet and by the feeding status [25]. The level of NAD^+ precursors may considerably affect the anti-tumor efficiency of NAMPT inhibitors. These inhibitors induce individually variable levels of side effects, including retinopathy and thrombocythemia [22, 26]. In this study, we investigated the effect of tumor metabolic environment and the influence of the interaction between NA/NAM and the gut microbiota on the anti-leukemic activity of APO866. We show that tumor environments that are enriched in NA/NAM from the diet, markedly affect APO866 therapeutic efficacy. Furthermore, we show that when the tumor

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environment is specifically enriched in NAM, the anti-leukemic effect of APO866 is modulated by the levels of intestinal bacteria.

MATERIALS AND METHODS

Cell lines, primary cells and culture conditions

A panel of 21 hematopoietic cancer cell lines and primary cells from patients (listed in Table S1 and S2), was evaluated. *Escherichia coli* and *Saccharomyces cerevisiae* strains were kindly supplied by Dr Philippe Hauser (Institute of Microbiology, Lausanne University Hospital, Lausanne, Switzerland). All cells were cultured in RPMI (Invitrogen AG, 61870-01) supplemented with 10% heat inactivated fetal calf serum (Amimed, 2-01F30-I) and 1% penicillin/streptomycin at 37 °C (Amimed, 4-01F00-H) in a humidified atmosphere of 95% air and 5% CO2. To eliminate mycoplasma from cell culture, mycoplasma-infected leukemic cells, were cultured in the medium (as mentioned above), supplemented with BM-cyclin (Roche, Mannheim, Germany; Cat. No. 10799050001) according to the manufacturer's instructions.

Cell death characterization

Uninfected or bacteria-infected leukemic cells were cultured without or with APO866 (as indicated in each figure) in presence or absence of NAD^+ precursors. APO866-induced cell death was determined using ANNEXIN-V (ANN; eBioscience, BMS306FI/300) and 7 aminoactinomycin D (7AAD; Immunotech, A07704) stainings as described by the manufacturer and analyzed using flow cytometry. Dead cells were identified as ANN+ and/or 7AAD+.

Genome editing by CRISPR method

Single guide RNAs targeting the early exon (exon number 2) of PARP1 were chosen in the sgRNA library [27]. LentiCRISPR plasmid specific for NAPRT gene was created according to the provided instructions. Oligonucleotides were designed as follow: Forward 5'- CACCGCC-CACCTGGCGTAGCTGACC-3'; Reverse 3'- AAACGGTCAGCTACGC-CAGGTGGGC-5'. Oligonucleotides were synthetized, then phosphorylated and annealed to form oligo complex. LentiCRISPR vector was BsmBI digested and dephosphorylated. Linearized vector was purified, and gel extracted and ligated to oligo complex. The lentiCRISPR vector containing the sgRNA was then used for virus production. Cells were infected and selected with the appropriate dose of puromycin (1 µg/ml). Clone isolation was performed by limiting dilution in 96 well-plates.

TA cloning

TA cloning kit (Life technologies, K202020) was used according to manufacturer's instructions to sequence DNA fragment containing the region where Cas9 was guided by a sgRNA.

Immunoblotting

Protein samples were harvested in lysis buffer containing 20 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 2.5 mM EGTA, 0.1 mM dithiothreitol, 50 mM NaF, 1 mM Na_3VO_4. A protease inhibitor cocktail (Roche, 11873580001) was added. Lysates were sonicated and protein concentration was determined using a Bradford assay. Proteins (25–40 µg) were separated by SDS-PAGE on a 10% polyacrylamide gel and analyzed by immunoblotting. The mouse anti-NAPRT (#86634) and the rabbit anti-actin antibodies were purchased from Cell Signaling. After incubation with primary antibodies, the following secondary antibodies were applied: polyclonal goat anti-mouse or goat anti-rabbit IgG conjugated with IRDye 680 (LI-COR, B70920-02) or IRDye 800 (LI-COR, 926-32210). Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR). Full and uncropped western blots were uploaded as 'Supplemental Material'.

Quantification of NAD^+ metabolites using LC-MS/MS

Sample extraction. Serum samples (25 µL) were extracted with 225 µl of ice-cold methanol containing stable isotope-labeled metabolites. Sample extracts were centrifuged (15 min, 14000 rpm at 4 °C). The supernatant was collected and evaporated to dryness in a vacuum concentrator (LabConco, Missouri, US). Then sample extracts were reconstituted in 75 µL of water prior to LC-MS/MS analysis.

LC-MS/MS method. Extracted samples were analyzed by Liquid Chromatography coupled with tandem mass spectrometry (LC - MS/MS) in positive electrospray ionization (ESI) mode. An Agilent 1290 Infinite (Agilent Technologies, Santa Clara, California, US) ultra-high performance liquid chromatography (UHPLC) system was interfaced with Agilent 6495 LC-MS QqQ system equipped with an Agilent Jet Stream ESI source. This LC-MS/MS was used for the quantification of the intermediates implicated in NAD^+ *de novo synthesis* and *salvage* pathways.

The separation of NAD^+ metabolites implicated in salvage and Preiss-Handler pathway was carried out using the Scherzo SMC18 (3 µm 2.0 mm × 150 mm) column (Imtakt, MZ-Analysentechnik, Mainz, Germany). The mobile phase was composed of $A = 20$ mM Ammonium Formate and 0.1% formic acid in H_2O and B = Acetonitrile: Ammonium formate 20 mM and 0.1% formic acid (90:10, v/v). The gradient elution started at 100% A (0–2 min), reaching 100% B (2 min–12 min), then 100% B was held for 3 min and decreased to 100% A in 1 min following for an isocratic step at the initial conditions (16 –22 min). The flow rate was 200 µL/min, column temperature 30 °C and the sample injection volume was 2 µL. To avoid sample carry-over injection path was cleaned after each injection using a strong solvent (methanol 0.2% formic acid) and weak solvent (0.2% formic acid in water).

AJS ESI source conditions operating in positive mode were set as follows: dry gas temperature 290 °C, nebulizer 45 psi and flow 12 L/min, sheath gas temperature 350 °C and flow 12 L/min, nozzle voltage +500 V, and capillary voltage +4000 V. Dynamic Multiple Reaction Monitoring (DMRM) acquisition mode with a total cycle of 600 ms was used operating at the optimal collision energy for each metabolite transition.

Data processing. Data were processed using Mass Hunter Quantitative (Agilent). For absolute quantification, the calibration curve and the internal standard spike were used to determine the response factor. Linearity of the standard curves was evaluated using a 14-point range; in addition, peak area integration was manually curated and corrected where necessary. The concentrations of metabolites were corrected for the ratio of peak area between the analyte and the ISTD, to account for matrix effects.

Stool collection, bacterial DNA detection and quantification

Bacterial DNA from collected mouse stools were extracted using the Power Fecal PRO kit (ref. 51804). The quantitative PCR was performed with 5 µL DNA, 200 nM of each primer Eubact_27F (AGAGTTTGATCMTGGCTCAG) and Eubact_244R (ACTGCTGCCTCCCGTAG) [28] and 10 µL iTaq Universal SYBR Green Supermix (BioRad, Switzerland, ref. 172–5122) as follows: start 95 °C for 5', denaturation at 95 °C for 15" and hybridization at 60 °C for 1' repeated for 40 cycles. The analyses were performed on the StepOne Plus. Ten-fold dilutions of a control plasmid prepared by RDBiotech (France) was used to calibrate the qPCR.

Generation of intestinal microbiota-depleted mice and evaluation of therapeutic efficacy of APO866 in mouse xenograft with intact or depleted intestinal bacteria

Six- to 8-week female non-leaky C.B.-17 severe combined immune deficiency (SCID) mice (Iffa Credo, L' Arbresle, France) were bred and housed in micro-isolator cages in a specific pathogen-free room within the animal facilities at the University Hospital of Lausanne. Animals were allowed to acclimatize to their new environment for 1 week prior to use. All animals were handled according to the respective institutional regulations after approval of the animal ethics committee of the University of Lausanne. Depletion of the intestinal microbiota was performed as described elsewhere [29] using an antibiotic cocktail of streptomycin (500 mg/kg, Sigma-Aldrich, Saint-Louis, USA; Cat. No. S9137), gentamicin (125 mg/kg, Sigma-Aldrich, Saint-Louis, USA; Cat. No. G1264), bacitracin (250 mg/kg, Sigma-Aldrich, Saint-Louis, USA; Cat. No. 11702) and ciprofloxacin (67.5 mg/kg, Sigma-Aldrich, Saint-Louis, USA; Cat. No. 17850) in NaCl 0.9% administered daily by oral gavage. Stools were routinely collected. The sample size was chosen on the basis of an adequate power using a student test (T-test), between 2 means (Control vs treated), based on data from our previous studies [30]. The accepted statistical significativity (alpha) was 0.05, with an obtained adequate power of 0.85.

The in vivo evaluation of APO866 was carried out using a xenograft model of ML-2 human AML. SCID mice with intact or depleted intestinal bacteria were fed with various diets, enriched without or with either NAM or NA, at least one week before being transplanted subcutaneously into the right flank with ML-2 cells (10^7). Once the tumor reached a volume of 100–150 mm^3, mice were randomly subdivided into untreated (control or vehicle) and APO866-treated groups. Mice were administered

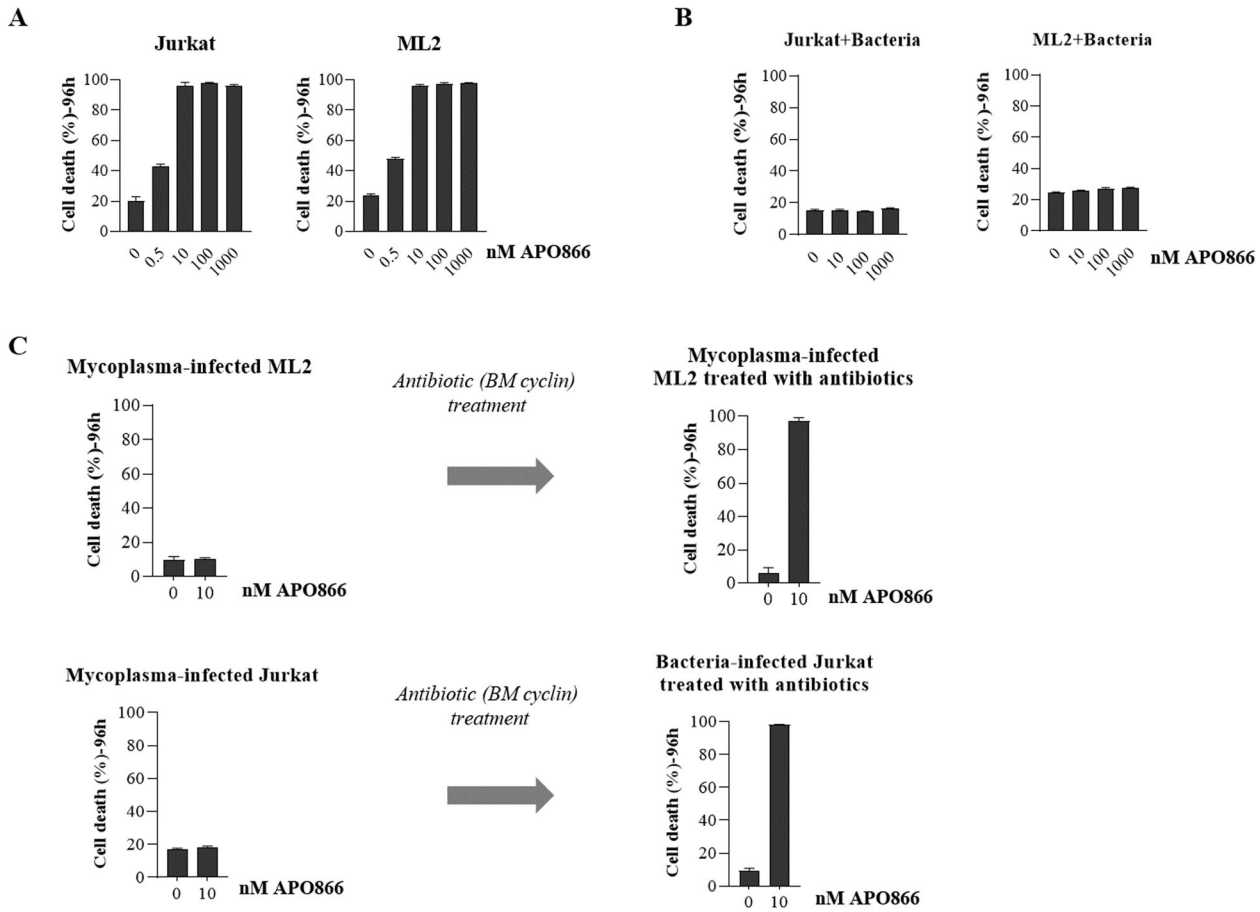

Fig. 1 Bacteria protect leukemic cells from the anti-tumor activity of APO866 without affecting the drug uptake, the capacities to proliferate or to form colonies. Dose dependent analysis of cell death on two unrelated uninfected (**A**) and bacteria-infected (**B**) leukemic cells (Jurkat and ML2) exposed to APO866 for 96 h. Cell death was assessed using annexin and 7AAD stainings and analyzed by flow cytometry. Cells stained positively for either annexin or 7AAD alone or both were considered dead cells. **C** APO866 sensitivities of bacteria-infected ML2 and Jurkat cells, before and after antibiotic treatment. Data are mean ± SD, derived from at least three independent experiments.

intraperitoneally with APO866 (15 mg/kg body weight) in 200 µL 0.9% saline, twice a day for 4 days, repeated weekly over 3 weeks. Control groups were treated similarly with saline solution. All animals were monitored daily for signs of illness and killed immediately if tumor size reached a diameter of 15 mm. It is noteworthy to mention that depletion of the intestinal microbiota was confirmed before mice were xenografted. The investigator was not blinded to the group allocation during the experiment.

Statistical analysis

Data are expressed as mean plus or minus standard error of the mean (SEM) unless otherwise noted. Values between groups were compared using non-parametric test. The Kaplan-Meier method using long rank test was applied for the analyses of animal survival studies. GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. P values less than .05 were considered statistically significant.

RESULTS

Bacteria abrogate the anti-tumor activities of APO866 in several hematopoietic malignant cells

To examine the effect of bacteria on the anti-leukemic effects of NAMPT inhibitors, we took advantage of cell lines that were infected or not with Mycoplasma. First, we sought to identify the Mycoplasma species infecting our cell cultures. Using a high-throughput detection and multiplex identification of cell contamination test [31], Table S3 shows that the cell cultures were infected with *Mycoplasma arginini*. Supernatant from Mycoplasma-contaminated cell cultures were used to infect other leukemic cell cultures (dilution factor: 100–200). Next, two unrelated uninfected and Mycoplasma-infected leukemia cell lines were incubated with various concentrations of APO866 (0–1000 nM) for 96 h and subsequently double stained using annexin V/7AAD to monitor cell death. As shown in Fig. 1A, APO866 effectively killed malignant hematopoietic cells: specifically, 10 nM APO866 was sufficient to kill 100% of the leukemic cells, which is in line with our previous studies [30, 32, 33]. This dose was therefore chosen for subsequent experiments. In contrast, the presence of Mycoplasma in cell culture fully abolished the anti-leukemia effects of APO866 (Fig. 1B). Of note, one can observe that Mycoplasma contamination per se did not affect neither drug uptake (Fig. S1A), nor cell viability (Fig. S1B), whereas it abrogated the capacity of APO866 to inhibit cell proliferation (Fig. S1C) and to block clonogenicity (Fig. S1D). Mycoplasma-infected leukemia cells were re-sensitized to APO866 after bacterial elimination with BM cyclin antibiotic treatments (Fig. 1C), thus confirming that bacteria were responsible for the lack of APO866 anti-tumor effect. It is noteworthy to mention that BM cyclin treatment eliminated Mycoplasma from infected leukemic cells, as shown in Fig. S2A. We extended our observation on a wide range of hematopoietic malignant cells and confirmed that the presence of bacteria in culture can abrogate the anti-tumor activity of APO866 on all tested cell lines and primary hematopoietic malignant cells (Table S4 and Fig. 2).

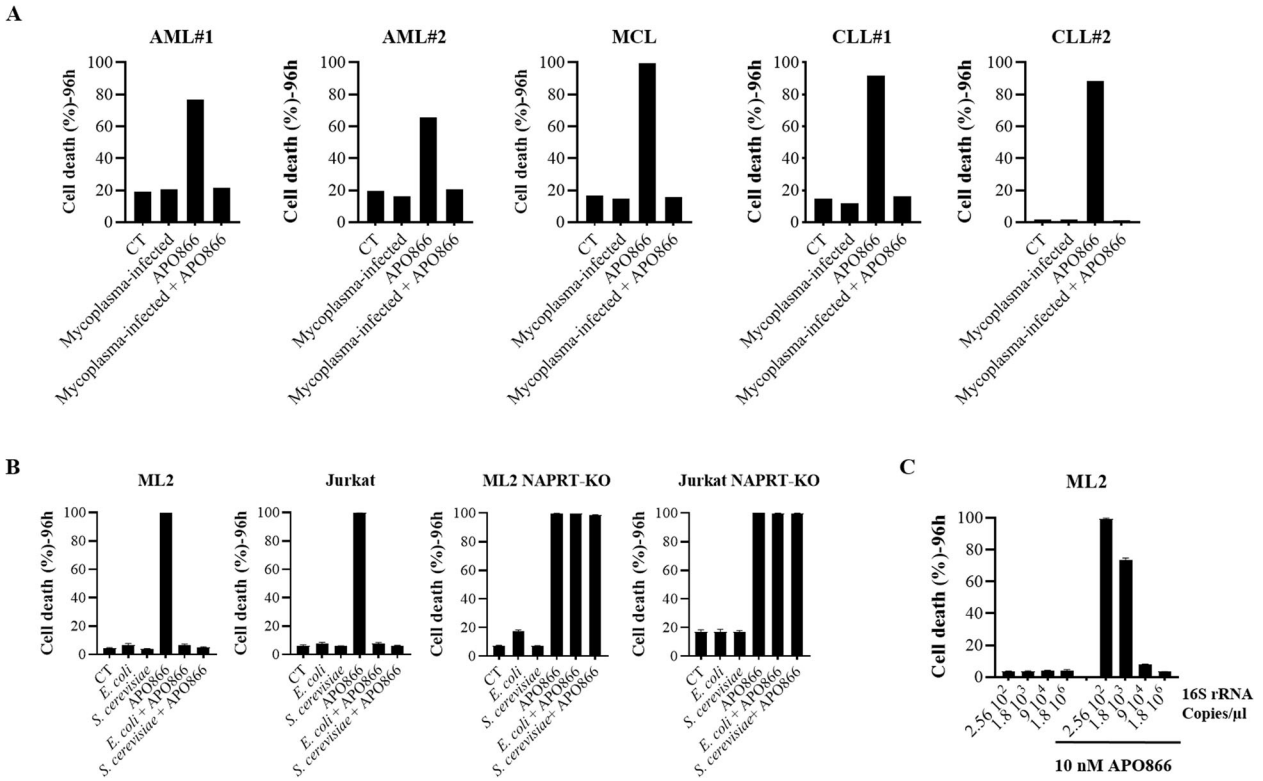

Fig. 2 Bacteria abrogate the anti-leukemic/lymphoma effects of APO866 in primary cells from patients diagnosed with various hematological malignancies and in several leukemia/lymphoma cell lines. Cell death analysis on several uninfected and bacteria-infected primary leukemic cells (**A**) and ML-2 cells infected with different cell number of *E. coli* exposed to 10 nM APO866 was monitored as mentioned in Fig. 1. APO866 sensitivities of (**B**) ML-2 and Jurkat cells (both either WT or NAPRT-KO) infected with *E.coli* or *S.cerevisiae* and of (**C**) ML-2 cells exposed to different *E. coli* quantities (inocula). Cell death was assessed as described in Fig. 1. Data are mean ± SD, derived from at least three independent experiments.

