APO866 INCREASES ANTI-TUMOR ACTIVITY OF CYCLOSPORIN-A BY INDUCING MITOCHONDRIAL AND ENDOPLASMIC RETICULUM STRESS IN LEUKEMIA CELLS

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1
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Footnotes

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Abbreviations: Pgp, P-glycoprotein; NAMPT, nicotinamide phosphoribosyltransferase; Δψm, mitochondrial transmembrane potential; PBMCs, peripheral blood mononuclear cells; PBPs, peripheral blood precursor cells; B-CLL, B-cell chronic lymphocytic leukemia; AML, acute myeloid leukemia; TMRE, tetramethylrhodamine ethyl ester; CI, co-operative index; ER, endoplasmic reticulum; UPS, unfolded protein response; UPR, ubiquitin-proteasome system.
Translational Relevance

The rate-limiting enzyme in NAD$^+$ biosynthesis from nicotinamide, NAMPT, regulates growth and metastatic potential of tumor cells. Leukemic cells show a higher NAD$^+$ turnover rate than normal cells, suggesting that NAD$^+$ biosynthesis could be critically required in hematologic malignancies, too. Here we show that the NAMPT inhibitor APO866 is active, but only achieves a partial cell killing in primary leukemia cells. Inhibition of P-glycoprotein 1 (Pgp), which is one of the key factors mediating multidrug resistance, is shown to potentiate the cytotoxic effects of APO866 in leukemia cells, but not in healthy leukocytes and hematopoietic progenitor cells, by increasing intracellular APO866 concentration and thereby exacerbating ATP shortage and endoplasmic reticulum stress. Our data indicate a possible, new, safe and widely applicable approach for treating hematologic malignancies.
Abstract

Purpose: The nicotinamide phosphoribosyltransferase (NAMPT) inhibitor, APO866, has been previously shown to have anti-leukemic activity in preclinical models, but its cytotoxicity in primary leukemia cells is frequently limited. The success of current anti-leukemic treatments is reduced by the occurrence of multidrug resistance, which, in turn, is mediated by membrane transport proteins, such as P-glycoprotein-1 (Pgp). Here we evaluated the anti-leukemic effects of APO866 in combination with Pgp inhibitors and studied the mechanisms underlying the interaction between these two types of agents.

Experimental Design: The effects of APO866 with or without Pgp inhibitors were tested on the viability of leukemia cell lines, primary leukemia cells (AML, n=6; B-CLL, n=19), and healthy leukocytes. Intracellular NAD\(^+\) and ATP levels, mitochondrial transmembrane potential (\(\Delta \Psi_m\)), markers of apoptosis and of endoplasmic reticulum (ER) stress were evaluated.

Results: The combination of APO866 with Pgp inhibitors resulted in a synergistic cytotoxic effect in leukemia cells, while sparing normal CD34\(^+\) progenitor cells and peripheral blood mononuclear cells. Combining Pgp inhibitors with APO866 led to increased intracellular APO866 levels, compounded NAD\(^+\) and ATP shortage, and induced \(\Delta \Psi_m\) dissipation. Notably, APO866, Pgp inhibitors and, to a much higher extent, their combination induced ER stress and ER stress inhibition strongly reduced the activity of these treatments.

Conclusions: APO866 and Pgp inhibitors show a strong synergistic cooperation in leukemia cells, including AML and B-CLL samples. Further evaluations of the combination of these agents in clinical setting should be considered.
Introduction

Intracellular nicotinamide adenine dinucleotide (NAD+) is essential for several cellular processes, acting either as a coenzyme in redox reactions or as a substrate for NAD+-degrading enzymes. Cancer cells are highly dependent on NAD+ to face increased metabolic demands and high proliferation rates (1). Tryptophan, nicotinic acid (NA), nicotinamide (NAM) and nicotinamide ribose are the main NAD+ precursors in mammals. Specifically, NAD+ production from NAM via nicotinamide phosphoribosyltransferase (NAMPT) appears to play a major role in lymphocytes and hematopoietic cells and to be further upregulated in leukemia cells, justifying their susceptibility to NAMPT inhibitors such as APO866 (formerly known as FK866 or WK175) (2-9). Based on its promising preclinical activity, APO866 was proposed as novel drug for different hematological malignancies (4, 7, 10, 11). Nevertheless, this agent exhibits variable and frequently limited cytotoxicity against primary leukemia cells, which limits its applicability as a single agent. In the attempt to obviate to such limitation, APO866 has been combined with Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) (12), DNA damaging agents (daunorubicin, cisplatin, Ara-C and melphalan) (13, 14), ionizing radiations (15), rituximab (16) and proteasome inhibitors (17), frequently achieving remarkable anticancer effects.