Collectively, these results suggest that the efficacy of NAMPT inhibitors may be strongly reduced by bacteria and that such efficacy could possibly be restored by antibiotic treatment.

Bacteria circumvent APO866-induced anti-leukemic activities by activating the Preiss-Handler pathway of NAD^+ synthesis

Bacteria possess NMASE, which converts NAM to NA that serves as an NAD^+ precursor for leukemic cells to synthesize NAD^+ via the Preiss-Handler pathway. The resulting NAD^+ production may therefore provide a mechanism to bypass the anti-cancer effects of NAMPT inhibition. To test this hypothesis, we measured by LC-MS/MS the content of NAD^+ metabolites in conditioned medium (CM) from uninfected and from Mycoplasma-infected leukemia cells, in the absence or presence of 10 nM APO866. The CM from uninfected leukemic cells contained mainly NAM and just faint traces of NA (Fig. 3A). In the CM from Mycoplasma-infected leukemia cells, NAM was converted into NA, as evidenced by a significant increase in NA that correlated with a corresponding decrease in NAM. We next evaluated whether the presence of NA in the CM was followed by activation of the Preiss-Handler pathway for NAD^+ production in leukemia cells. As shown in Fig. 3B and summarized in Fig. 3C, in comparison with uninfected leukemia cells, Mycoplasma-infected cells displayed a significant increase in NAD^+ intermediate metabolites that are involved in NAD^+ synthesis from NA. Consistent with our observation, Fig. 4A shows that supplementation with NA could abrogate the anti-tumor activity of APO866 and that less than 1 µM of NA is sufficient to protect cells against APO866-induced cytotoxicity. To demonstrate further that bacteria protect leukemic cells via the Preiss-Handler NAD^+ production pathway, we generated leukemic cells in which NAPRT is knocked-out (KO) (Fig. 4B). As shown in Fig. 4C, in these NAPRT-KO cells, NA failed to prevent APO866-induced cell death, but the downstream product of NAPRT, nicotinic acid mononucleotide (NAMN), fully reversed APO866-mediated cell killing. In agreement with this observation, NAPRT-KO leukemic cells were highly sensitive to APO866 treatment despite the presence of Mycoplasma in cell culture (Fig. 4D). Finally, we measured the NAD^+ metabolites in the CM from uninfected and Mycoplasma-infected NAPRT-KO leukemic cells treated with APO866. As expected, in these cells the presence of bacteria increased NA in the CM (Fig. 5A) but failed to increase intracellular NAD^+ metabolites involved in the Preiss-Handler pathway (Fig. 5A–C). In agreement with the above-mentioned data, Mycoplasma-infected leukemic cells displayed an increased NMASE activity compared to uninfected ones (Fig. S2B). To extend this finding to any organism that possesses NMASE, we used unrelated microorganisms possessing the latter enzyme, namely *Escherichia coli* or *Saccharomyces cerevisiae*, to infect leukemic cells that were next incubated with or without APO866. Figure 2B shows that both *E. coli* and *S. cerevisiae* fully abrogated the APO866-induced cell death in untransfected but not in NAPRT-KO leukemic cells. To demonstrate that bacteria confer resistance to APO866 treatment, ML-2 cells exposed to different *E. coli* quantities (inocula), were treated without or with APO866 and cell death monitored. As expected, Fig. 2C shows that the protective effect to APO866 treatment correlated with bacterial copy number in culture. Of great interest, hematopoietic malignant cells supplemented with filtered (through a PVDF filter 0.1 µM) CM from either *E. coli* or *S. cerevisiae*-infected leukemic cell culture abolished APO866 cytotoxic activity (Fig. S2C), indicating that both microorganisms produce a soluble factor that could blunt the anti-tumor activity of NAMPT inhibitor.

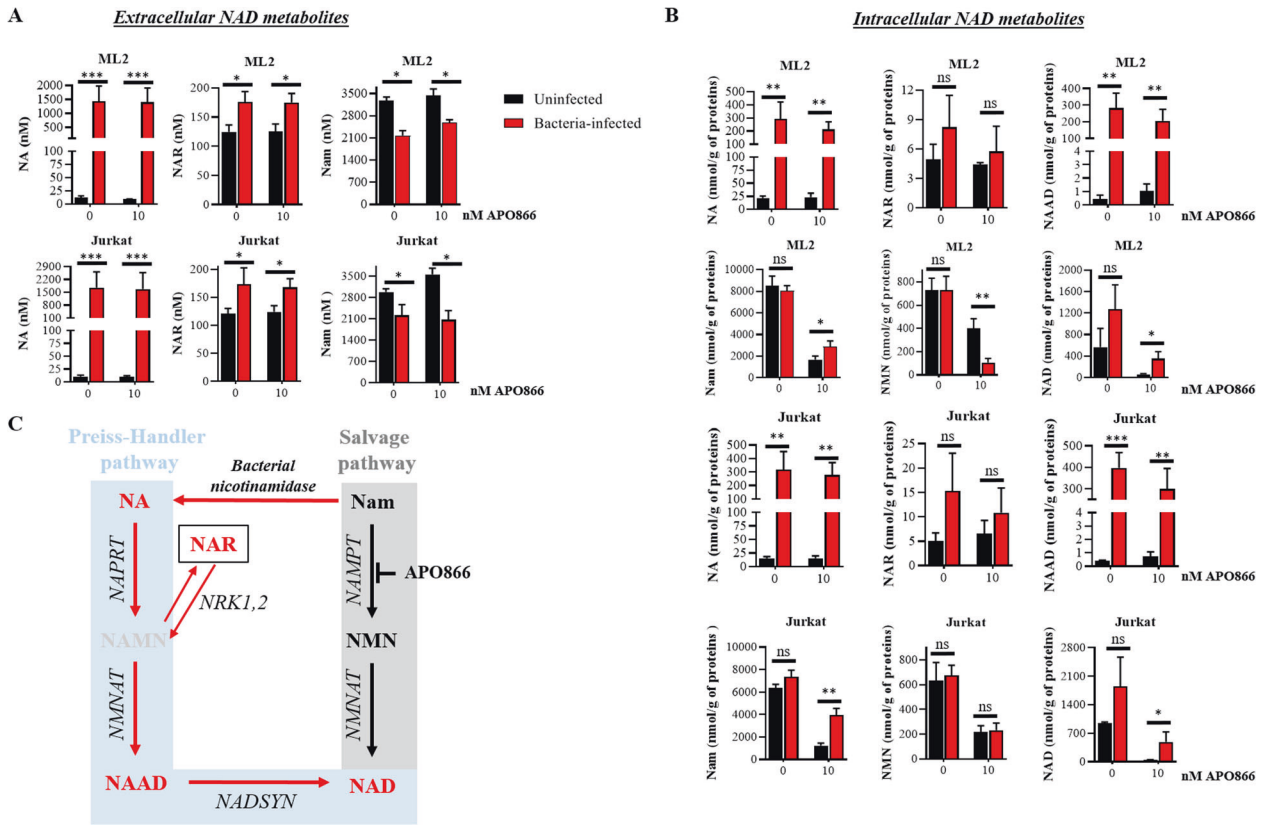

Fig. 3 Bacteria circumvent APO866-induced anti-leukemic activities by activating the Preiss-Handler pathway to synthesize NAD^+. Quantification of extracellular (**A**) or intracellular (**B**) NAD^+ metabolites in cell-conditioned medium (or supernatant) or within cells from uninfected- or bacteria-infected ML-2 and Jurkat cells, using LC-MS/MS. **C** Summary of identified NAD^+ metabolites: in red, metabolites significantly increased; black, no changes; and grey, metabolites, not detected (below the limit of detection). Data are derived from at least three independent experiments. Data are mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns Not significant.

Cancer stem cells (CSC) play a major role in drug resistance (or relapse) and are also known to significantly affect cancer therapy [34]. We examined whether CSC could be involved in the resistance to APO866 treatment. To this end, we examined the major CSC properties, such as stem cell markers (CD34, CD117, and CD123) expression and quiescence status of uninfected vs bacteria-infected leukemic cells. No stem cell markers were found on both uninfected and bacteria-infected leukemic cells (Fig. S3A) and there was no difference in terms of quiescent status between uninfected vs bacteria-infected leukemic cells (Fig. S3B). The results strongly suggest that CSC are not involved in APO866 resistance in our experimental model.

Altogether, the data suggest that whenever NAM is available, any NA-producing microorganisms such as various bacteria species (for instance gut microbiota), may strongly reduce the efficacy of NAMPT inhibitors by activating NAD^+ production through the Preiss-Handler pathway in cancer cells. Silencing NAPRT in tumor cells could restore the therapeutic efficacy of NAMPT inhibitors even in the presence of bacteria and NAM.

Tumor metabolic environment and intestinal bacteria inhibit the in vivo efficacy of APO866 in xenograft model of human leukemia

To examine the influence of the tumor environment on the anti-cancer activity of APO866, mice were fed with a diet that was poor in vitamin B3 (a standard diet contains 130 mg/kg of food NA), or with diets that were either enriched in NAM (1 g/kg of food) or in NA (1 g/kg of food) for two weeks before being grafted subcutaneously with ML-2 cells. In mice receiving the standard diet, treatment with APO866 cleared tumor cells and resulted in 100% survival for more than 5 weeks of observation, whereas all mice from the untreated group died within 2 weeks with tumors (Fig. 6A). In contrast, feeding mice with diet enriched in either NAM or NA abrogated the therapeutic efficacy of APO866 (Fig. 6B, C), strongly suggesting that the nutritional content of vitamin B3 should be considered to optimize the efficacy of NAMPT inhibitors. We next evaluated whether the intestinal microbiota could affect the efficacy of NAMPT inhibitors in leukemia treatment using the above-described in vivo xenograft model of human AML. Mice were fed with different diets (as described above) and were treated with a cocktail of antibiotics that was recently reported to be efficient at depleting intestinal bacteria [29]. Microbiota depletion was assessed weekly by analyzing bacterial content in stools, using universal 16S rRNA bacterial primers. As reported in Fig. 6D, antibiotic treatment decreased bacterial PCR copy number by 2 logs in comparison to stools collected before initiation of antibiotic treatment. As shown in Fig. 6E, in the control group (standard diet and without APO866 treatment), all mice died within 2 weeks post-transplantation, with a median survival of 8 days (95% confidence interval [3.8–60.5]). In the group of microbiota-depleted mice fed with the same standard diet, APO866 exerted a strong therapeutic effect, with 100% of mice surviving and remaining disease-free for the 40-day duration of the study. When antibiotic-treated mice were put on a NAM-enriched diet, administration of APO866 prevented tumor development and significantly prolonged mouse survival, with a median survival of 23 days (95% confidence interval [0.9 to 6.7]) compared with control which presented a median survival of 9 days (95% confidence interval [0.15–1.02]) (Fig. 6F). The result

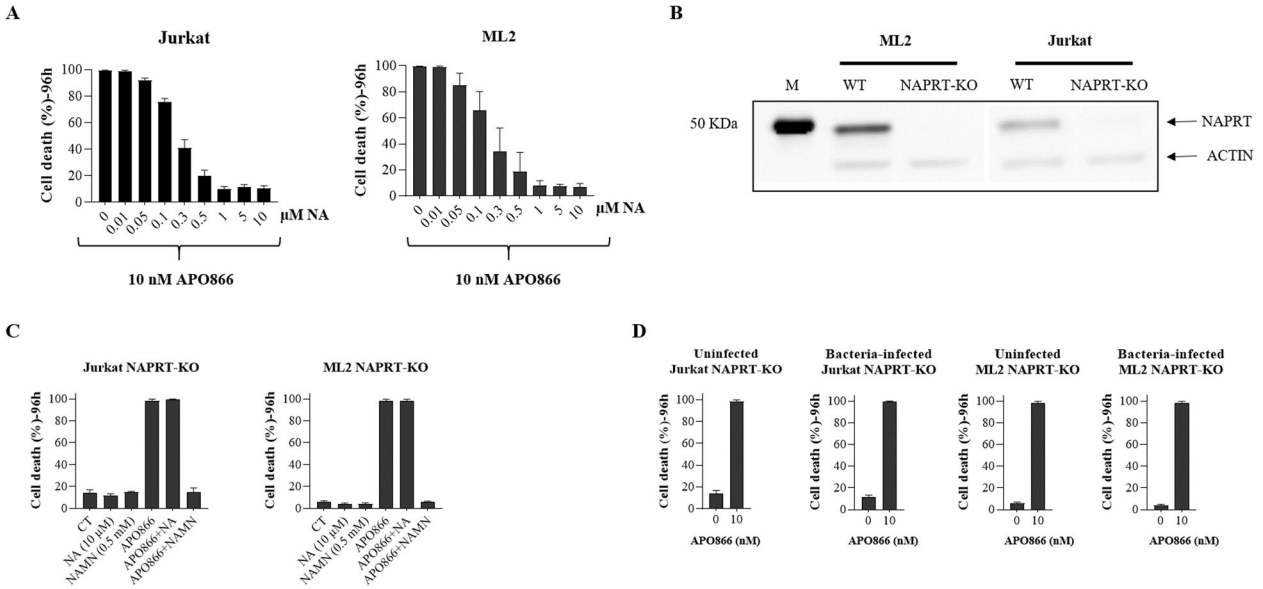

Fig. 4 The abrogation of the anti-leukemic effect of APO866 in bacteria-infected leukemic cells requires the integrity of NAPRT. A ML-2 or Jurkat cells were incubated with or without various concentration of NA in presence of 10 nM APO866 for 96 h and cell death was assessed as described in Fig. 1. **B** NAPRT was knocked-out in wild type ML-2 and Jurkat cells using CRISPR/Cas9 technology; loss of expression was confirmed by Western blotting. **C** Jurkat NAPRT-KO and ML2 NAPRT-KO cells were pre-incubated with or without NA (10 μM) or NAMN (0.5 mM) before exposure to 10 nM APO866 for 96 h and cell death was assessed as described in Fig. 1. **D** Uninfected and bacteria-infected Jurkat/ML2 NAPRT-KO cells were treated without or with 10 nM APO866 and cell death monitored as described in Fig. 1. Data are derived from at least three independent experiments, and they are shown as mean ± SD.

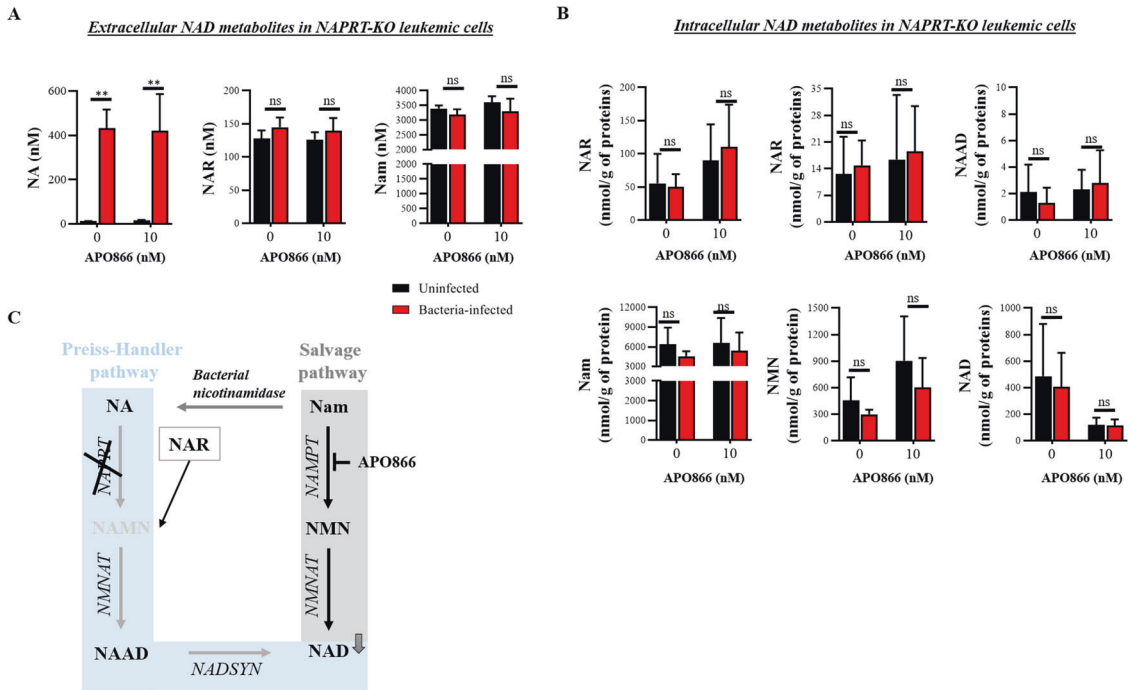

Fig. 5 Bacteria-infected NAPRT-KO leukemic cells do not lead to activation of the Preiss-Handler pathway despite the significant increase of NA in the conditioned medium. Quantification of extracellular (**A**) or intracellular (**B**) NAD^+ metabolites in cell-conditioned medium or within cells from uninfected or bacteria-infected NAPRT-KO ML-2 cells, using LC-MS/MS. **C** Summary of identified NAD^+ metabolites: in red, metabolites significantly increased; black, no changes; and grey, metabolites not detected (below the limit of detection). Data are derived from at least three independent experiments. Data are mean ± SD, $^{**}p < 0.01$, ns not significant.