Multidrug resistance (MDR) limits the benefit of different types of anticancer agents (18). Its etiology is multifactorial, but overexpression of membrane transport proteins, such as 170-kDa P-glycoprotein-1 (Pgp), represents a leading cause (19). By extruding drugs across the plasma membrane, Pgp reduces their intracellular concentration and, thus, their efficacy (23-28). Consistent with this biological function, high levels of Pgp are frequently observed in hematologic (lymphomas, multiple myeloma, and leukemia) (20, 21), as well as in solid
(neuroblastoma and soft tissue sarcoma) tumors (22) and Pgp overexpression has been frequently associated with a poor prognosis. Several natural and synthetic Pgp inhibitors have been identified, including drugs in clinical use, such as calcium channel blockers (verapamil and nifedipine), indole alkaloids (reserpine), steroids (progesterone and tamoxifen) and the immunosuppressive agents cyclosporin A and rapamycin (29). A recent clinical study evaluated PSC-833 (valspodar), a second-generation Pgp inhibitor, in combination with chemotherapy in patients with acute myeloid leukemia (AML) (30). Disappointingly, this trial failed to show any clinical advantage from the use of the Pgp inhibitor, suggesting that the clinical settings and the anticancer treatments that are going to benefit the most from the use of Pgp inhibitors may still have to be identified.

Here we demonstrate that Pgp inhibitors, such as cyclosporin A (CsA), verapamil and PGP-4008, synergistically increase the anti-leukemic activity of APO866. This effect is shown to reflect an increased intracellular concentration of APO866, which, in turn, increases its ability to block growth- and survival-promoting pathways in leukemia cells.
Materials and Methods

Cell lines and reagents
The leukemia (OCI/AML2, OCI/AML3, HL-60, HEL, KG1a, SET1, MV4-11, MEC.1, MEC.2 and LAMA-84 imatinib-sensitive or -resistant) multiple myeloma (RPMI-8226 and Dox40) and lymphoma (Daudi, U937, Raji and SU-DHL1) cell lines were provided by collaborators or purchased from ATCC or DSMZ (Braunschweig, Germany). All cell lines were grown in RPMI 1640-based medium supplemented with 10% FBS (GIBCO, Life Technologies, Carlsbad, CA), 2 μM l-1 glutamine, 100 U ml-1 penicillin, and 100 μg ml-1 streptomycin (GIBCO, Life Technologies, Carlsbad, CA). Nilotinib was supplied by Novartis and was stored at 10 mM in DMSO at -20°C. CsA, verapamil, melphalan, doxorubicin, fludarabine and velcade were obtained from the pharmacy of the S. Martino Hospital in Genoa, Italy. PGP-4008 was purchased from Alexis Biochemicals (Plymouth Meeting, PA USA). Tetramethylrhodamine ethyl ester (TMRE), 4-PBA, NAM and NA were obtained from Sigma-Aldrich (Sigma Aldrich Italia, Milano, Italy). APO866 was generously provided by the NIMH Chemical Synthesis and Drug Supply Program.

Primary cell isolation from patient samples
Following written consent obtainment, peripheral blood samples were obtained from a cohort of 25 patients (19 B-CLL and 6 AML) and healthy donors (n = 3) at the Department of Internal Medicine of the University of Genova (Genova, Italy), according to the Declaration of Helsinki. The clinical and laboratory features of B-CLL and AML patients are summarized in Tables 1 and 2, respectively. For B-CLL cell isolation, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll-Hypaque (Biotest, Dreieich, Germany).
B-CLL phenotype of the obtained cell preparations was confirmed by immunostaining with anti-CD19, anti-CD5, and anti-CD23 (Immunotech, Marseille, France), followed by the flow cytometric analysis. The purity of the isolated B-CLL cells was typically >85%. AML blasts were isolated by adding a 6% dextran solution (Fresenius Kabi, Varese, Italy) to the blood specimens at a ratio of 4:5, followed by a 1-h incubation at room temperature. Thereafter, the leukocytes-enriched supernatants were transferred to a 50 ml conical centrifuge tube and centrifuged at 300xg for 10 min. Residual red blood cells were lysed by suspending the cell pellets in 4 ml 0.2% NaCl for 30 seconds followed by addition of 4 ml 1.6% NaCl and immediate centrifugation at 300xg for 10 min. Normal PBMCs were isolated from healthy donor blood samples by density gradient centrifugation on Ficoll-Hypaque. Cells were either used immediately for viability assays or for mRNA isolation, or stored at -80° in medium containing 20% FBS and 10% DMSO. CD34+ peripheral blood precursor cells (PBPCs) were obtained from the excess PBPC concentrates (1-2ml) of G-CSF-mobilized patients undergoing autologous PBPC transplantation (n=3), after obtaining informed consent according to the Declaration of Helsinki. CD34+ cells were purified using the CD34 MicroBead Kit from Miltenyi Biotec (Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Using this method, CD34+ cells were typically >80% pure and >80% viable as detected by propidium iodide (PI) staining and flow cytometry (see below).