shows that NAM-enriched diet cannot fully abrogate APO866 anti-tumor effect when microbiota is depleted, hampering any conversion of NAM to NA to bypass NAMPT inhibition in tumors. In contrast, feeding mice with a NA-enriched diet completely abrogated the therapeutic efficacy of APO866, despite the depletion of gut microbiota with antibiotics (Fig. 6G). Antibiotic treatment per se had no effect on tumor growth. As in our previous studies [30, 32, 35–37], the chosen dose of APO866 (15 mg/kg) was well tolerated as demonstrated by no premature deaths and by the survival of all mice treated with APO866

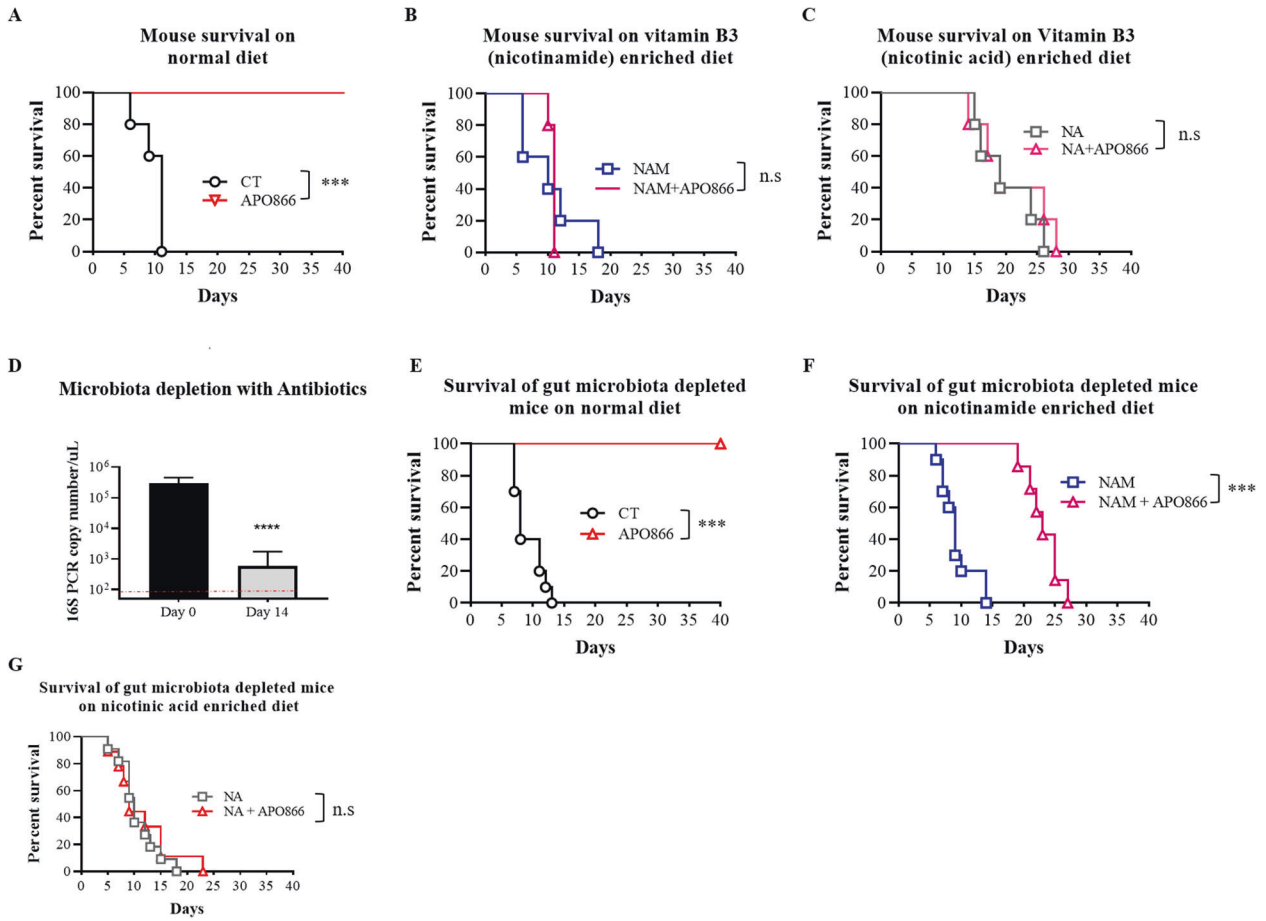

Fig. 6 Tumor metabolic environment and intestinal bacteria severely hamper the in vivo efficacy of APO866 in xenograft model of human leukemia. In vivo efficacy of APO866 on SCID mice with intact (**A**–**C**; $n = 5$) or depleted intestinal bacteria (**E**–**G**; $n \geq 7$), xenografted with ML-2 cells and fed with normal diet (**A**, **E**), enriched diet with NAM (**B**, **F**), or NA (**C**, **G**). 6- to 8-week-old C.B.-17 SCID mice were fed with different diets either poor in vitamin B3 (normal diet) or enriched in vitamin B3 (NAM or NA) for two weeks and subsequently treated with or without a cocktail of antibiotics to deplete intestinal microbiota before being transplanted subcutaneously with ML-2 cells. Once tumor sizes of 100–150 mm^3 were reached, mice were randomized into a control group (black line) and a treated group (colored line). APO866 administration and tumor volume assessment were conducted as described in "Methods" section. All animals were monitored daily, and the study was ended when tumor volume reached around 1000 mm^3. ***$P < 0.001$. **D** 16S rRNA PCR analysis on murine stool samples before and after antibiotic cocktail treatment using universal bacterial primers. Data are mean ± SD, $n \geq 9$. Dot line indicates lower limit detection for PCR.

throughout the 40 days of observation, without signs of toxicity including loss of body weight, lethargy or rough coat.

Collectively, these data underscore that the tumor metabolic environment as well as gut microbiota can interfere with the therapeutic efficacy of a NAMPT inhibitor.

DISCUSSION

Here we show that the amount of NAM or NA contained in food and of bacteria from the gut microbiota can severely modulate the anti-leukemia activity of a NAMPT inhibitor. Numerous bacteria species, including those found in gut microbiota, contain *PncA* [38], enabling them to convert NAM into NA, which in turn activates an alternative route of NAD^+ biosynthesis, "the Preiss-Handler pathway". This circumvents the NAD^+ depletion that would normally be induced by the blockade of the NAD^+ salvage pathway by NAMPT inhibitors. Accordingly, as an example, CM from Mycoplasma-infected cells displays high levels of NA that correlate with a decrease of NAM. Furthermore, supplementation with filtered CM from bacteria-infected cells or exogenous NA protects from APO866-induced tumor cell cytotoxicity both in vitro and in vivo. APO866 significantly depletes intracellular NAD^+ content in both uninfected and bacteria-infected malignant hematopoietic cells but the residual NAD^+ synthesized from NA is sufficient to prevent reactive oxygen species (ROS, Fig. S4A, S4B) production, preserve mitochondrial integrity (Fig. S5A), blunt the ATP depletion (Fig. S6) and thus protect leukemic/lymphoma cells from APO866-induced cell death (Fig. S5B). We provide evidence using xenograft model of human leukemia that gut microbiota can protect tumor cells from APO866-induced cell death if the diet is enriched in NAM. Importantly, our data strongly suggest that silencing/inhibition of NAPRT or antibiotic therapy could restore the anti-leukemia efficacy of APO866 despite the presence of bacteria.

The loss of the anti-tumor activity of NAMPT inhibitors in presence of bacteria supports the recent study by Shats et al. [24] reporting that bacteria can boost the mammalian host NAD^+ and thereby confer resistance to NAMPT inhibitors in an in vivo setting of colon cancer. Here, we provide for the first time in vivo evidence that gut bacteria, despite being hosted at distant sites from a tumor, can protect leukemia/lymphoma cells from NAMPT inhibitor-induced death. This finding could be explained by the fact that bacteria produce a soluble factor (Fig. S2C) that circulate through the body and thus, confers resistance at distant sites. NMASE, which is present in many bacteria species [38–40], converts NAM to NA whenever NAM is made available to such bacteria, providing an alternative

NAD^+ precursor for cancer cells. Any therapeutic strategy aiming to block only one route of NAD^+ biosynthesis should therefore be expected to fail in tumor cells in which the enzymatic apparatus of both NAD^+ production routes (via NAMPT and via NAPRT) is expressed. Specifically, our data strongly suggest that two conditions are required for gut bacteria to protect tumor cells from APO866-induced cell death: 1) the expression of NAPRT and 2) the availability of sufficient amounts of NAM to bacteria. We demonstrate the possibility of reversing bacterial protection of leukemia cells by optimizing the NAD^+-lowering therapy via two alternative strategies: i) simultaneously inhibiting both NAMPT and NAPRT or ii) combining NAMPT inhibition with oral antibiotic therapy aiming at depleting gut microbiota. Both approaches warrant being tested in clinical studies.

Our data indirectly support the concept that sensitizing cancer cells to NAMPT inhibitors in humans may need to combine them with additional inhibitor(s). We recently showed that inhibiting NAPRT could prove useful for sensitizing several cancer cells to NAMPT inhibitors [36]. Furthermore, these inhibitors could be specifically targeted to tumoral cells to prevent toxicity due to profound NAD^+ depletion in healthy cells. Of note, NAMPT inhibition can be combined with inhibitors of metabolic or signaling pathways that act synergistically to kill tumoral cells. This was exemplified by Zhang et al. [41], who provided evidence that inhibition of two histone deacetylases, HDAC8 and SIRT6, enhances the anti-leukemic effect of NAMPT inhibitor in AML with minimal toxicity to healthy cells.

Although in our context, bacteria confer resistance to NAMPT inhibitors mainly via modulation of NAD^+ metabolites, there is growing evidence indicating that host commensal microbiota can also modulate the efficacy of cancer therapy via other mechanisms that include immunomodulation, activation of autophagy, translocation, and drug metabolism [42–45]. We cannot firmly rule out the possibility that the resistance to NAMPT inhibitors observed in the present study could also reflect, at least in part, such additional mechanisms and thus, further experiments are warranted to elucidate this issue.

In conclusion, we demonstrate that a NAM-rich diet, through its take-up by the gut microbiota, can confer resistance to APO866, a very potent NAMPT inhibitor. Lowering specific NAD^+ precursors in the diet, silencing or inhibiting NAPRT in cancer cells or depleting the gut microbiota using antibiotics are all potential avenues for optimizing the activity of NAMPT inhibitors in hematological malignancies.

DATA AVAILABILITY

The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

OEM, SM, PB, AB, HGA and JI performed, analyzed data and wrote the paper. KS, FP, IS, and ANe analyzed results and wrote the paper. ANa designed, executed and analyzed experiments, coordinated the project, and wrote the paper. MAD designed and analyzed experiments, coordinated the project, and wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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PROJECT 2 - SUPPLEMENTARY DATA

Table S1. Cell line description

Disease diagnosis	Cell line	Provider	Catalogue no.
Acute Myeloid Leukemia (AML)	HEL (M6)	DSMZ	ACC 011
	MV4-11 (M5)	DSMZ	ACC 102
	THP-1	ATCC	TIB-202
	SKM-1 (M5)	DSMZ	ACC 547
	NOMO-1 (M5)	DSMZ	ACC 542
	ML-2 (M5)	DSMZ	ACC 015
	NB-4 (M3)	DSMZ	ACC 207
	HL-60 (M2)	ATCC	CCL-240
	Kasumi (M2)	DSMZ	ACC 220
T-Acute Lymphoblastic Leukemia (T-ALL)	Jurkat	ATCC	TIB-152
	CCRF-CEM	DSMZ	ACC 240
	MOLT-4	DSMZ	ACC 362
B-Chronic Lymphoblastic Leukemia (B-CLL)	Mec-1	DSMZ	ACC 497
Burkitt Lymphoma	Ramos	DSMZ	ACC 603
Multiple Myeloma (MM)	RPMI-8226	ATCC	CCL-155
	MOLP-8	DSMZ	ACC 569

ATCC: American Type Culture Collection; DSMZ: German Collection of Microorganisms and Cell cultures

Table S2. Primary cells from patients

Patient diagnosis	Sample no	Place
Acute Myeloid Leukemia (AML)	AML #1	CHUV
	AML#2	CHUV
Mantle Cell Lymphoma (MCL)	MCL	CHUV
B-Chronic Lymphoblastic Leukemia (B-CLL)	B-CLL #1	CHUV
	B-CLL #2	CHUV

CHUV: Centre Hospitalier Universitaire Vaudois

Table S3. Leukemic cells are infected with Mycoplasma arginini

Cell line	Species	Myoplasma species	Conclusion
Uninfected ML2 WT	Human		clean
Uninfected Jurkat WT	Human		clean
Mycoplasma-infected ML2	Human	*Mycoplasma arginini*	Contaminated
Mycoplasma-infected Jurkat	Human	*Mycoplasma arginini*	Contaminated

Table S4. Bacteria abrogate the anti-leukemic effects of APO866 in different primary leukemic cells

	Cell death (mean +/-SD); 10nM APO866, 96h	
Disease fiagnosis/ cell line	**Uninfected**	**Bacteria-infected**
Acute Myeloid Leukemia (AML)		
MV4-11	98.5 +/- 1.2	4.6 +/- 0.3
NOMO-1	98.2 +/- 1.1	4 +/- 0.1
SKM-1	98.1 +/- 0.05	4.3 +/- 0.3
THP-1	98.7 +/- 0.9	4.1 +/- 0.4
NB-4	96.8 +/- 0.5	3.8 +/- 0.5
HL-60	87.7 +/- 0.7	3.8 +/- 0.5
Acute lymphoblastic leukemia		
CCRF-CEM	82.6 +/- 4.6	14.4 +/- 0.3
MOLT-4	87.7 +/- 0.6	13.6 +/- 0.7
B-Chronic Lymphocytic Leukemia (B-CLL)		
Mec-1	60.4 +/- 1.3	9.1 +/- 0.7
Burkitt lymphoma		
Ramos	93 +/- 3	11.1 +/- 1.5
Multiple myeloma		
RPMI-8226	80.4 +/- 1.3	9.5 +/- 0.8
MOLP-8	95 +/- 1.2	9 +/- 0.2

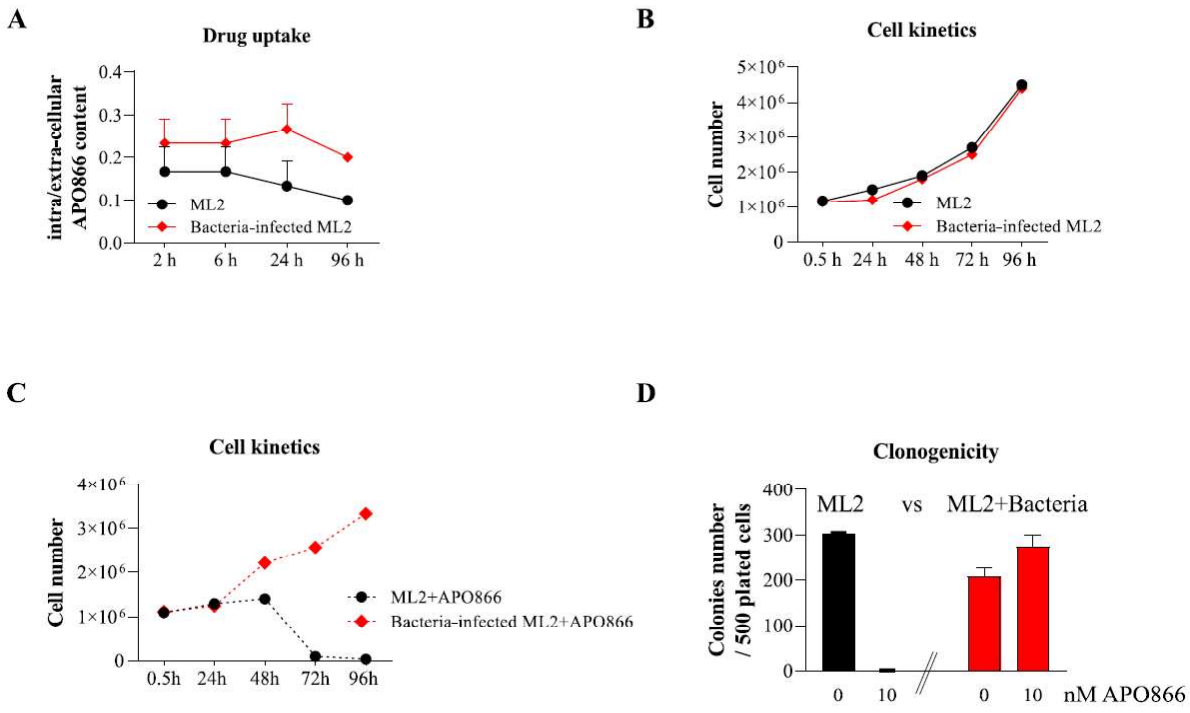

Figure S1. Bacteria protect leukemic cells from the anti-tumor activities of APO866 without affecting their drug uptake, capacity to proliferate, and propensy to form colonies.

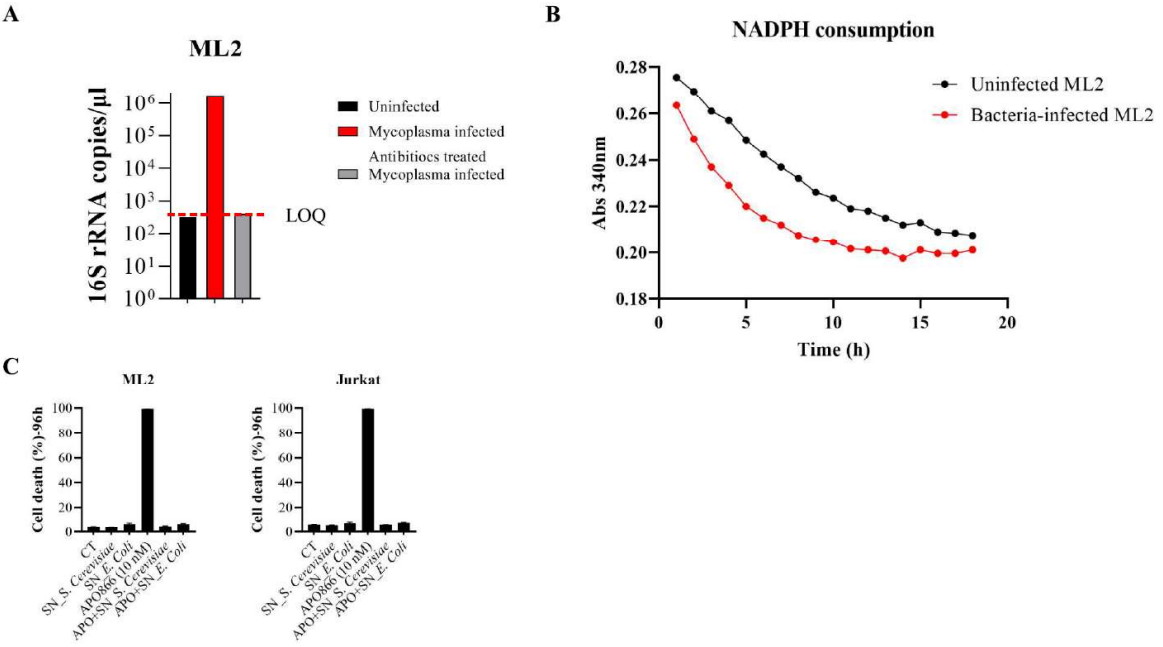

Figure S2. Antibiotic (BM cyclin) treatments eliminate Mycoplasma from infected-leukemic cells (A). Bacteria-infected cells display an increased nicotinamidase activity compared to uninfected ones (B) . Filtered supernant from *E. coli* infected leukemic cells confers resistance to APO866 (C).

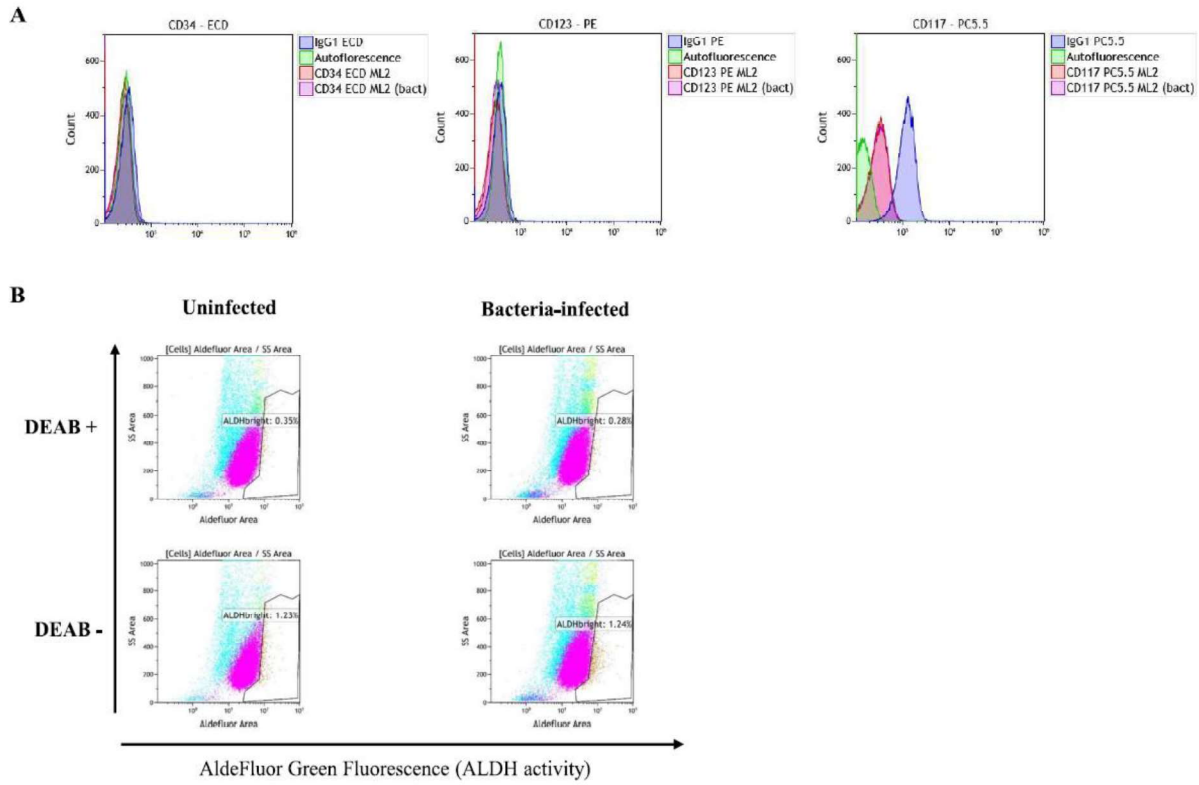

Figure S3. Uninfected and bactera-infected leukemic cells do not present in stem cell markers expression (A) as well as in quiescent status evaluated by AldeFluor Assay (Stem Cell techonologies) (B).

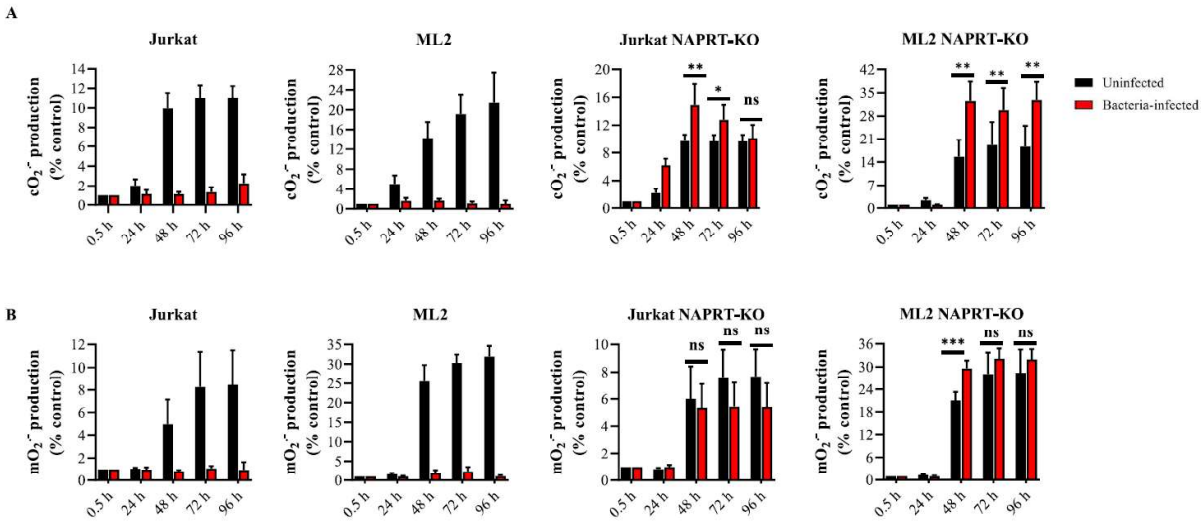

Figure S4. Bacteria blunt APO866-induced oxidative stress in WT but not in NAPRT-KO leukemic cells.

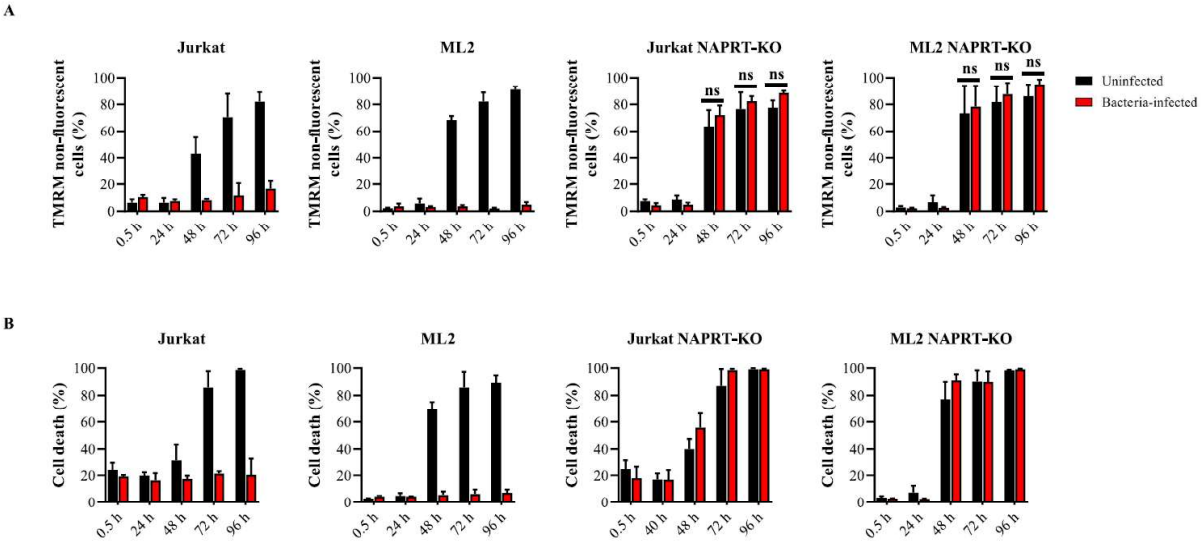

Figure S5. Bacteria abrogate loss of mitochondrial membrane potential and cell death induced by APO866 in WT but not in NAPRT-KO leukemic cells

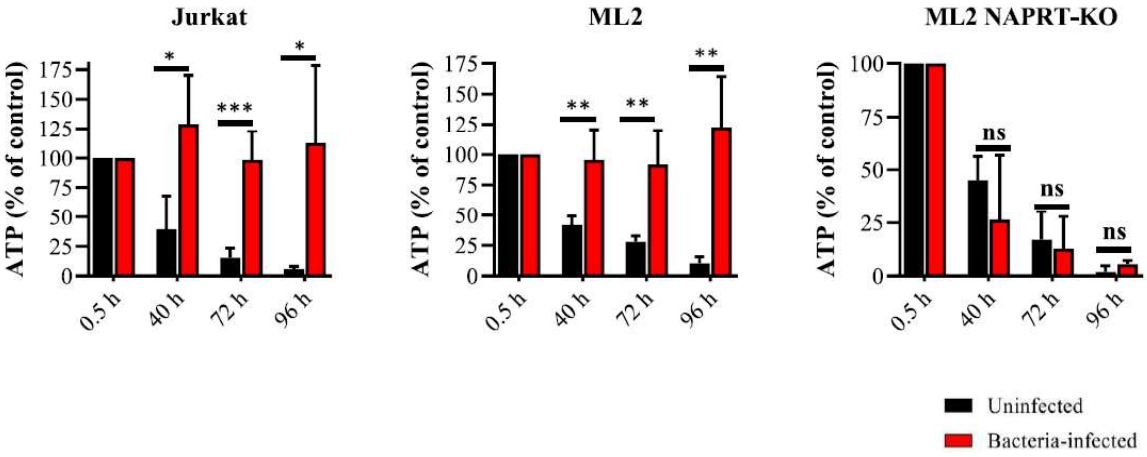

Figure S6. Bacteria prevent ATP depletion induced by APO866 in WT but not in NAPRT-KO leukemic cells

PROJECT 3

The following chapter is a reprint from the article Matsumoto *et al.* (2023) published in *Cancers*.

Matsumoto, S., *et al*. Nicotinaldehyde, a Novel Precursor of NAD Biosynthesis, Abrogates the Anti-Cancer Activity of an NAD-Lowering Agent in Leukemia. *Cancers*. 2023; 15(3):787.

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Summary of the results

In mammalian cells, NAD is synthesized through multiple pathways via the activities of different enzymes. Growing evidence demonstrate the existence of novel precursors and pathways contributing to NAD homeostasis. Depicting the global picture of NAD metabolome is of major interest, as these precursors may affect the therapeutic efficacy of NAD-targeting anticancer agent. In this study, we identified nicotinaldehyde as a novel precursor for NAD synthesis in leukemia cells. We showed that nicotinaldehyde is converted to nicotinic acid by leukemia cells, and further exploited through the Preiss-Handler pathway to increase intracellular NAD levels in cells under APO866 treatment. Consequently, nicotinaldehyde abrogates the antitumor toxicity of APO866 both *in vitro* and in preclinical settings. This study shows for the first time the function of nicotinaldehyde in the NAD metabolome and highlights the modulatory effect of tumor metabolic environment on the therapeutic efficacy NAD-targeting compounds.

Personal Contributions

I designed and performed *in vitro* experiments and conducted the study with mouse xenograft models. I analysed the experimental data and wrote the manuscript under the supervision of Dr. Aimable Nahimana and Prof. Duchosal.

cancers

MDPI

Article

Nicotinaldehyde, a Novel Precursor of NAD Biosynthesis, Abrogates the Anti-Cancer Activity of an NAD-Lowering Agent in Leukemia

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Simple Summary: Cancer cells are reliant on a sufficient amount of nicotinamide adenine nucleotide (NAD) to sustain their altered metabolism and proliferation. Targeting NAD depletion with inhibitors of NAD biosynthesis has therefore emerged as a promising approach for cancer treatment. Growing evidence demonstrates the existence of multiple precursors and alternative pathways for NAD biosynthesis, and that many parameters that include gut microbiota may negatively affect the therapeutic efficacy of NAD lowering agents in cancer treatment. These findings raise the need to depict further the NAD biogenesis in mammalian cells in order to improve the efficacy of NAD targeting anti-cancer treatment. In the present study, we report the identification of nicotinaldehyde as a novel NAD precursor in leukemia cells. Our findings reveal the implication of a novel NAD metabolite in modulating the anti-cancer efficacy of an NAD-lowering agent and suggest potential strategies to enhance its therapeutic effect.

Abstract: Targeting NAD depletion in cancer cells has emerged as an attractive therapeutic strategy for cancer treatment, based on the higher reliance of malignant vs. healthy cells on NAD to sustain their aberrant proliferation and altered metabolism. NAD depletion is exquisitely observed when NAMPT, a key enzyme for the biosynthesis of NAD, is inhibited. Growing evidence suggests that alternative NAD sources present in a tumor environment can bypass NAMPT and render its inhibition ineffective. Here, we report the identification of nicotinaldehyde as a novel precursor that can be used for NAD biosynthesis by human leukemia cells. Nicotinaldehyde supplementation replenishes the intracellular NAD level in leukemia cells treated with NAMPT inhibitor APO866 and prevents APO866-induced oxidative stress, mitochondrial dysfunction and ATP depletion. We show here that NAD biosynthesis from nicotinaldehyde depends on NAPRT and occurs via the Preiss–Handler pathway. The availability of nicotinaldehyde in a tumor environment fully blunts the antitumor activity of APO866 in vitro and in vivo. This is the first study to report the role of nicotinaldehyde in the NAD-targeted anti-cancer treatment, highlighting the importance of the tumor metabolic environment in modulating the efficacy of NAD-lowering cancer therapy.

Keywords: NAD; nicotinaldehyde; APO866; NAMPT inhibitor; acute myeloid leukemia

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1. Introduction

Nicotinamide adenine dinucleotide (NAD) is an essential metabolite in cell life, participating in myriads of cellular fundamental processes. NAD (referring to both the oxidized NAD^+ and reduced NADH forms) has a major role in the energy metabolism by serving as a co-factor to catalyze redox reactions involved in several metabolic pathways, including glycolysis, TCA cycle and oxidative phosphorylation. It also serves as a substrate for enzymes such as sirtuins, poly-ADP-ribose polymerases (PARPs), and cyclic ADP ribose

synthases (such as CD38 and CD157), and therefore plays essential roles in gene expression, DNA damage repair, stress response signaling, or regulation of apoptosis [1]. A sustained production of NAD and the maintenance of its sufficient level are crucial for the survival and proliferation of both healthy and malignant cells. Due to their rapid proliferation, cancer cells are notably more exposed to environmental and intrinsic threats, such as metabolic stresses and DNA synthesis needs or DNA damaging insults. Moreover, they are in continuous need of producing energy and rely on a sustained biosynthesis of NAD [2]. For these reasons, targeting NAD depletion in malignant cells has emerged as an attractive therapeutic strategy for anti-cancer treatment.

Mammalian cells can produce NAD through three major pathways from different NAD precursors [3]: (i) nicotinamide (NAM) or its related riboside form (NR), through the "salvage" pathway; (ii) nicotinic acid (NA) or its related riboside form (NAR), through the "Preiss–Handler" pathway; or (iii) tryptophan through the so-called "de novo" pathway. NAM and NA are the most important precursors and are widely used as NAD precursors in mammals [4–7]. Within the salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT) acts as the rate-limiting enzyme and converts NAM to NMN, which is then converted to NAD by NMN adenylyltransferase (NMNAT). This pathway is of major importance as it enables the recycling of NAM, the end product of NAD utilization by NAD-consuming enzymes, back to NAD.

In line with this notion, the inhibition of NAMPT with small molecule inhibitors has been extensively studied as a promising anti-cancer treatment. Several inhibitors of NAMPT have been developed to date, among which APO866 (also known as FK866 or Daporinad) was the first reported and the most extensively studied [8–10]. In several mouse xenograft models, NAMPT inhibitors induced the inhibitory effect on tumor initiation and progression, leading to the prolongation of overall survival of the animals [11,12]. These promising preclinical results led to their assessment in clinical trials, however, none of them succeeded in showing an objective and consistent tumor response in patients [13,14]. This implies a strong need to optimize the use of NAMPT inhibitors in clinical settings. In the attempt to explain the lack of efficacy in clinical trials, recent studies demonstrated that gut microbiota and metabolites available in the tumor environment could greatly contribute to counteracting the antitumor activity of NAMPT inhibitors. This was operating through the activation of NAD biosynthetic pathways alternative to that blocked by NAMPT inhibitors, enabling the replenishment of intracellular NAD [15,16]. These reports highlight the complexity of the inter-conversion of substrates for NAD biosynthesis and the importance of controlling the tumor metabolic environment for optimizing the therapeutic efficacy of NAMPT inhibitors.

Nicotinaldehyde is a natural compound found in plants and bacteria, and it is used as an intermediate for molecule synthesis in the pharmaceutical and agronomical industries. It can also be contained in foods of plant origin such as honey [17]. To date, only a few studies have investigated the role of nicotinaldehyde in NAD metabolism in humans.

In this study, we report the identification of nicotinaldehyde as a novel NAD precursor that can be used for NAD generation by leukemia cells. Notably, we show that nicotinaldehyde supplementation abrogates the antitumor properties of NAMPT inhibition with APO866 both in vitro and in vivo, through an enzymatic conversion to NA and activation of an alternative NAPRT-dependent biosynthesis of NAD. Of interest, we provide the first description of the potential negative impact of nicotinaldehyde on the anti-leukemia activities of NAMPT inhibitors.