Viability assays

2 x 10^5 cells/well (primary leukemia cells, PBMCs and PBPCs) or 5 x 10^4 cells/well (OCI/AML3 and MEC.1) were plated in 96 well plates in a final volume of 200 μl in the presence or absence of the indicated stimuli. Dead cells were quantified 96 h later by PI staining (2 μg/ml) and flow
cytometry (FACS Calibur, Becton Dickinson, BD Italia, Milan, Italy). Specific death was calculated as follows: \[\frac{(% \text{experimental death} - % \text{spontaneous death})}{(100 - % \text{spontaneous death})} \times 100.\]

For Annexin-V/PI staining 3 x 10⁶ leukemic cells were plated in 1 ml medium in 24-well plates in the presence of the indicated stimuli and for the indicated amounts of time. Afterwards, cells were washed, stained with Annexin-V-FITC (Becton Dickinson) and PI and analyzed by flow cytometry. For the detection of hypodiploid cell nuclei, cell pellets were suspended in a buffer containing 0.1% sodium citrate, 0.1% Triton-X 100, and 50 μg/ml PI. Thereafter, cells were analyzed by flow cytometry.

**Mitochondrial transmembrane potential ($\Delta \psi_m$) determination**

$\Delta \psi_m$ was determined as previously described (4, 17). Briefly, cells were harvested, washed and incubated in the presence of 50 nM TMRE in regular RPMI-based medium for 15′ at 37°C. Thereafter, cells were analyzed by flow cytometry.

**Immunoblotting**

Whole-cell lysates and cell fractions were prepared as previously described (4, 17). Protein concentrations were determined by Bradford assay (Bio-Rad, CA) and 10-50 μg proteins were subjected to SDS-PAGE, transferred to PVDF membranes, and detected with the following antibodies: anti-NAMPT (Bethyl Laboratories, Inc., Montgomery, TX), anti-BIP, -IRE1α, -ubiquitin, -CHOP, -MDR1/ABCB1 (Cell Signaling Technology, Beverly, MA), anti-γ tubulin (mouse monoclonal, Sigma Aldrich) and anti-nucleolin (Santa Cruz Biotech, CA). Standard enhanced chemiluminescence (ECL by Thermo Fisher Scientific, IL) was used for protein bands detection.
**Determination of NAD⁺ and ATP levels**

Intracellular NAD(H) content was determined with a NAD(H) Quantification Kit by BioVision following the instructions of the manufacturer. Intracellular ATP content was determined with Cell titer Glo Luminescent Cell Viability Assay (Promega). NAD⁺ and ATP values were normalized to the number of viable cells as determined with Trypan Blue (Lonza).

**Intracellular APO866 measurement**

20x10⁶ primary B-CLL cells were plated in 6-well plates and treated for 24 h with 3 nM APO866 in presence or absence of CyA 0.3 μM. Thereafter, cells were harvested and lysed in water. The extracted material was analyzed on an Agilent 1100 capillary chromatography system, equipped with a diode array detector and coupled to a mass spectrometer Agilent 1100 series LC/MSD Trap, equipped with an orthogonal geometry electrospray ion source and an ion-trap analyzer. HPLC separation was performed on a Waters Atlantis TM dC18 column (150x1 mm; particle size, 3 μm) at a flow rate of 30 µl/min; eluent A was 0.1% formic acid in water, eluent B was acetonitrile containing 0.1% formic acid, and the applied gradient was as follows: during the first 5 min eluent B was maintained at 5%. Subsequently, between min 5 and min 35 , eluent B was progressively increased to 100%. Detection wavelength was set at 220 and 260 nm. MS spectra were acquired in positive ion mode in the m/z range 100-400. APO866 concentration values were normalized to a protein concentration.

**Immunohistochemistry (IHC)**

Sections of BM samples from patients diagnosed with hematologic malignancies at the IRCCS AOU San Martino-IST were stained with anti-NAMPT (clone H-300; sc-67020) (Santa Cruz, 11
CA, United States) (31). IHC was performed using the Ventana BenchMark XT automated immunostainer. Tissue sections were deparaffinized and rehydrated. After antigen retrieval, sections were incubated with primary antibodies at a dilution of 1:200 and 3,3’-diaminobenzidine (DAB) was used as a chromogen. Sections were counterstained with May-Grünwald-Giemsa.

**RNA interference (RNAi)**

RNAi was performed with an ON-TARGET PLUS SMART pool targeting ABCB1 (GE Dharmacon, Lafayette, CO). A non-targeting scrambled negative control siRNA was used as negative control (GE Dharmacon, Lafayette, CO). Briefly, OCI/AML3 and MEC-1 cells were transiently transfected with MDR-1 siRNA with the Amaxa technology (V-solution with X-001 or U-013 program, respectively).

**Statistical Analyses**

Each experiment was repeated at least three times. Statistical analyses were performed with GraphPad Prism software 6 using one-way ANOVA for multiple-group comparison or unpaired t-test for two-group comparison. P values below 0.05 were considered significant. Expression levels of NAMPT in human cancer cell lines were obtained from data sets collected in Oncomine portals at [http://www.oncomine.org](http://www.oncomine.org) (Barretina Cell Line data set). Copy number data for the human 7q22.3 locus (where NAMPT maps) in tumor cell lines was downloaded from the publicly available database ([http://www.broadinstitute.org/ccle](http://www.broadinstitute.org/ccle)). Next, data were analyzed using the Integrative Genomics Viewer (IGV) analysis software. Expression levels of NAMPT in different hematological tumors, were obtained from publicly available Gene Expression
Omnibus (GEO) datasets (accession numbers GSE12417 for AML; GSE4475 for DLBCL and GSE22762 for CLL). Differences in median-centered transcript levels between different groups of samples were evaluated by unpaired Student’s t test. Drug synergism was analyzed by isobologram analysis using the CalcuSyn Version 2.0 software program (Biosoft). A Combination index (CI) less than 1.0 indicates synergism; CI =1, additive effect; and CI > 1, no significant combination effect (32).
Results

NAMPT is overexpressed and has adverse prognostic relevance in hematologic malignancies

We first investigated the relevance of NAMPT in hematological malignancies by characterizing its expression in a wide range of cell lines and patient samples. In line with previously published data (4, 7, 12, 17, 33), an analysis of the Cancer Cell Line Encyclopedia (CCLE) database (34) revealed higher NAMPT expression levels in hematologic malignancies (including lymphomas, leukemias and multiple myeloma - MM) compared with cancer cell lines of epithelial origin (Figure 1A). An array-based comparative genomic hybridization (aCGH) analysis of the same dataset showed focal amplification of the NAMPT locus (mapping on 7q22.3) and NAMPT transcript levels were found to correlate with the DNA copy number, particularly in cell lines derived from hematologic malignancies (Pearson value= 0.391; p=0.002) (Supplementary Figure 1A-B). These findings, supported by the strong NAMPT expression in leukemia cell lines (Figure 1B) and by our previous study of NAMPT’s role in MM (4, 17, 35), prompted us to further investigate the role of this enzyme in leukemogenesis. Using IHC, we were able to confirm that BM biopsies from newly diagnosed acute myeloid leukemia (AML) and B-cell chronic lymphocytic leukemia (B-CLL) patients exhibit significantly increased NAMPT levels than samples obtained from healthy donors (Figure 1C).

We also retrospectively analyzed the prognostic relevance of baseline NAMPT expression by interrogating microarray datasets of AML, DLBCL and B-CLL patients. As shown in Figure 1D, high NAMPT expression in hematologic malignancies was significantly associated with poor overall survival. Thus, altogether, these data supported the notion that NAMPT plays an
important role in the pathophysiology of hematologic malignancies and that it represents an attractive therapeutic target (11, 16, 34, 36).

**Pgp inhibitors synergistically cooperate with APO866 to the killing of human leukemia cells**

The variability of APO866 anti-tumor effects prompted us to search for drugs that, when combined with this NAMPT inhibitor would enhance its efficacy (12, 14, 16, 17, 37). To this end, we screened several agents that are widely used in hematology, monitoring their effects on viability of primary leukemia cells (either as single agents or in combination with APO866). As shown in Figure 2A, CsA was readily identified as one of the best sensitizers of primary B-CLL cells to the activity of APO866. CsA is an immunosuppressant whose mechanism of action entails the obstruction of calcineurin and, thereby, inhibition of NF-AT (nuclear factor of activated T-cells). However, when treating primary B-CLL cells with FK506 (tacrolimus), an unrelated calcineurin inhibitor, we found that this agent failed to enhance APO866 activity (Supplementary Figure 2), suggesting that the potentiation effect observed with Csa may reflect an alternative mode of action of the latter. In addition to inhibiting calcineurin, Csa is also a well-known Pgp inhibitor. Thus, we reasoned that Pgp inhibition may be the mechanism underlying Csa-mediated enhancement of APO866 anti-leukemic activity and tested other, unrelated Pgp inhibitors in combination with APO866 to see whether they would recreate the effects of Csa. Indeed, both verapamil and PGP-4008 strongly enhanced APO866 activity in two leukemia cell lines (OCI/AML3 - AML - and MEC.1 - B-CLL) (Figure 2B, C) and in primary leukemia cells from a cohort of patient that included cases of AML (n=6) and B-CLL (n=19) (Figure 2C and Tables 1-2).
Consistent with the hypothesis that APO866 may be a Pgp substrate, utilizing HPLC/MS, we were able to show that both Csa and PGP-4008 increase APO866 intracellular levels in primary B-CLL cells (Figure 2D and Supplementary Figure 3). Finally, additional evidence in support of the notion that APO866 anti-leukemic activity is regulated by Pgp activity was obtained in RNAi experiments and in studies with Pgp-overexpressing cell lines. In a first set of experiments, the leukemia cell lines OCI-AML3 and MEC1 were transfected with Pgp siRNA and Pgp silencing was verified by WB two days after transfection (Figure 3A, upper insets). As shown in Figure 3A (lower insets), Pgp depletion significantly enhanced leukemia cell death upon APO866 treatment vs. control, non-targeting siRNA. Notably, the addition of CsA in leukemia cells in which Pgp was previously silenced further enhanced APO866 activity, suggesting that reducing Pgp protein levels by RNAi may be used to further increase the efficacy of pharmacological Pgp inhibitors. Subsequently, Doxo40 (44) and LAMA84-r (45), two well-characterized Pgp-overexpressing cell lines, were compared with their parental cell lines (RPMI8226 and LAMA84, respectively) in terms of susceptibility to APO866, Csa, and their combination. As predicted, cells overexpressing Pgp were found to be more resistant to APO866 than the non-Pgp-overexpressing cells (Figure 3B). Therefore, collectively, these findings are consistent with the hypothesis that APO866 is a substrate of Pgp and that APO866 activity can be with Pgp inhibitors, including Csa.