2. Materials and Methods

2.1. Cell Lines, Primary Cells and Culture Conditions

Six hematological cancer cell lines were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) or ATCC and include Jurkat (T-acute lymphoblastic leukemia); ML2, SKM-1 and NOMO-1 (acute myeloid leukemia); and Namalwa and Ramos (Burkitt lymphoma). The molecular profiles are available at DSMZ or ATCC. CD38 expres-

sion profiles are provided in Figure S1. ML2 NAPRT KO cells were generated by the CRISPR genome editing method as described previously [16]. All cells were cultured in RPMI (Invitrogen AG, 61870-01, Life Technologies, Zug, Switzerland) supplemented with 10% heat inactivated fetal calf serum (FCS, Amimed, 2-01F30-I) and 1% penicillin/streptomycin (PS) (Amimed, 4-01F00-H, BioConcept, Allschwil, Switzerland) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.2. Flow Cytometry

To evaluate the cytotoxic effect of APO866 (kindly provided by TopoTarget, Switzerland) alone or in combination with nicotinaldehyde (Sigma, P62208, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), cancer cells were incubated without or with 10 nM (if not specified otherwise) of APO866 in the absence or presence of 10 µM of nicotinaldehyde and stained with annexin-V (eBioscience, BMS306FI/300, Life Technologies) and 7-aminoactinomycin D (7AAD, A07704, Beckman Coulter, Nyon, Switzerland) as described by the manufacturer and analyzed using flow cytometry. Dead cells were identified as $7AAD^+$ and early apoptotic cells as annexin$^+$ $7AAD^-$. Live cell number was counted with CountBright™ Plus Absolute Counting Beads (Invitrogen, C36995). Mitochondrial membrane potential (MMP) was evaluated with Tetramethylrhodamine methyl ester (TMRM) staining (Invitrogen, T668, Thermo Fisher Scientific, Reinach, Switzerland). After a 96 h treatment with APO866 and/or nicotinaldehyde, cells were washed with warm PBS and resuspended in PBS containing 100 nM TMRM dye for 30 min at 37 °C, and the fluorescence level was measured by flow cytometry. The levels of intracellular ROS, namely mitochondrial and cytosolic superoxide anion radicals $O_2{}^-$ and total H_2O_2, were assessed using specific fluorescent probes, Mitosox (Molecular Probes, M36008, Life Technologies), DHE (Marker Gene Technologies, MGT-M1241-M010, Abcam, Amsterdam, Netherlands) and carboxy-H2DCFDA (Molecular Probes, C-400, Life Technologies), respectively. After a 96 h treatment with APO866 and/or nicotinaldehyde, cells were washed with PBS (or DPBS for carboxy-H2DCFDA) and resuspended in PBS containing 2.5 µM, 10 µM and 20 µM of specific probes, respectively, for 30 min at 37 °C, and the fluorescence level was assessed by flow cytometry.

2.3. Determination of Intracellular NAD and ATP Content by Biochemical Assay

Moreover, 3×10^5 cells were seeded in 24-well plates in the presence or absence of APO866 or nicotinaldehyde supplementation. At 96 h of incubation at 37 °C, cells were harvested by centrifugation at 12,000 rpm at 4 °C for 5 min followed by a wash with cold PBS and suspension in lysis buffer (20 mM $NaHCO_3$, 100 mM Na_2CO_3). Total intracellular NAD content was measured using a colorimetric cycling assay. Briefly, cell lysates were incubated in the presence of Phenazine Ethosulfate (PES), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium Bromide (MTT), Alcohol Dehydrogenase (ADH) and ethanol. When ADH oxidizes ethanol, NAD^+ is reduced to NADH, which further allows the reduction/oxidation of PES and MTT. The resulted formation of reduced MTTH can be monitored as an increase of absorbance at 570 nm. The rate of MTTH appearance is proportional to the concentration of NAD(H) present in the assay mixture. The values were normalized by protein concentrations determined with BCA protein assay. Total ATP content was measured using the ATP determination Kit (Life Technologies, A22066) according to the manufacturer's instructions.

2.4. Quantification of NAD Metabolome Using LC-MS/MS

For NAD-related metabolites quantification, cells were incubated at 37 °C for 96 h in the presence or absence of APO866 (10 nM), without or with nicotinaldehyde supplementation (10 µM) in the RPMI culture medium containing 10% FCS and 1% PS. After centrifugation at 14,000× *g* for 15 min, the conditioned medium (or the supernatant) and the cell pellets were collected separately. RPMI medium (without cells) was collected similarly after supplementation with nicotinaldehyde (10 µM) and incubation at 37 °C for 96 h.

NAD-related metabolites levels in the obtained samples were evaluated by LC-MS/MS as described previously [16].

2.5. Evaluation of In Vivo Antitumor Efficacy of APO866 in Mouse Xenograft Models

Severe combined immune deficiency (SCID) mice (Iffa Credo, L'Arbresle, France) were housed in micro-isolator cages in a specific pathogen-free room within the animal facilities at the University Hospital of Lausanne. Animals were allowed to acclimatize to their new environment for 1 week prior to use. All animal experiments were conducted according to the respective institutional regulations after the approval of the animal ethics committee of the University of Lausanne. Mice were transplanted subcutaneously into the right flank with 1×10^7 ML2 human AML cells. Once the tumor reached a volume of 100 mm^3, mice were subdivided into groups of treatments (vehicle, nicotinaldehyde, APO866, nicotinaldehyde and APO866). APO866 (15 mg/kg body weight) was administered intraperitoneally in 200 µL 0.9% saline, twice a day for 4 days, repeated weekly for 3 weeks. Nicotinaldehyde (30 mg/kg body weight) was administered intraperitoneally in 200 µL 0.9% saline, once a day for 4 days, repeated weekly for 3 weeks. Control groups were similarly treated but with saline solution. All animals were monitored daily for signs of illness and killed immediately if the tumor size reached a volume of 1000 mm^3.

2.6. Immunoblotting

Protein samples were separated by SDS-PAGE on a 10% polyacrylamide gel and analyzed by immunoblotting. The mouse anti-NAPRT (HPA023739) and the rabbit anti-actin (ab1801) antibodies were purchased from Abcam (Amsterdam, The Netherlands). After incubation with primary antibodies, polyclonal goat anti-mouse IgG conjugated with IRDye 680 (LI-COR, B70920-02, LI-COR Biosociences, Lincoln, NE, USA) or goat anti-rabbit IgG conjugated with IRDye 800 (LI-COR, 926-32210) were applied. Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR).

2.7. Statistical Analyses

Unless specified otherwise, data are expressed as mean ± SD. The experiments were repeated independently at least three times. Statistical analyses were conducted using GraphPad Prism 8.0 Software (GraphPad Software, San Diego, CA, USA). Unpaired t-tests were used for evaluating differences between groups. For animal survival analyses, Kaplan–Meier method using the log rank test was applied. Statistical significance was established for $p < 0.05$.

3. Results

3.1. Nicotinaldehyde Abrogates the Anti-Leukemic Effect of APO866

Recently, we showed that bacteria present in the cell culture or in gut microbiota can significantly lower the antitumor efficacy of APO866 through the catalytic activity of nicotinamidase that converts NAM into NA, thus fueling an alternative NAD synthesis pathway [16]. In continuation with our previous study, here we used nicotinaldehyde (or 3-pyridinecarboxaldehyde), known as a potent inhibitor of nicotinamidase [18,19] (Figure 1A), with the attempt to reverse the bacterial protective effect in APO866-treated malignant cells. Surprisingly, nicotinaldehyde treatment did not resensitize mycoplasma-infected leukemia cells to APO866, but rather abrogated APO866-antitumor activity in uninfected cells (Figure S2).

Nicotinaldehyde is the aldehyde form of the well-described NAD precursor NA, however, the implication of nicotinaldehyde in the NAD metabolome in mammalian cells has not been explored so far. To evaluate the potential effect of nicotinaldehyde on the antitumor activity of APO866, we treated two human leukemia cell lines, Jurkat and ML2 cells, with APO866 at 10 nM for 96 h without or with nicotinaldehyde (10 µM) and monitored the cell viability. As shown in Figure 1, the supplementation of nicotinaldehyde did not affect cell viability (Figure 1B) nor proliferative capacity (Figure 1C), indicating that this dose of

nicotinaldehyde is well tolerated by leukemia cells. While treatment with APO866 killed 100% of leukemia cells, co-treatment with nicotinaldehyde allowed cells to survive and proliferate at the same rate as the untreated cells (Figure 1B,C). Accordingly, we showed that nicotinaldehyde is able to abrogate cell death induction by NAMPT inhibition. A time course analysis of cell death rate demonstrated that nicotinaldehyde effectively protects leukemia cells from the APO866 cytotoxic effect from the early phase of the treatment (Figure 1D). We also showed that the supplementation with nicotinaldehyde conferred a dose-dependent protection in APO866-treated leukemia cells (Figure 1E). The half maximal effective concentration, defined as the concentration of nicotinaldehyde that reverses 50% of APO866-induced cell death, was estimated to be 0.47 µM in both Jurkat and ML2 cells. To further extend these observations, the same analysis was performed on additional hematologic malignant cell lines. Consistent with our initial finding, the supplementation with nicotinaldehyde abrogated the antitumor activity of APO866 on all tested cell lines (Figure S3).

Overall, the results indicate that nicotinaldehyde supplementation protects leukemia cells from APO866-induced cytotoxicity.

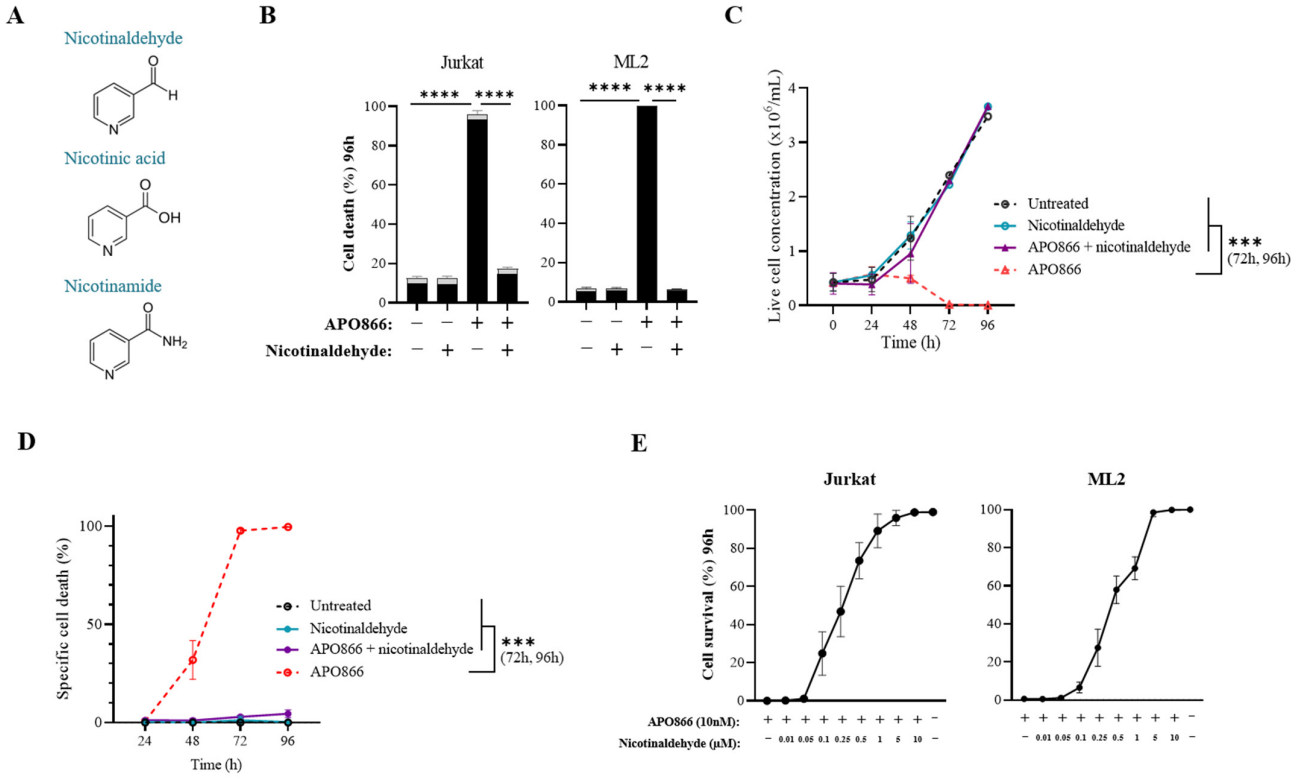

Figure 1. Nicotinaldehyde supplementation abrogates APO866 cytotoxicity in leukemia cells. (**A**) Chemical structures of nicotinaldehyde (3-pyridinecarboxaldehyde) and of NAD precursors nicotinic acid and nicotinamide. (**B**) Cell death of Jurkat and ML2 cells at 96 h of APO866 (10 nM) treatment with or without 10 µM of nicotinaldehyde supplementation (black box = $7AAD^+$, late apoptosis and necrosis. Grey box = annexin V^+ $7AAD^-$, early apoptosis). (**C**) Time course cell growth evaluated from live cell number and (**D**) time course cell death rate of Jurkat cells treated with APO866 (10 nM) for 96 h, with or without 10 µM nicotinaldehyde supplementation. (**E**) Cell survival of Jurkat and ML2 cells treated with APO866 (at 10 nM) and nicotinaldehyde at concentrations ranging from 0.01 to 10 µM for 96 h. Error bars represent SD of at least 3 independent replicates. *** $p < 0.001$, **** $p < 0.0001$.

3.2. Nicotinaldehyde Prevents APO866-Induced Intracellular NAD Depletion, Subsequent Oxidative Stress, Mitochondrial Membrane Depolarization and ATP Depletion

The primary effect of APO866, causing subsequent cancer cell death, is the depletion of the intracellular NAD content (by approximately >95%) [20]. Therefore, we next evaluated the intracellular levels of NAD in APO866-treated leukemia cells in the absence or presence of nicotinaldehyde. As shown in Figure 2A and in line with previous studies, APO866 fully depleted intracellular NAD content in Jurkat cells. Contrastingly, APO866-treated leukemia cells that received nicotinaldehyde supplementation showed partial but significant maintenance of intracellular NAD at approximately 13% of the amount in untreated control cells. These observations suggest that nicotinaldehyde enables the replenishment of intracellular NAD despite the inhibition of NAMPT, indicating that nicotinaldehyde could be a potential novel source of NAD biosynthesis. Supplementation of nicotinaldehyde alone had no effect on the intracellular NAD level, suggesting that leukemia cells already had enough substrates and that their NAD levels were in equilibrium. This observation is consistent with the global notion that the intracellular NAD metabolome is tightly regulated in the cells. Additionally, we tested whether nicotinaldehyde can still protect leukemia cells from APO866-induced cell death if it is supplemented only when NAD is already depleted by APO866. We first treated leukemia cells with APO866 (10 nM) alone, and at 24 h after drug treatment, nicotinaldehyde (10 μM) was supplemented and the cells were further incubated for an additional 72 h. As shown in Figure S4A, nicotinaldehyde was able to protect leukemia cells from APO866-induced cell death, although NAD depletion was already induced at 24 h when nicotinaldehyde was added to the cell cultures (Figure S4B). These observations suggest that nicotinaldehyde can be rapidly exploited by leukemia cells to replenish intracellular NAD and inhibit APO866-induced cell death.

The cytotoxic events involved in the tumor-killing activity of APO866 have been well described in a previous study [21]. Notably, NAD depletion induced by APO866 triggers oxidative stress with the generation of high levels of reactive oxygen species (ROS), mitochondrial membrane depolarization and subsequent ATP depletion. Next, we investigated whether nicotinaldehyde supplementation prevents these cytotoxic events in APO866-treated leukemia cells. As reported in Figure 2B, we observed that treatment with APO866 alone significantly increased intracellular ROS levels in Jurkat cells: cytosolic and mitochondrial superoxides increased, respectively, by 15- and 13-fold compared to the untreated conditions, and hydrogen peroxide increased 13-fold. Mitochondrial membrane depolarization was enhanced 7-fold compared to the control (Figure 2C). Intracellular ATP dropped to an undetectable level with APO866 treatment (Figure 2D). Supplementation with nicotinaldehyde maintained intracellular ROS, mitochondrial membrane potential and ATP content at control levels (Figure 2B–D). Similarly, nicotinaldehyde abrogated these events in ML2 cells (Figure 2E–G). Collectively, the data demonstrate that nicotinaldehyde supplementation protects leukemia cells from the subsequent cytotoxic events induced by NAD depletion upon treatment with APO866.

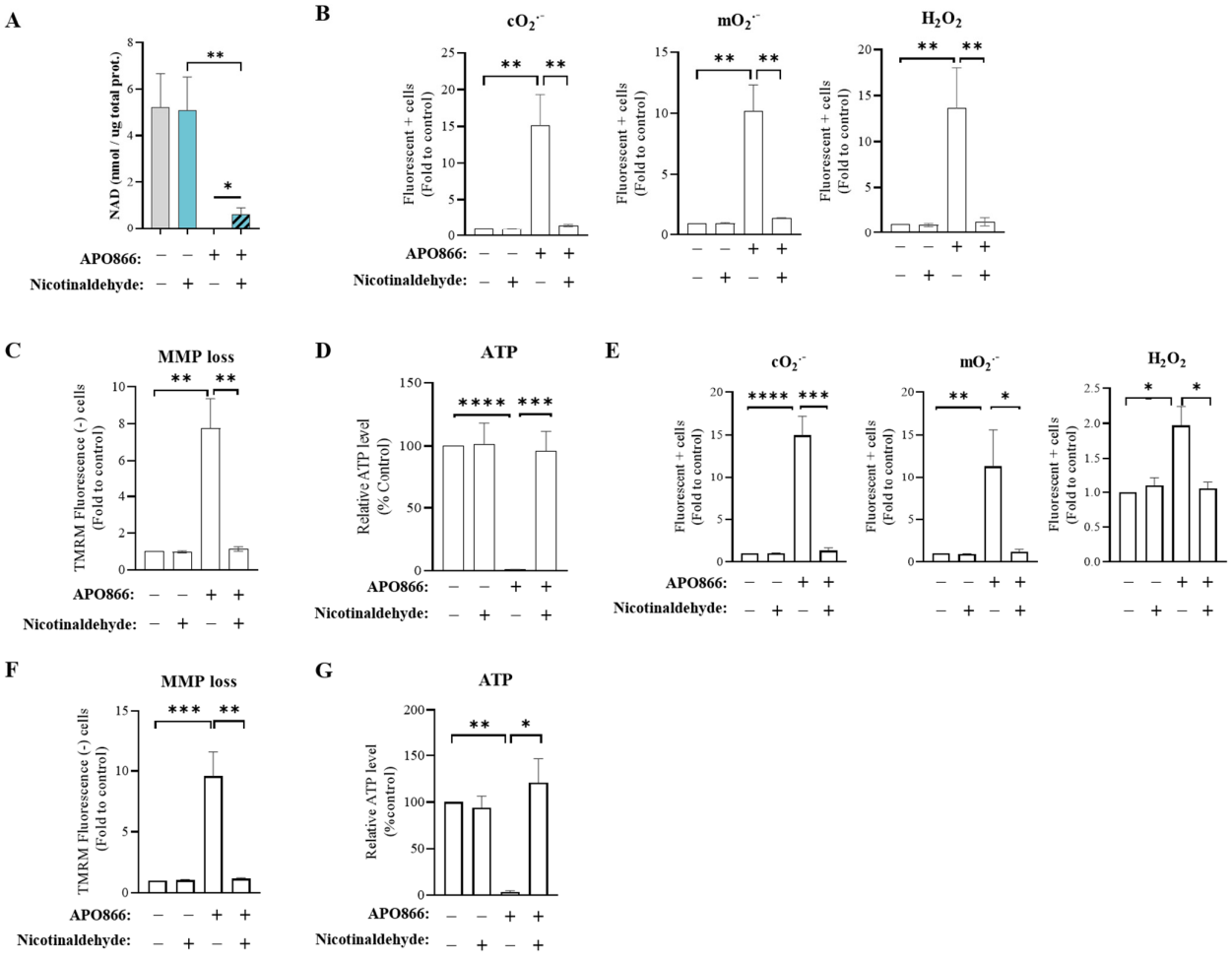

Figure 2. Nicotinaldehyde supplementation counteracts NAD depletion and abrogates cytotoxic events induced by APO866. Jurkat and ML2 cells were treated without or with 10 nM of APO866 in absence or presence of 10 µM of nicotinaldehyde supplementation for 96 h to assess various cytotoxic signatures of APO866. (**A**) Intracellular total NAD (NAD^+ and NADH) measured by biochemical assay and normalized to protein concentration, in Jurkat cells at 96 h of treatment. (**B**) Intracellular ROS (cytosolic and mitochondrial superoxide O_2^- and cellular hydrogen peroxide H_2O_2) levels measured with specific probes (DHE, mitosox and H2DCFDA, respectively) by flow cytometry in Jurkat cells. (**C**) Mitochondrial membrane potential (MMP) determined with TMRM staining, using flow cytometry in Jurkat cells (**D**) ATP concentration measured by luminescence assay, relative to the concentration in untreated cells. (**E**) ROS, (**F**) MMP and (**G**) ATP concentration in ML2 cells. Data are mean ± SD, n ≥ 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3. Nicotinaldehyde Protective Function Requires the Integrity of NAPRT

Next, we sought to investigate how nicotinaldehyde contributes to the maintenance of intracellular NAD. The oxidation of aldehydes to their related carboxylic acids is a well-known phenomenon. This conversion can occur either via the action of enzymes with aldehyde oxidizing and reducing activities or spontaneously in the presence of oxygen [22,23]. Based on this notion, we hypothesized that nicotinaldehyde can be converted to NA either spontaneously or enzymatically. In this scenario, there might be an involvement of NAPRT, the rate-limiting enzyme of the NAD biosynthesis pathway that utilizes NA as the starting substrate (Figure 3A), in the replenishment of intracellular NAD from nicotinaldehyde. To test this hypothesis, we used cancer cells that were either endogenously deficient in functional NAPRT (Namalwa) (Figure 3B) or that lacked the NAPRT gene by genetic knock-out (Jurkat NAPRT KO and ML2 NAPRT KO). As shown in Figure 3C, the measurement of cell death in APO866 and nicotinaldehyde-co-treated Jurkat

NAPRT KO, ML2 NAPRT KO and Namalwa cells revealed that nicotinaldehyde could not protect these cells from APO866-induced cell death. We next evaluated the impact of nicotinaldehyde supplementation on intracellular metabolites and ROS levels in these cells. The measurement of intracellular NAD level in Jurkat NAPRT KO cells showed that nicotinaldehyde supplementation failed to counteract the depletion of NAD upon treatment with APO866 (Figure 3D). Consequently, the cytotoxic events induced by APO866 were not prevented, as illustrated by the increase of ROS, the mitochondrial membrane depolarization and the depletion of ATP (Figure 3E–G). In a similar manner, in Namalwa cells nicotinaldehyde supplementation could not counteract APO866-induced cytotoxic events (Figure S5). Additionally, in order to assess whether the catalytic function of NAPRT is essential, we tested the effect of the potent NAPRT inhibitor 2-hydroxynicotinic acid (2-HNA) on the nicotinaldehyde tumor-protective function against APO866. Similar to NAPRT genetic knock-out, the chemical inhibition of NAPRT with 2-HNA (2 mM) abrogated the protection by nicotinaldehyde from APO866-induced cell death (Figure 3H). Given that 2-HNA acts as a competitive inhibitor of the enzymatic binding pocket of NAPRT [24,25], the protective function of nicotinaldehyde seems to mediate the enzymatic activity of NAPRT. Altogether, the data demonstrate that the integrity of NAPRT status is essential for the replenishment of NAD, prevention of oxidative stress, ATP depletion, and consequent rescue from APO866-induced cell death with nicotinaldehyde supplementation.

3.4. Nicotinaldehyde Boosts the Level of NA and Activates the Preiss–Handler Pathway as an Alternative Route of NAD Biosynthesis to Circumvent the Anti-Leukemic Activity of APO866

To provide evidence of the conversion of nicotinaldehyde to NA, we next investigated whether a spontaneous oxidation of nicotinaldehyde to NA can occur in RPMI culture medium supplemented with nicotinaldehyde. To this end, we quantified the NAD-related metabolites by LC-MS/MS in RPMI medium supplemented or not with 10 μM of nicotinaldehyde. As reported in Figure 4A, the supplementation with nicotinaldehyde yielded a significant increase of NA from 0.003 μM to 0.3 μM in RPMI culture medium, suggesting a spontaneous oxidation of nicotinaldehyde to NA. No significant variation in any other NAD metabolites was observed (Figure S6A). Secondly, mammalian cells possess multiple enzymes that are capable of catalyzing the oxidation of aldehydes to their corresponding carboxylic acids [22]. This notion suggests that these enzymes in leukemia cells can significantly contribute to the conversion of nicotinaldehyde to NA. To test this hypothesis, we quantified NAD metabolites in the conditioned media (CM) of ML2 NAPRT KO cells cultured in the absence or presence of nicotinaldehyde. As ML2 NAPRT KO cells are not able to consume NA, the extent of conversion can be expected to be strongly evidenced when compared to that in ML2 WT cells, which are potentially able to consume the generated NA. In accordance with this hypothesis, Figure 4A shows that the CM of ML2 NAPRT KO cells supplemented with nicotinaldehyde displayed a tremendous increase of NA (2.8 μM) when compared with the CM without supplementation (0.003 μM). The amount of converted NA upon supplementation in the presence of leukemia cells corresponds to a significant increase that is almost 10 times higher than in the absence of cells (0.28 μM). No significant variation of any other metabolite was observed (Figure S6B). With ML2 WT cells, supplementation with nicotinaldehyde resulted in a higher level of extracellular NA in the CM compared to control (from 0.015 μM to 0.003 μM, non-significant change) (Figure 4A), but to a much less extent when compared with the increase with ML2 NAPRT KO cells (2.8 μM), indicating that the generated NA is presumably consumed by ML2 WT cells. In addition, in the CM of ML2 WT cells, supplementation with nicotinaldehyde led to high levels of other NAD precursors including NaR (0.095 μM compared to 0.044 μM), and NAM (4.9 μM compared to 3.1 μM) (Figure 4B, Figure S6C). Moreover, we measured the NAD metabolites in the cell pellets of ML2 cells cultured without or with nicotinaldehyde supplementation and observed that intracellular NA in ML2 NAPRT KO cells was also increased by 6-fold (Figure 4C, Figure S6D). This indicates that considerable amounts of nicotinaldehyde have been transformed to NA and uptaken within cells. We did not

observe any other significant variations in the intracellular metabolite levels within the ML2 WT cells (Figure S6E), consistent with the notion of a tight regulation of NAD metabolites levels in the cells with sufficient substrates. Of note, nicotinaldehyde supplementation did not affect the levels of any metabolite of the de novo pathway.

Collectively, our observations indicate that the supplemented nicotinaldehyde is converted to NA in a cell-dependent manner and is used as an alternative precursor for NAD biosynthesis through the NAPRT-dependent Preiss–Handler pathway.

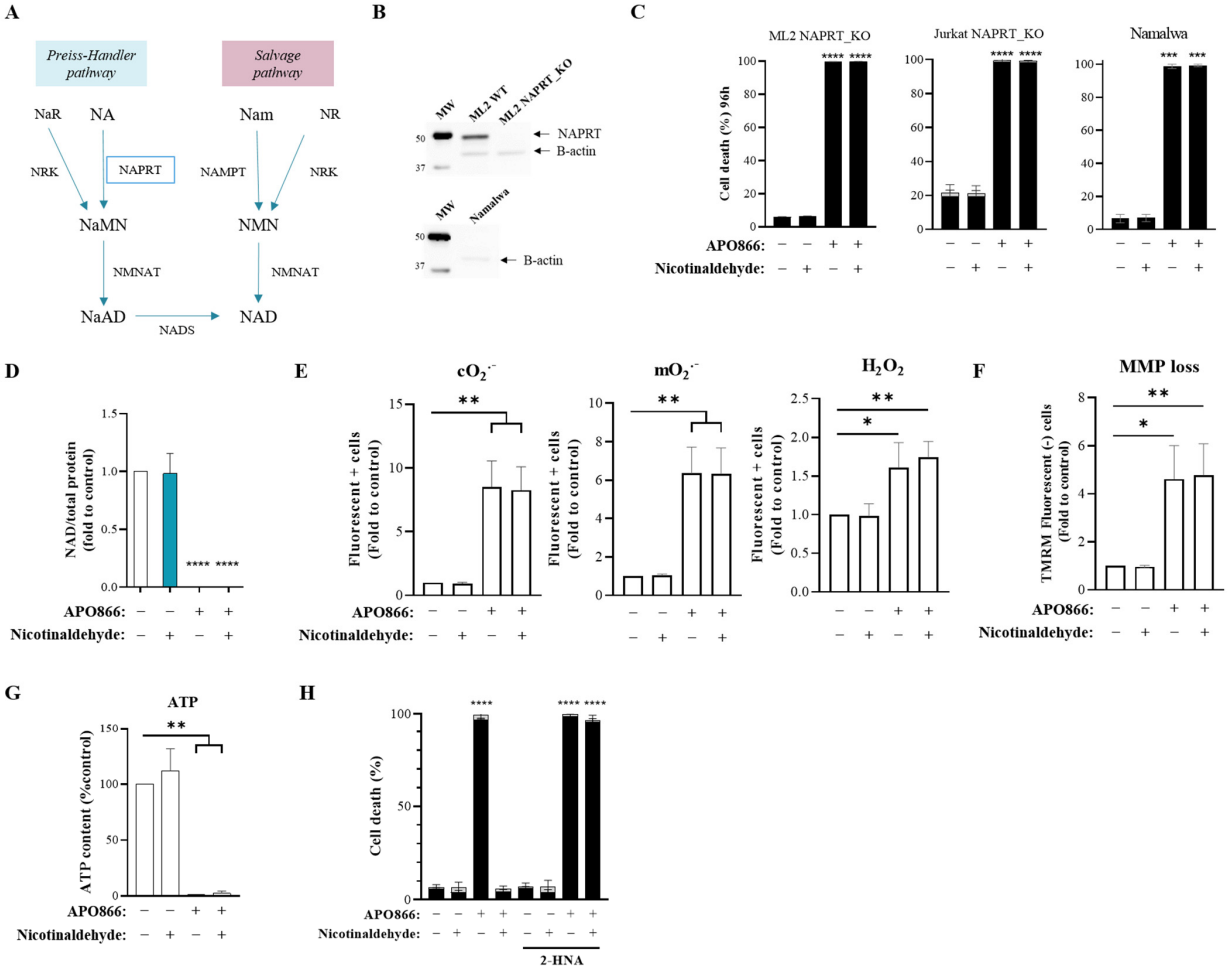

Figure 3. The nicotinaldehyde protective effect is dependent on NAPRT. (**A**) Schematic representation of major NAD biosynthetic pathways. Nam, nicotinamide; NR, nicotinamide riboside; NMN, Nam mononucleotide; NAMPT, Nam phosphoribosyltransferase; NRK, NR kinase; NMNAT, NMN adenylyltransferase; NA, nicotinic acid; NaR, nicotinic acid riboside; NaMN, NA mononucleotide; NAPRT, NA phosphoribosyltransferase; NADK, NAD kinase. (**B**) Western Blot images of NAPRT protein in ML2 WT, ML2 NAPRT KO and Namalwa cells. MW, molecular weight. (**C**) Cell death (black box = $7AAD^+$, late apoptosis and necrosis. Grey box = annexin V^+ $7AAD^-$, early apoptosis) of NAPRT-lacking Jurkat NAPRT KO, ML2 NAPRT KO and Namalwa cells at 96 h of APO866 10 nM treatment. Uncropped Western Blots are available in Figure S7 (**D**) Intracellular NAD level in Jurkat NAPRT KO cells treated without or with APO866 (10 nM) in absence or presence of nicotinaldehyde supplementation (10 µM) for 96 h. (**E**) Intracellular levels of ROS (cytosolic and mitochondrial $O_2{}^{\cdot 4}$ and cellular H_2O_2 measured with specific probes (DHE, mitosox and H2DCFDA). (**F**) MMP determined using flow cytometry, after cell staining with TMRM. (**G**) ATP concentration measured by luminescence assay and normalized to the protein concentration in untreated cells at the corresponding time point. (**H**) Cell death at 96 h of ML2 cells treated without or with APO866 and nicotinaldehyde, in the absence or presence of 2 mM 2-hydroxynicotinic acid (2-HNA). Data are mean ± SD, n ≥ 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

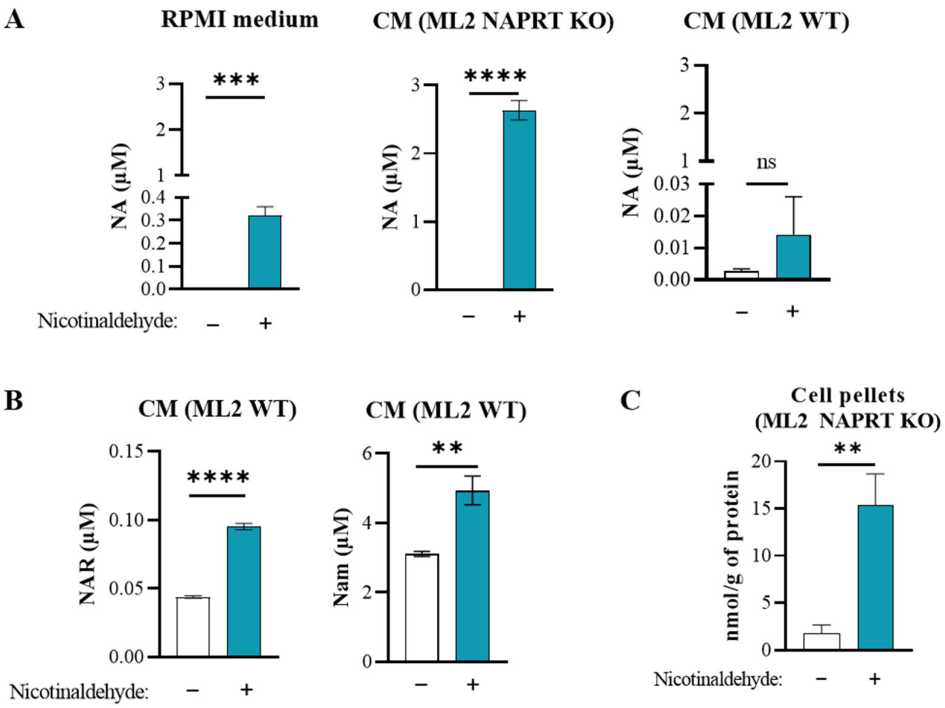

Figure 4. Nicotinaldehyde supplementation modifies NAD-related metabolite levels in the culture medium and within cells. NAD metabolites were quantified by LC-MS/MS in cells and conditioned media (CM) after 96 h of incubation at 37 °C. (**A)** NA concentration in the culture medium supplemented without (−) or with (+) nicotinaldehyde (10 µM), in absence of cells or in presence of ML2 WT or ML2 NAPRT KO cells, after 96 h of incubation at 37 °C. (**B**) NAM and NAR concentrations in the conditioned media of ML2 WT cells without or with nicotinaldehyde supplementation (10 µM). (**C**) Intracellular concentration of NA normalized to protein concentration in ML2 NAPRT KO cells cultured in medium supplemented without or with nicotinaldehyde (10 µM). n = 3 error bars = SD, ns = non-significant. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (vs. not supplemented medium if not specified otherwise).

3.5. Administration of Nicotinaldehyde Blunts the Antitumor Activity of APO866 in Mouse Xenograft Model of Human Leukemia

Next, we evaluated the impact of nicotinaldehyde on the therapeutic efficacy of APO866 in vivo using a mouse xenograft model of human leukemia. To this end, we subcutaneously injected ML2 cells in SCID mice. Once the tumor appeared and reached a volume of 100–150 mm^3, mice were randomized into control and treated groups. Mice were administered intraperitoneally with (i) saline solution (control); (ii) APO866 (15 mg/kg); (iii) nicotinaldehyde (30 mg/kg); and (iv) APO866 + nicotinaldehyde. Of note, we did not observe any signs of toxicity in all mice, including those that were treated with nicotinaldehyde, indicating that nicotinaldehyde treatment was safe at the chosen dose. Without any treatment (control group), or with nicotinaldehyde treatment alone, tumors grew rapidly and reached the endpoint volume of 1000 mm^3 within two weeks after tumor appearance (Figure 5A). APO866 treatment eradicated tumor growth within one week. However, when mice were co-treated with APO866 and nicotinaldehyde, the tumor growth was comparable to that of the control group, demonstrating that nicotinaldehyde fully blunted the APO866 antitumor effect. This led to overall survival rates that are comparable between the control, nicotinaldehyde-administered and nicotinaldehyde + APO866 co-treated groups, where all mice reached the endpoint within four weeks after tumor injection (Figure 5B). Contrastingly, APO866-administered mice survived for more than eight weeks of observation after tumor injection and remained disease-free without any appearance of a palpable tumor. Taken together, these results in a mouse xenograft model demonstrate that nicotinaldehyde abrogates in vivo the therapeutic efficacy of APO866.