Finally, we assessed whether combining APO866 with Pgp inhibitors would result in an increased cytotoxicity in healthy PBMC and PBPC, too. However, neither APO866 nor Pgp inhibitors or their combination induced cell death in these cells, suggesting that these treatments could have a favorable therapeutic index in vivo, too (Supplementary Figure 5A, B).
CsA enhances NAD⁺ shortage, ∆Ψₘ loss and apoptosis triggered by APO866

It is well established that APO866-mediated cytotoxicity largely relies on the depletion of intracellular NAD⁺ stores, which, in turn, ultimately leads to ATP shortage (2, 36, 46). Utilizing a cycling enzymatic assay we monitored the metabolic changes occurring in primary leukemia cells (AML and B-CLL), as well as in leukemia cell lines, following their exposure to APO866, Pgp inhibitors, and their combinations. In line with our previous studies, APO866 treatment alone consistently reduced intracellular NAD⁺ content, as well as ATP, in cell lines and in primary leukemia cells (Figure 4A, B, and Supplementary Figure 6). Interestingly, Pgp inhibitors alone were found to also slightly reduce both NAD⁺ and ATP. However, in response to a treatment with combined APO866 and Pgp inhibitors, both NAD⁺ and ATP depletion were exacerbated. To gain further insight into the mechanism of cell death occurring in response to combined APO866 and Pgp inhibitors, we monitored ∆Ψₘ and the occurrence of apoptosis (by PI/Annexin-V staining), as well as of hypodiploid cell nuclei, in leukemia cells over time. With this combined approach we were able to show that APO866 causes ∆Ψₘ dissipation, an apoptotic cell phenotype (AnnexinV⁺/PI⁻ or AnnexinV⁺/PI⁺), as well as a strong increase in hypodiploid (apoptotic) cell nuclei in primary B-CLL cells (Figure 5A-C). Taken as single agents, Pgp inhibitors (Csa, PGP-4008 and nilotinib) were much less effective than APO866. However, adding a Pgp inhibitor to APO866 consistently led to a much more pronounced ∆Ψₘ loss and apoptotic phenotype. Since previous studies showed autophagy to be frequently associated with APO866-induced leukemia cell death (4, 11, 16, 17), we also investigated whether an aberrant activation of the autophagic machinery would also be involved in the cooperation between APO866 and Pgp inhibitors. However, addition of a Pgp inhibitor failed to increase the expression of LC3B-II (a marker of autophagy activation) above the levels detected with
APO866 alone (data no shown). In addition, autophagy inhibition with 3-methyl adenine (3-MA) failed to protect leukemia cells from APO866 in combination with Pgp inhibitors (data not shown). Therefore, these data essentially rule out a major role of autophagy in the observed synergistic effects between APO866 and Pgp inhibitors.

**Evidence for an involvement of ER stress and UPR in leukemia cells sensitization to APO866 by Pgp inhibitors**

Previous studies by our groups showed that APO866 negatively affect ER physiology in susceptible cells. In addition, recent studies have also linked the anticancer activity of Pgp inhibitors (including Csa and verapamil) to the induction of ER stress and of a terminal unfolded protein response. Thus, since ER stress is a main trigger for apoptotic responses, we assessed its potential relevance in the anti-leukemic effects of APO866, Pgp inhibitors and their combination (13, 47-51). ER stress inhibition with the chemical chaperone 4-phenyl butyric acid (4-BPA) (52) significantly reduced the cytotoxic effects of APO866, CsA and of the two combined agents in OCI/AML3 and MEC.1 cells, as well as in primary B-CLL cells (Figure 6A). An analysis of ER stress-related markers was also performed. As predicted, in cells that were co-treated with APO866 and Csa a stronger increase in IRE1α, C/EBP-homologous protein (CHOP) and BIP levels as compared with the single-agent treatments was observed (Figure 6B and Supplementary Figure 7). A marked increase in the molecular chaperone BIP was also detected in primary B-CLL cells treated with APO866 and CsA (Figure 6C). Finally, a considerable accumulation of misfolded proteins, detected as a smear of high-molecular weight adducts (Figure 6B) in response to combined Csa and APO866 was also documented, whilst a weaker smear, previously identified as made of poly-ubiquitinated proteins (53), was observed in response to APO866.
alone. Thus, overall, these findings indicate a novel role for ER stress and unfolded protein accumulation in the anti-leukemic activity of APO866 and of its combination with Pgp inhibitors.

**Nicotinamide and Nicotinic acid completely abolish activity of co-treatment in leukemia cells**

The Preiss Handler pathway (2, 8) for NAD$^+$ biosynthesis sees the conversion of nicotinic acid to nicotinic acid mononucleotide (NaMN) by the enzyme Nicotinic Acid Phosphoribosyl Transferase (NAPRT1). NaMN is subsequently converted to NAD$^+$ by an additional enzymatic reaction. In NAPRT1 proficient cells, NA addition is typically sufficient to rescue the cytotoxic activity of APO866, allowing to verify that APO866-induced cell death is indeed mediated by reduced NAD$^+$ availability (54). Based on these premises, we first determined $NAPRT1$ expression in primary leukemia cells by Q-PCR and essentially found that $NAPRT1$ was ubiquitously expressed. Thereafter, we investigated the specific role of NAD$^+$ depletion in the observed synergism (between APO866 and Pgp inhibitors) by rescuing NAD$^+$ biosynthesis with NA supplementation. Consistent with our previous findings (37), NA supplementation completely abrogated the anti-leukemic activity of APO866, both as a single agent and in combination with Csa (Supplementary Figure 8 A, B), confirming the role of NAD$^+$ depletion in the activity of these anti-leukemic treatments. Interestingly, no protection from APO866, Csa, or their combination was conferred to leukemia cells by tryptophan supplementation, essentially ruling out a major role for the $de$ $novo$ NAD$^+$ biosynthetic pathway in this type of cancer (data not shown).
Discussion

Here we show that the anti-leukemic activity of the NAD⁺-lowering agent APO866 is strongly enhanced by combining it with Pgp inhibitors. The latter are shown to increase APO866 intracellular concentration, exacerbating APO866’s effects on cellular energetics. In addition, a key role for ER stress in the anticancer activity of APO866 and of its combination with Pgp inhibitors is demonstrated for the first time.

We first documented that high levels of NAMPT are expressed in several types of hematologic malignancies and demonstrated the prognostic relevance of this protein. Next, in the attempt to identify agents that, when combined with APO866, would enhance its anti-leukemic activity, we discovered Csa as a highly effective potentiator. Notably, the combination of Csa with APO866, while highly active in different types of leukemias, was found to spare healthy PBMCs and PBPC suggesting that this novel regimen should have a favourable therapeutic index in patients, too.

At the molecular level, the ability of Csa to boost APO866 activity is shown to reflect its function as a Pgp inhibitor, instead of as a calcineurin inhibitor. Consistent with this notion, Csa and other Pgp inhibitors increase intracellular APO866 levels, thereby enhancing APO866-induced NAD⁺ depletion, ΔΨm loss, and ATP shortage. (Supplementary Figure 8 C) Activation of the apoptosis machinery is one of the downstream events leading to leukemia cell death in response to combined APO866 and Pgp inhibitors, as detected by Annexin-V/propidium iodide staining and by the occurrence of hypodyplid cell nuclei. In addition, we show here for the first time that APO866 and, to a higher extent, its combination with Pgp inhibitors induce ER stress and that this type of response plays a role in the anticancer activity of these agents. Notably, this type of cell death appears to be distinct from the autophagic-cell death that was previously
observed with single agent APO866 treatment (11, 16). The lack of effect of autophagy inhibition on the cell demise that occurs in response to APO866 plus Csa could reflect the fact that, by strongly increasing the intracellular levels of APO866 and, consequently, its metabolic effects (i.e. NAD+ and ATP shortage), Csa leads to a shift in the cell death programs activated by the NAMPT inhibitor, preferentially activating apoptosis and ER stress (51).

In conclusion, our data indicate that APO866 is a bona fide Pgp substrate and that combining this agent with Pgp inhibitors (including Csa) strongly potentiate its cytotoxic activity on leukemia, but not on healthy cells. We demonstrate a key role for endoplasmic reticulum (ER) stress in the observed synergistic interaction between APO866 and Pgp inhibitors. Our data provide the biological rationale for combining Pgp inhibitors with APO866 in leukemia patients.
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Author contributions

A.C., M.C. and A.N. designed the research, performed experiments, analyzed the data and wrote the manuscript; S.B. performed experiments, and analyzed the data; D.S., C.A., I.C., P.A., A.S., C.Z., G.D., A.S. and A.G. performed experiments; A.P and F.M. edited manuscript; L. M. and G. F. performed IHC analysis; I.P., M.B., F.P., A.B. and M.G. provided patient samples. The authors have no competing financial interest to declare.
Legends to the Figures

Figure 1. NAMPT expression and its prognostic relevance in hematologic malignancies. A) NAMPT mRNA expression in solid tumors and haematological cancers across lines included in the Cancer Cell Line Encyclopedia (Oncomine at http://www.oncomine.org: Barretina Cell Line data set). In the box plots, the middle bars represent the median value, the boxes encompass the values from the lower to the upper quartile (25th to 75th percentile), and the whiskers encompass the 25th percentile minus 1.5-times the interquartile range and the 75th percentile plus 1.5-times the interquartile range. B) Whole cell lysates from AML (OCI-AML3, OCI-AML2, HEL, KG1a, MV4-11, SET1 and HL60), B-CLL (Mec.1 and Mec.2) and Lymphoma (DAUDI, U937, SU-DHL1 and Raji) cell lines were analyzed for NAMPT expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. C) IHC analysis on three representative BM specimens derived from healthy donors (HD), AML and B-CLL affected patients, show NAMPT expression (positive cells are brown) and H&E. Magnification: 60x. D) Kaplan-Meier plots showing prognostic relevance of NAMPT expression on overall survival of acute myeloid leukemia (n=79; GSE12417) (59), chronic lymphocytic leukemia (n=148; GSE22762) (60) and diffuse large B-cell lymphoma (n=396; GSE4475) (61) patients. Medians±S.D. are presented. The group of patients with higher SIRT6 expression (red line) had shorter overall survival than patients with lower SIRT6 expression (blue line) (log-rank test).