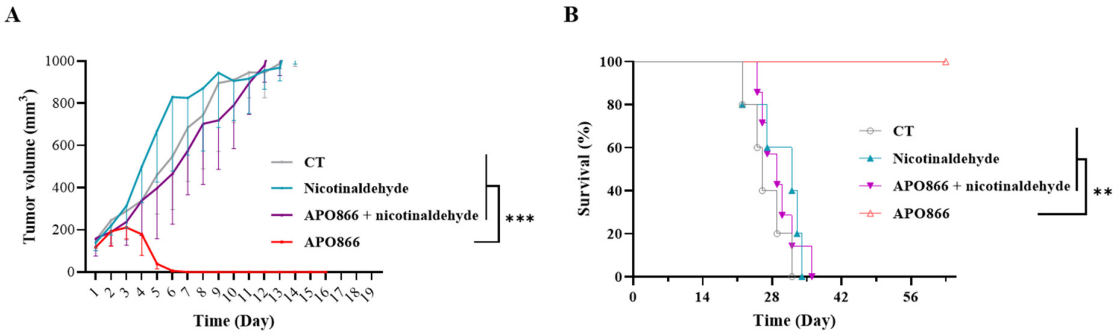

Figure 5. Nicotinaldehyde abrogates APO866 antitumor effect in a mouse xenograft model of human leukemia. (**A**) Evolution of tumor growth and (**B**) survival rate of mice xenografted with ML2 cells and treated without or with APO866 in absence or presence of nicotinaldehyde. Control group received vehicle (grey line). Nicotinaldehyde (30 mg/kg; blue line), APO866 (10 mg/kg; red line) or nicotinaldehyde plus APO866 (purple line) were administered as described in "Materials and Methods". Day 1 corresponds to the beginning of the treatment (when tumor volume reached >100mm^3) (**A**) or the day of tumor injection (**B**). n = 5 per group, error bars = SD. ** $p < 0.01$, *** $p < 0.001$. Data were analyzed by Kaplan–Meier survival analysis with log-rank test.

4. Discussion

Here, we report a previously undescribed function of nicotinaldehyde in NAD metabolism and its negative impact on the therapeutic efficacy of NAMPT inhibitors in leukemia treatment. We demonstrated that nicotinaldehyde can be used as a novel NAD precursor by leukemia cells to circumvent the NAD depletion typically induced by NAMPT inhibition blocking the NAD salvage pathway. Mechanistically, our data demonstrate that when present in a tumor environment, nicotinaldehyde is converted to NA either spontaneously or in a cell-dependent manner. NA converted from nicotinaldehyde is exploited through the NAPRT-dependent Preiss–Handler pathway. Although nicotinaldehyde supplementation only partially restores intracellular NAD decreased by APO866 in leukemia cells, the residual NAD synthesized from the converted NA is sufficient to prevent ROS production, preserve mitochondrial integrity, and abrogate ATP depletion and thus protects leukemia cells from APO866-induced cell death. Furthermore, using a xenograft model of human leukemia, we provide evidence that nicotinaldehyde can severely hamper the in vivo efficacy of APO866.

Very few studies on nicotinaldehyde are reported in the scientific literature. Nicotinaldehyde is a natural compound found in plants and bacteria, which can also be contained in foods of plant origin such as honey [17]. However, little is known about the role of nicotinaldehyde in the NAD metabolism in humans. A previous study by Shibata et al. [26] demonstrated that administering nicotinaldehyde in an NA-free Tryptophan-limiting diet to rats can significantly boost the NAD level in the blood and liver. These authors also showed that nicotinaldehyde can be used as a precursor of NAD with the same efficiency as NA. Our study supports this previous report and complements it by demonstrating that nicotinaldehyde is exploited through conversion to NA and further transformation in the Preiss–Handler pathway.

The conversion of nicotinaldehyde to NA is presumably catalyzed by enzyme(s) with aldehyde oxidative activity. Several enzymes such as aldo-keto reductases, alcohol dehydrogenase (ADH), or aldehyde dehydrogenase (ALDH) demonstrated catalytic activities toward nicotinaldehyde in vitro [27–29]. Although we sought to identify the responsible enzymes for this conversion, we have not obtained conclusive results so far. The precise mechanism remains to be established and is an objective of future research in the laboratory.

The data also suggest that nicotinaldehyde can be administrated safely in animals at the chosen doses as demonstrated by the absence of any signs of toxicity such as loss of body weight, lethargy, rough coat and no premature deaths. The safety of nicotinaldehyde could be most probably explained by the existence, in mice as well as in humans, of a

large number of aldehyde metabolizing enzymes, as mentioned above. In line with this assumption, several reported studies in which rats were fed diets supplemented with nicotinaldehyde have been carried out without toxicity while showing significant activities comparable to NA at the same doses [26,30].

It is noteworthy to mention that nicotinaldehyde is mostly reported as a potent inhibitor of nicotinamidase, an enzyme that is absent in mammals but widely distributed in several species of bacteria, plants and many metazoan species [18]. This enzyme converts NAM to NA, and its importance in mammalian systemic NAD homeostasis has emerged recently through several studies showing the implication of gut microbial enzymes in the modulation of host NAD metabolite levels [15,16,31]. Nicotinaldehyde could therefore be considered to block the conversion of NAM to NA and its subsequent use through the NAPRT pathway. However, our results strongly suggest that nicotinaldehyde can act as an alternative precursor for NAD biosynthesis, rather than blocking the above-mentioned biosynthetic route through nicotinamidase. This opposite function should be considered carefully when using nicotinaldehyde as an inhibitor of nicotinamidase.

Importantly, although our data revealed a negative impact of a novel NAD precursor on the anti-leukemic activity of APO866, we also demonstrated that the sensitivity of leukemia cells can be restored by genetically silencing or pharmacologically inhibiting NAPRT. These results support the strategy of a dual targeting of the two major NAD biosynthesis pathways to induce robust NAD depletion and anti-cancer therapeutic efficacy, despite the potential interference of NAD-related metabolites present in the tumor environment.

Our metabolomic analysis revealed a significant increase of NaR in the conditioned medium of ML2 WT cells upon supplementation with nicotinaldehyde. A study from Kulikova et al. [32] showed that NaR can be generated from NA via NaMN formation (by NAPRT) and its dephosphorylation by cytosolic 5′-nucleotidases (NT5), under conditions that lead to the excessive formation of NaMN. We can speculate that the successive conversions of nicotinaldehyde to NA, and NA to NAMN, may have led to the observed increase of NaR released in the culture medium through a similar mechanism via NT5. Although additional investigation is needed to confirm this hypothesis, this observation illustrates the complex network and inter-conversions of NAD precursors, and the potential importance of riboside NAD precursors, which are worth exploring further.

5. Conclusions

These findings identify nicotinaldehyde as a novel member of the NAD metabolome that can contribute to NAD biosynthesis and modulate the response to NAMPT inhibition in leukemia cells. Our study suggests the complexity of the NAD metabolome and therefore stresses the need to further explore the interactions between NAD precursors. The results highlight the crucial role of the NAPRT-dependent pathway in cell survival and demonstrate the importance of targeting both NAMPT- and NAPRT-dependent pathways in order to optimize the therapeutic efficacy of NAMPT inhibitors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15030787/s1, Figure S1: CD38 expression status of ML2, Jurkat and Namalwa cells; Figure S2: Nicotinaldehyde does not resensitize mycoplasma-infected leukemia cells to APO866, but rather abrogates APO866-antitumor activity in uninfected cells; Figure S3: Nicotinaldehyde supplementation abrogates APO866 cytotoxicity in several hematological cancer cell lines; Figure S4: Nicotinaldehyde supplementation at 24 h of APO866 treatment rescues ML2 and Jurkat cells from APO866-induced cell death; Figure S5: Nicotinaldehyde supplementation does not counteract cytotoxic events induced by APO866 in Namalwa cells; Figure S6: NAD-related metabolite levels in the culture medium, cells and conditioned media upon nicotinaldehyde supplementation; Figure S7: Original Western Blot for Figure 3C.

Author Contributions: Conceptualization, S.M., M.A.D. and A.N.; Methodology, S.M. and A.N.; Validation, S.M.; Formal analysis, S.M., P.B., A.B. and A.N.; Investigation, P.B., A.B. and A.N.; Resources, M.A.D. and A.N.; Data curation, S.M., M.A.D. and A.N.; Writing—original draft, S.M.; Writing—review and editing, P.B., A.B., M.A.D. and A.N.; Supervision, A.N.; Project administration, A.N.; Funding acquisition, M.A.D. and A.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of the University of Lausanne (Cantonal number VD3039x1a and National number 31354, approval date 23 September 2019).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no competing financial interest.

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cancers

Supplementary Materials: Nicotinaldehyde, a Novel Precursor of NAD Biosynthesis, Abrogates the Anti-Cancer Activity of an NAD-Lowering Agent in Leukemia

Saki Matsumoto, Paulina Biniecka, Axel Bellotti, Michel A Duchosal and Aimable Nahimana

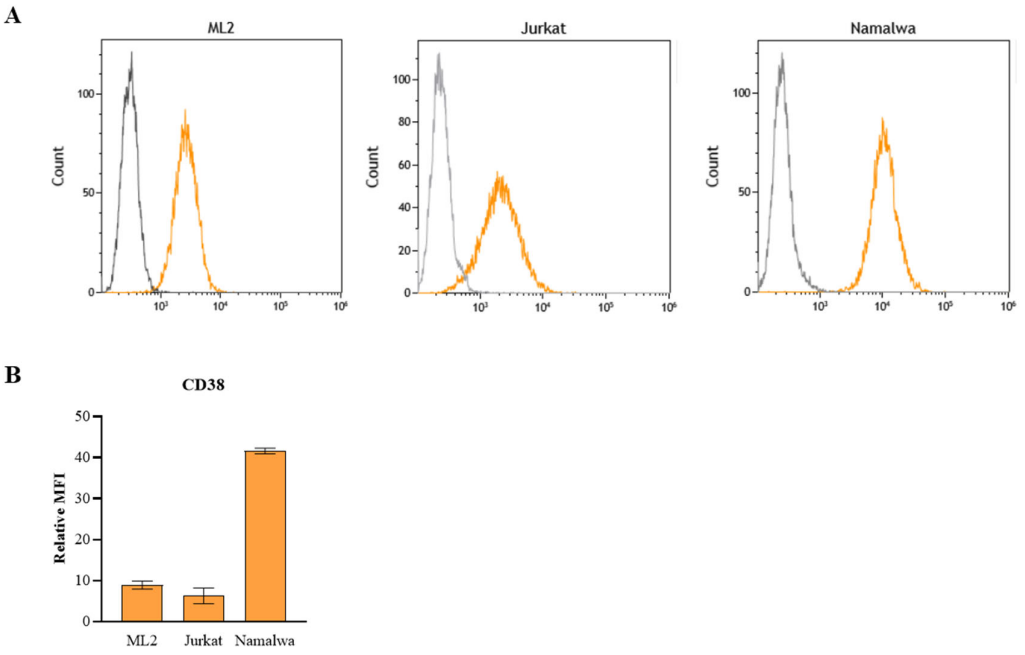

Figure S1. CD38 expression status of ML2, Jurkat and Namalwa cells.CD38 expression was evaluated in ML2, Jurkat and Namalwa cells by flow cytometry. (**A**) Representative fluorecence levels of cells labelled with CD38-specific (orange) and matched isotype control-targeted (grey) antibodies. (**B**) Relative fluorecence intensities calculated as the ratio of mean fluorescence intensities (MFI) of cells labelled with CD38-specific antibody and the control. Data are mean ± SD, $n = 3$.

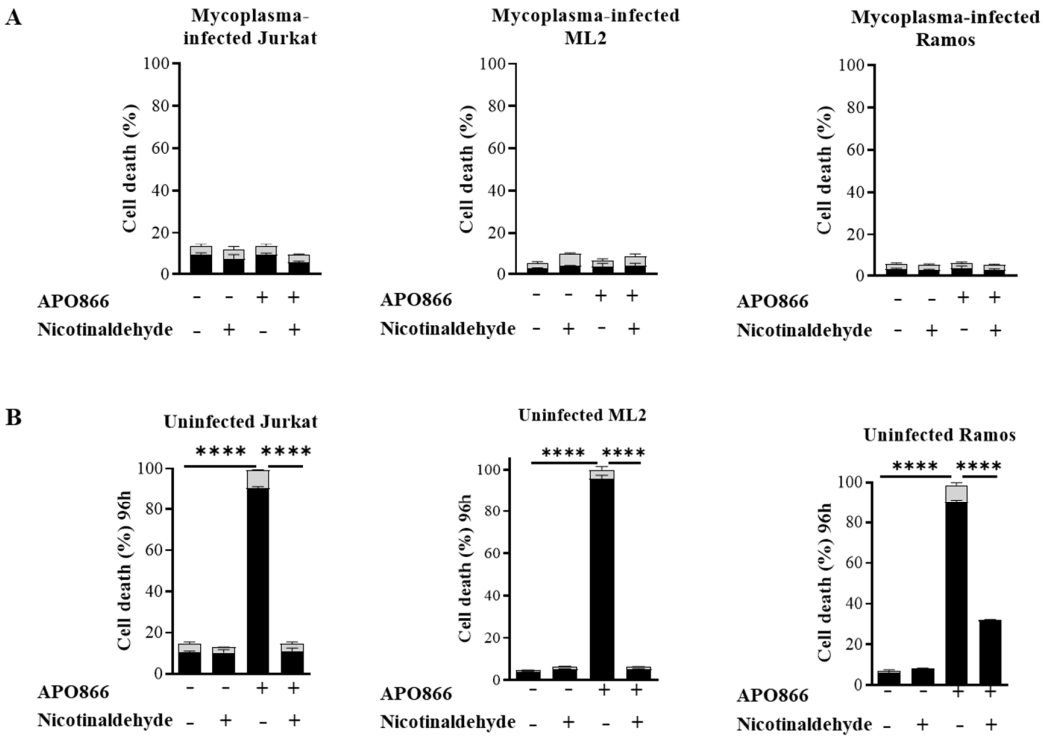

Figure S2. Nicotinaldehyde does not resensitize mycoplasma-infected leukemia cells to APO866, but rather abrogates APO866-antitumor activity in uninfected cells. ML2, Jurkat and Ramos cells were infected (**A**) or unifected (**B**) with mycoplasma as described previously [16]. Cell death was assessed at 96 h of APO866 (10 nM) treatment with or without 250 µM of nicotinaldehyde supplementation (black box = 7AAD+, late apoptosis and necrosis. Grey box = annexin V+ 7AAD− , early apoptosis). Data are mean ± SD, $n = 3$. **** $p < 0.0001$.

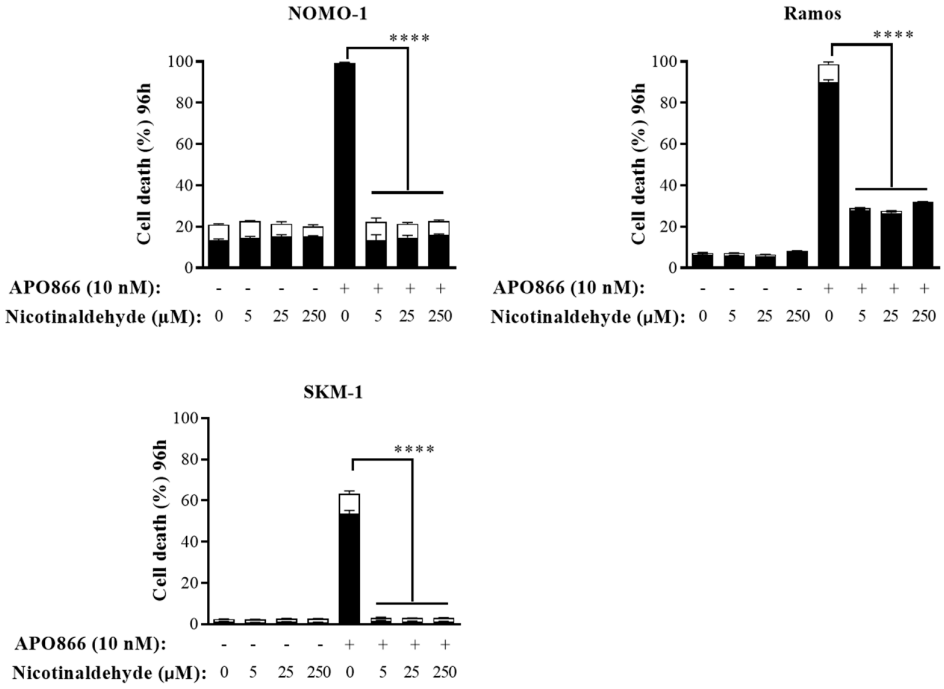

Figure S3. Nicotinaldehyde supplementation abrogates APO866 cytotoxicity in several hematological cancer cell lines. Cell death of Ramos (Burkitt lymphoma), SKM-1 and NOMO-1 (acute myeloid leukemia) cells assessed at 96 h of APO866 treatment (10nM) with or without nicotinaldehyde supplementation at 5, 25 and 250 μM. Data are mean ± SD, $n = 3$. **** $P < 0.0001$.