Figure 2. NAMPT inhibition triggers synergistic anti-leukemia effect with CsA. A) 2.5 x 10^4 primary B-CLL cells were incubated in 96-well plates in the presence or absence of 3 nM APO866 and different anti-leukemia drugs (0.5 μM Melphalan, 0.3 μM Doxorubicin, 5 μM Fludarabine, 0.01 μM Velcade and 1 μM CsA) or their combination. Cell death was assessed 96 h later by PI staining and flow cytometry. Results are means of triplicates ± SD. ns: not
significant; **p=.01;***p=.002;****p<.0001. B) 2.5 x 10^4 OCI/AML3 (left) or Mec.1 (right) cells were plated in 96-well plates and treated with or without increasing doses of APO866 (3 nM) for 48 h, and then vehicle or Pgp modulators (10 μM Verapamil, 1 μM CsA and 10 μM PGP-4008) were added for further 48h. Viability was assessed using PI staining and FACS analysis. Data presented are means of triplicate ± SD (n=3; **p=.02,*** p<.001, **** p<.0001). C) Primary leukemic cells from 25 patients (19 B-CLL and 6 AML) were plated in 96-well plates and incubated with 3 nM APO866 and various Pgp modulators (verapamil, Nilotinib or PGP-4008). Cell death was assessed 96 h later by PI staining and flow cytometry. Results are means of triplicates ± SD. CI values < 1, =1 and >1 mean indicate synergistic, additive or infra-additive effect, respectively. D) 2x10^7 primary B-CLL cells/well were plated in 6-well plates and treated for 24 h with 3 nM APO866 in presence or absence of CsA 1 μM. Thereafter, cells were harvested and lysed in water. The extracted material was then analyzed by mass spectrometry. APO866 concentration in each extract was normalized to protein concentration.

**Figures 3. Modulation of P-glycoprotein (Pgp) activity affects APO866 sensitivity in leukemia cells.** A) OCI-AML3 (left) and MEC.1 (right) were transfected using different concentrations of ON-TARGET PLUS SMART pool targeting MDR-1 or non targeting negative control siRNA (nt siRNA). Twenty-four hours after transfection, leukemia cells were treated with 3 nM APO866, in the presence or absence of 1 μM CsA. Cell lysates were obtained 48 h after transfection and subjected to Western Blot analysis to confirm decrease in MDR1 protein expression. GAPDH was used as loading control. Cell death was assessed 72 h later by PI staining and flow cytometry. B) 2.5 x 10^4 LAMA-84r (left) or Doxo40 (right) cells, in parallel with their parental cell lines (LAMA84 and RPMI8226, respectively), were plated in 96-well
plates and treated with or without increasing doses of APO866 (3 nM) for 48 h. Then, vehicle or CsA (1 μM) were added for further 48 h. Viability was assessed using PI staining and FACS analysis. A-B) Data presented are means of triplicate ± SD (n=3; *p=0.05, **0.003<p<0.002, ***.001<p<.0003, **** p<.0001).

Figure 4. Pgp modulators treatment combined with APO866 enhances NAD\(^+\) and ATP depletion produced by APO866. A) 2 x 10\(^6\) primary leukemic (AML and B-CLL) cells/well were plated in 6-well plates and incubated with 3 nM APO866, 1 μM CsA and 6 μM Verapamil alone or in combination. After 24 h (left) or 48 h (right) later, cells were harvested and NAD\(^+\) or ATP levels were determined in cell extracts. B) 1 x 10\(^5\) Mec.1 cells/well were plated in 6-well plates and incubated with or without 3 nM APO866 and Pgp modulators (10 μM Verapamil, 1 μM CsA). After 24 h (left) or 48 h (right) later, cells were harvested and intracellular NAD (H)\(^+\) and ATP levels were evaluated and compared to those in control cells. A-B) means ± SDof at least three independent experiments are shown (*0.05<p<0.03; **0.004<p<0.002; ***0.001<p<0.0009; ****p<0.0001).