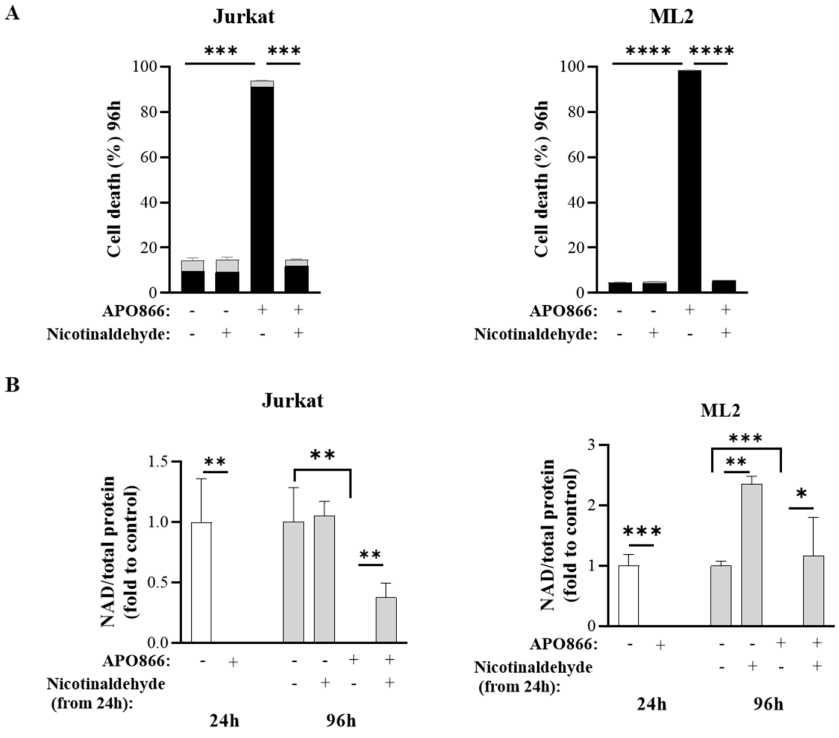

Figure S4. Nicotinaldehyde supplementation at 24 h of APO866 treatment rescues ML2 and Jurkat cells from APO866-induced cell death. ML2 and Jurkat cells were treated with APO866 (10 nM) and after 24 h of treatment, 10 μM of nicotinaldehyde was added to cell cultures that were further incubated for additional 72 h. (**A**) Cell death assessed at 96 h of APO866 treatment (black box = 7AAD+, late apoptosis and necrosis. Grey box = annexin V+ 7AAD− , early apoptosis). (**B**) Intracellular NAD levels evaluated at 24 h (before addition of nicotinaldehyde) and at 96 h. Data are mean ± SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

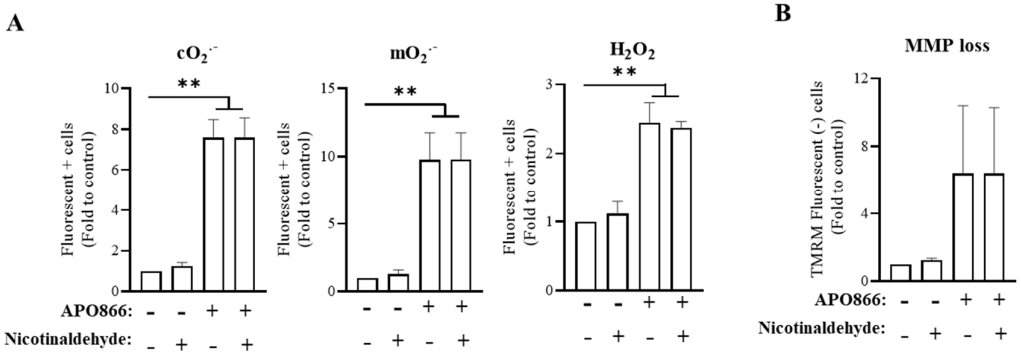

Figure S5. Nicotinaldehyde supplementation does not counteract cytotoxic events induced by APO866 in Namalwa cells. Namalwa cells were treated with APO866 (10 nM) in absence or presence of 10 μM of nicotinaldehyde supplementation and different cytotoxic signatures of APO866 were assessed at 96 h of treatment. (**A**) Intracellular ROS (cytosolic and mitochondrial superoxide O_2^- and cellular hydrogen peroxide H_2O_2) levels measured with specific probes (DHE, mitosox and

H2DCFDA respectively) by flow cytometry. (**B**) Mitochondrial membrane potential (MMP) determined with TMRM staining, using flow cytometry. Data are mean ± SD, $n = 3$. ** $P < 0.01$.

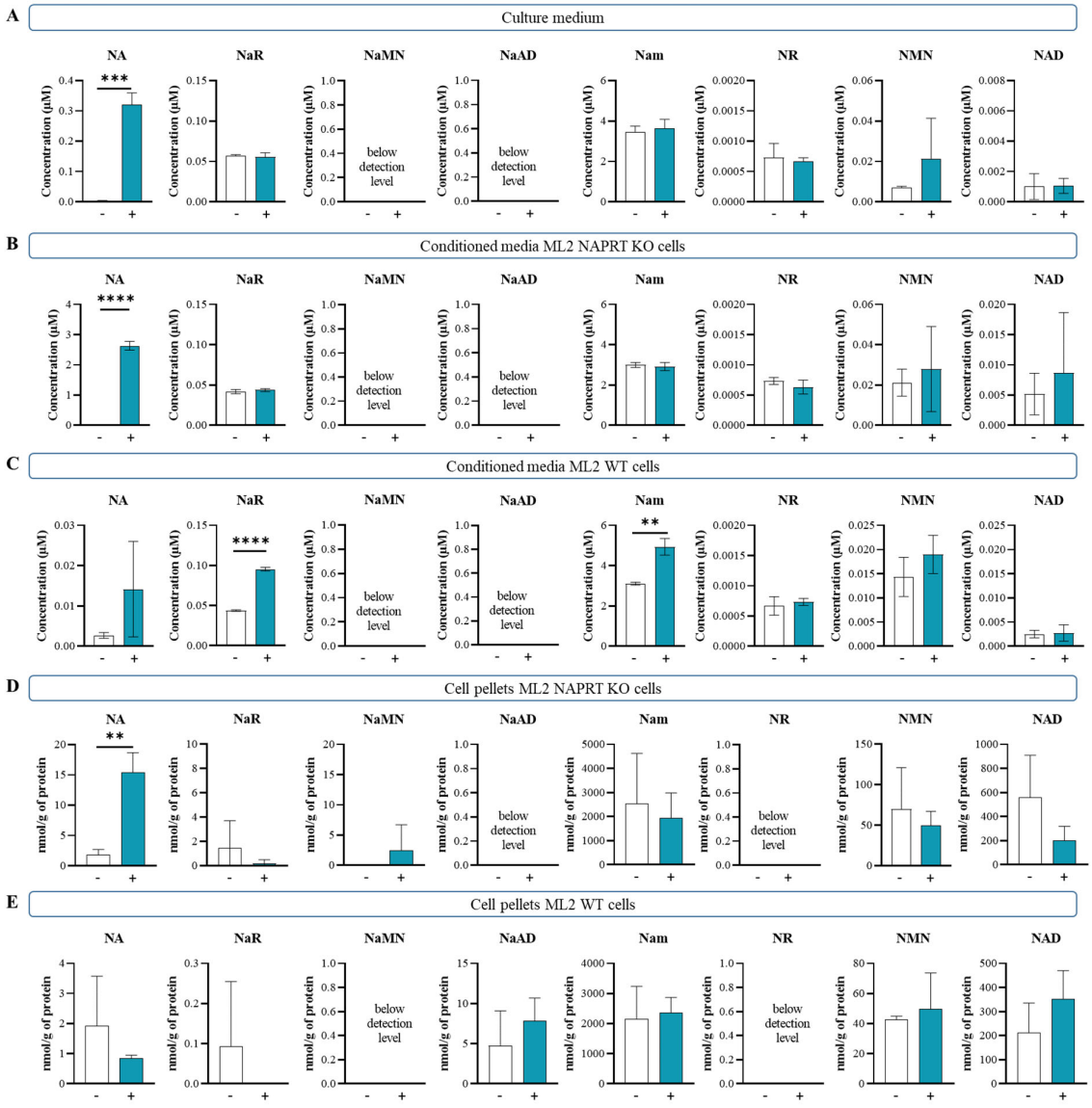

Figure S6. NAD-related metabolite levels in the culture medium, cells and conditioned media upon nicotinaldehyde supplementation. NAD-related metabolite levels were evaluated by LC-MS/MS without or with nicotinaldehyde supplementation (10 µM) and incubation for 96 h in (**A**) the RPMI culture medium (without cells), (**B**) conditioned media of ML2 NAPRT KO cells, (**C**) conditioned media of ML2 WT cells, **D**) cell pellets of ML2 NAPRT KO cells, and **E**) cell pellets of ML2 WT cells. Nam, nicotinamide; NR, nicotinamide riboside; NMN, Nam mononucleotide; NA, nicotinic acid; NaR, nicotinic acid riboside; NaMN, NA mononucleotide; NaAD, NA adenine dinucleotide. Data are mean ± SD, $n = 3$. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

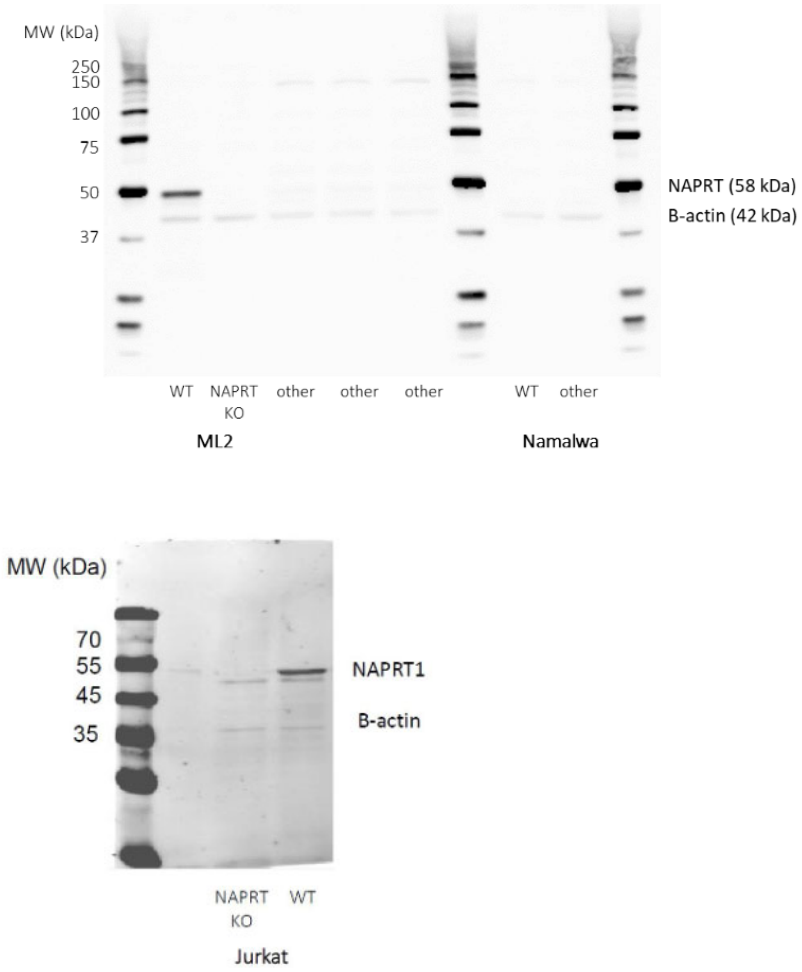

Figure S7. Original Western Blot for Figure 3C.

CHAPTER 3: DISCUSSIONS AND CONCLUSION

General discussions

In this study, we investigated multiple facets of cancer biology that influence the response of leukemia and lymphoma cells to NAD biosynthesis inhibitor APO866. In the first project, we characterized AML models of *in vivo*-acquired resistance toward APO866 and demonstrated a profound modification of their transcriptomic profiles. This reprogramming promoted the activation of pro-survival signaling pathways and adaptive metabolic changes, which altogether contributed to the acquired resistance. In the second project, we elucidated the role of gut microbiota in modulating NAD homeostasis within tumor cells and their potentiality to hinder the efficacy of NAMPT inhibitors in distant tumors. In the third project, we identified a novel member of human NAD metabolome, nicotinaldehyde, which can be exploited for NAD biosynthesis by leukemia cells treated with NAMPT inhibitors and therefore abrogate their anticancer effects. Collectively, our study provides comprehensive insights into the diverse mechanisms altering the response of leukemia and lymphoma cells to NAMPT inhibitors.

In the previous chapters dedicated to each project, we have provided discussions and future perspectives for the specific topics. In this section, we will discuss more in-depth and globally some aspects that have arisen from the projects.

The second and the third studies together demonstrated the complexity of NAD metabolome in the context of NAD-targeting cancer therapies. These studies demonstrated that the presence of NAD-related metabolites in the tumor environment and at systemic level can significantly impact treatment outcomes. Therefore, they highlight the importance of the availability of NAD-related metabolites in determining the efficacy of NAMPT inhibitors. Notably, diets or supplements can influence the levels of NAD-related metabolites in the blood circulation. For instance, NA was used for the treatment of dyslipidemia [98]. It is important to control the systemic levels of NAD precursors to optimize the effectiveness of NAMPT inhibitors for cancer treatment. Furthermore, the discoveries of nicotinaldehyde and of other potent precursors in other recent studies indicate the importance of gaining comprehensive understanding of the global landscape of human NAD metabolome. Further investigations are required to fully appreciate the intricate interactions between NAD-related metabolites, cancer cells and therapeutic interventions.

In addition, our studies have demonstrated the potential of blocking the NAPRT-dependent *Preiss-Handler* pathway as an effective approach to increase tumor sensitivity to

APO866. This strongly suggests the use of NAPRT inhibitor in combination with NAMPT inhibitors as a promising strategy to improve their efficacy. Several ongoing research efforts are focused on the identification and the development of NAPRT inhibitors [32], [99]. The newly identified NAPRT inhibitors exhibited significant anticancer activity when used in combination with FK866 [32]. Moreover, *NAPRT* depletion has been shown to induce the regression of ovarian cancer xenografts, suggesting that NAPRT inhibitors may also potentially consist of effective therapies as single agents in some cancers [100]. Indeed, some types of cancers have been demonstrated to be more dependent on the *Preiss-Handler* pathway than the salvage pathway for NAD biosynthesis [101]. Therefore, targeting NAPRT may be particularly efficient in such tumors. On the other hand, high expression of NAPRT may contribute to resistance toward NAMPT inhibitors. Hence, NAPRT expression level may also consist of a good predictive marker for the tumor response to NAMPT inhibitors. Taken together, consideration of both NAMPT and NAPRT pathways is crucial for optimizing the therapeutic effect of NAD-targeting anticancer treatment. The development of NAPRT inhibitors and the evaluation of NAPRT expression levels in tumors hold great promise in advancing the field of NAD-targeting therapies.

Both the salvage pathway and the *Preiss-Handler* pathways, as well as the *de novo* pathway rely on NMNAT activities for the conversion of the mononucleotides to NAD or NAAD. Targeting NMNAT represents a promising avenue for effectively inducing NAD depletion in tumors and should be investigated further. To date, gallotannin and few NAD analogs have been identified as NMNAT inhibitors [102], [103]. However, these inhibitors affect all isoforms of NMNAT, while growing evidence indicate that the three isoforms of NMNAT display different tissue distributions, subcellular localizations and functions [40]. Therefore, the development of selective isoform-specific inhibitors is of great interest to minimize the potential side effects on healthy tissues.

Our project on the characterization of *in vivo*-acquired resistance models is the first study in the existing literature to provide untargeted whole transcriptomic analyses of cancer cells resistant to NAD-targeting cancer therapies. This study offers interesting data on gene expression changes triggered by APO866 or associated with resistance to APO866. Exploring further these transcriptomic data will provide valuable insights into the mechanism of action of NAMPT inhibitors. For instance, in APO866-sensitive ML2, we observed upregulations of various functional pathways upon APO866 treatment. Several of them have been previously reported to be associated with NAMPT functions or APO866 cytotoxic effects, such as the DNA damage response, MAPK signaling or TGFβ signaling [41], [83], [104]. Additionally, other pathways appeared to be significantly altered by NAMPT inhibition but have not been extensively investigated so far. In-depth analysis will enable the identification of precise key

pathways or molecules, including transcription factors or signaling kinases that are involved in the anticancer effects of NAMPT inhibitors. Of note, the characterization of APO866-associated post-transcriptional and protein activity levels will complement the analysis and provide a more comprehensive understanding of the cellular response to NAMPT inhibitors.

Our study represents a significant advancement as it reports the first model of acquired resistance to APO866 generated in *in vivo* settings. We observed the activation of inflammatory signaling in the resistant leukemia cells, prompting further exploration into the potential contribution of the *in vivo* environment to this activation. In addition, it is worth investigating whether these pro-inflammatory features of resistant cells promote tumor immune escape.

Interestingly, the inflammatory signals induced in resistant leukemia cells, including IL1β, IL-8 and TNFα, align with those regulated by eNAMPT, which are notably reported to be released upon exposure to exogenous NAMPT. Indeed, eNAMPT can act as a pro-inflammatory cytokine, which binds to TLR4 and therefore activates NFκB, MAPK and STAT3, promoting the secretion of cytokines such as IL-1β, IL-8, IL-6 and TNFα [51]. Based on these observations, it is plausible to hypothesize a potential role of eNAMPT in the resistant leukemia cells. A secretion of eNAMPT by resistant ML2 may consist of a mechanism to enable sustained activation of cellular signaling pathways and inflammatory responses. Exploring this possibility could offer novel insights into the interplay between the tumor immune environment and the efficacy of NAMPT inhibitors, which warrant further investigation in future studies.

Metabolic adaptations were identified to be involved in the protective mechanism against APO866-induced stresses. Notably, our analysis revealed that resistant leukemia cells exhibited reduced mitochondrial OXPHOS activities. This reduction allows a higher availability of NAD in the cytosolic and nuclear compartments, where it can be used to fight cellular stresses. Consistent with this interpretation, previous studies have shown that cytosolic and nuclear NAD pools are more sensitive to NAMPT inhibitors compared to the mitochondrial pool [105]. In addition, a buffering function of mitochondria was observed in cells with excessive PARP activity and reduced NAD levels [106]. These findings highlight the close link between cellular metabolism and stress management, underlining the importance of subcellular distribution of NAD for the maintenance of cellular homeostasis.

The acquisition of resistance to therapies is a major issue in the treatment of AML. The dynamic clonal composition of AML is a key driver of the development of resistance in patients. Interestingly, many pathways identified to be involved in the resistance to APO866 in our study aligned with those reported to underlie the mechanisms of resistance to conventional or

targeted therapies in AML. For instance, venetoclax resistance is often associated with Mcl-1 upregulation, while resistance to FLT3 inhibitor may rely on the activation of alternative pro-survival pathways such as MEK/Erk or PI3K/Akt/mTOR pathways [107]. Therefore, these identified pathways appear to play a key role in the development of resistance to therapies in AML, and the exploration of combinatory approaches to target them holds great promise for overcoming therapy resistance and improving patient outcomes.

Conclusion

In this thesis, I elucidated the diverse mechanisms affecting the response of cancer cells to NAMPT inhibitors. Specifically, I deciphered the mechanism underlying resistance to NAMPT inhibitor APO866 in AML model. In addition, I demonstrated the modulatory effect of gut microbiota on the antitumor efficacy of APO866. Furthermore, I discovered a previously unidentified novel precursor of NAD biosynthesis in leukemia. These findings hold great potential for improving the limited efficacy of NAMPT inhibitors in clinical settings and overcoming resistance acquisition. The use of combinatory approaches with NAMPT inhibitors emerge as a promising strategy. I am confident that my findings will inspire future research on NAD biology and contribute to the development of innovative therapeutic strategies using NAMPT inhibitors.

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