Figure 5. The anti-leukemic effect of APO866 plus CsA occurs via apoptosis. A) 3 x 10\(^6\) primary B-CLL cells/well were plated in 6-well plates and incubated for 72 h with 3 nM APO866 and different Pgp modulators alone or their combination. Δψ\(_m\) was monitored at the indicated time points by TMRE staining and flow cytometry. B-C) 1 x 10\(^6\) primary B-CLL cells/well were plated in 6-well plates and treated for 48 h with or without 3 nM APO866 and Pgp modulators (1 μM CsA, 10 μM Verapamil, 10 nM Nilotinib or 10 nM PGP-4008). Thereafter, cells were harvested, washed, and used for Annexin-V/PI staining and flow cytometry (B), or for flow cytometric quantification of hypodiploid cell nuclei (C). The results are means ± SD of three separate experiments.
Figure 6. CsA plus APO866 anti-leukemic effect is mediated by ER stress-dependent UPR signaling. A) $1 \times 10^6$ OCI/AML3, Mec.1 or primary B-CLL cells/well were plated in 96-well plates and pre-incubated for 2 h with or without 4-phenyl butyric acid (4-BPA). Thereafter, leukemia cells were treated with APO866 (3 nM) for 48 h; CsA (1 µM) was then added for additional 48 h followed by cell death analysis using PI staining and FACS analysis. Data are mean ± SD of triplicate samples (*0.05<p<0.01; **.008<p<0.002; *** 0.0006<p<0.0002; ****p<0.0001). B) OCI/AML3 or Mec.1 cells were pretreated with or without a low dose of APO866 (1 nM) for 24 h, and then CsA (1 µM) was added for additional 24 h. Cells were then harvested, and cell lysates were subjected to immunoblot analysis using anti-BIP, anti-IRE1α, anti-ubiquitin, anti-CHOP, anti-tubulin or anti-nucleolin antibodies. C) B-CLL primary cells were treated with APO866 (10-100 nM), CsA (0.3-1 µM) or combined therapy for 48 h. Cell lysates were subjected to Western Blot analysis, using anti-BIP and anti-tubulin Abs. Blots shown are representative of 3 independent experiments. Relative expression was calculated by taking the ratio of the densitometry signal for BIP to tubulin in each sample using the Image J software (1.37v; National Institutes of Health, http://rsb.info.nih.gov/ij/) (bottom).
Table 1. Clinical and laboratory features of B-CLL patients

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ND: not determined; Nor, normal; +: trisomy; *: chemonaive patient.

Table 2. Clinical and laboratory features of AML patients

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PB: peripheral blood; BM: bone marrow
Table 3. Synergistic interactions between APO866 and efflux pump inhibitors in primary leukemia cell

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Primary B-CLL (#1-17) or AML (#18-25) cells were plated in 96 well plates and stimulated with 1μM CsA, 6 μM Verapamil and 3 nM APO866 alone or their combinations. Specific cell death was detected four days later by PI staining and flow cytometry. CIs are indicated in parenthesis. ND: not determined.
References


Acute Myeloid Leukemia
Chronic Lymphocytic Leukemia
Diffuse Large B-Cell Lymphoma
Figure 2

A

B

C

D

Primary leukemia cells

Intra-additive

Additive

Synergism

Combination Index

0.0
0.5
1.0
1.5
2.0

APO866
APO866+CsA
APO866+Verapamil
APO866+Nilotinib
APO866+PGP-4008

Author Manuscript Published OnlineFirst on May 11, 2015; DOI: 10.1158/1078-0432.CCR-14-3023

---

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Figure 3

A

Non-targeting
MDR-1

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B

LAMA84

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Figure 4

A

B-CLL patients

B

AML patients

B-CLL patients
Figure 5

A

- 24h $\Delta \psi_m$ 72h

CsA+APO866

CsA

APO866

control

N8+APO866

Nilotinib

AP0866

control

PGP+APO866

PGP-4008

APO866

control

24h $\Delta \psi_m$ 72h

B

- 14.5 4.7

CsA

14.3 6.7

Nilotinib

9.3 12

Verapamil

16.4 10.8

PGP-4004

19.6 10

PL

10.1 10.6

Annexin-V

C

- 20.1% 21.4%

CsA

37.3%

Nilotinib

25.6%

Verapamil

29.2%

PGP-4008

59.3%

77.5%

68%

66.3%

61.5%

DNA content (log)
Figure 6

A

OCI/AML3  

Specific cell death (%)  

BIP  
IRE1α  

APO866  
CsA  

+ 4-PBA  

Primary B-CLL cells

Specific cell death (%)  

B

MEC.1  

OC/AML3  

Primary B-CLL cells

BIP  
IRE1α  

APO866  
CsA  

+ 4-PBA  

Ubiquitin  
Tubulin  

Whole cell lysates  

Primary B-CLL cells

BIP  

Tubulin  

Nuclear cell lysates

B

Primary B-CLL cells

APO866  
CsA  

+ 4-PBA

BIP  

Tubulin  

Nuclear cell lysates

B
Clinical Cancer Research

APO866 INCREASES ANTI-TUMOR ACTIVITY OF CYCLOSPORIN-A BY INDUCING MITOCHONDRIAL AND ENDOPLASMIC RETICULUM STRESS IN LEUKEMIA CELLS

Antonia Cagnetta, Debora Soncini, Irene Caffa, et al.

*Clin Cancer Res* Published OnlineFirst May 11, 2015.

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