APO866 Increases Antitumor Activity of Cyclosporin-A by Inducing Mitochondrial and Endoplasmic Reticulum Stress in Leukemia Cells

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Abstract

Purpose: The nicotinamide phosphoribosyltransferase (NAMPT) inhibitor, APO866, has been previously shown to have antileukemic activity in preclinical models, but its cytotoxicity in primary leukemia cells is frequently limited. The success of current antileukemic 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 antileukemic 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 nicotinamide adenine dinucleotide (NAD+) and ATP levels, mitochondrial transmembrane potential (Δψm), 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 Δψm 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 acute myelogenous leukemia (AML) and B-cell chronic lymphocytic leukemia (B-CLL) samples. Further evaluations of the combination of these agents in clinical setting should be considered. Clin Cancer Res; 21(17); 3934–45. ©2015 AACR.

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; refs. 2–9). On the basis of its promising preclinical activity, APO866 was proposed as novel drug for different hematologic 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 TRAIL (12), DNA-damaging agents (daunorubicin, cisplatin, Ara-C, and melphalan; refs. 13, 14).
The rate-limiting enzyme in nicotinamide adenine dinucleotide (NAD⁺) biosynthesis from nicotinamide, NAMPT (nicotinamide phosphoribosyltransferase), 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.

**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), 2 μmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin ( Gibco; Life Technologies). Nilotinib was supplied by Novartis and was stored at 10 mmol/L 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). Tetramethylrhodamine ethyl ester (TMRE), 4-PBA, NAM, and NA were obtained from Sigma-Aldrich (Sigma-Aldrich Italia). 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-cell chronic lymphocytic leukemia (B-CLL) and AML patients are summarized in Tables 1 and 2, respectively. For B-CLL cell isolation, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Hypaque (Biotest). The B-CLL phenotype of the obtained cell preparations was confirmed by immunostaining with anti-CD19, anti-CD5, and anti-CD23 (Immunotech), 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) to the blood specimens at a ratio of 4:5, followed by a 1-hour incubation at room temperature. Thereafter, the leukocytes-enriched supernatants were transferred to a 50 mL conical centrifuge tube and centrifuged at 300 × g for 10 minutes. 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 300 × g for 10 minutes. 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°C in medium containing 20% FBS and 10% DMSO. CD34⁺ peripheral blood precursor cells (PBPC) were obtained from the excess PBPC concentrates (1–2 mL) 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) 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**

A total of 2 × 10⁵ cells per well (primary leukemia cells, PBMCs and PBPCs) or 5 × 10⁵ cells per 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.
Clinical and laboratory features of B-CLL patients

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

Mitochondrial transmembrane potential (ΔΨm) determination

ΔΨm was determined as previously described (4, 17). Briefly, cells were harvested, washed and incubated in the presence of 50 nmol/L TMRE in regular RPMI-based medium for 15 minutes 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) and 10 to 50 μg proteins were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected with the following antibodies: -NAMPT (Bethyl Laboratories, Inc.), anti-BIP, -IRE1α, -ubiquitin, -CHOP, -MDR1/ABCB1 (Cell Signaling Technology), -α-tubulin (mouse monoclonal; Sigma Aldrich) and anti-nucleolin (Santa Cruz Biotechnology). Standard enhanced chemiluminescence (ECL by Thermo Fisher Scientific) was used for protein bands detection.

Determination of NAD+ and ATP levels

Intracellular NAD(H) content was determined with an 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

A total of 20 × 10^6 primary B-CLL cells were plated in 6-well plates and treated for 24 hours with 3 nmol/L APO866 in presence or absence of CsA 0.3 μmol/L. 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 T3 column (150 × 1 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 minutes eluent B was maintained at 5%. Subsequently, between 5 and 35 minutes, 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 to 400. APO866 concentration values were normalized to a protein concentration.

Immunohistochemistry

Sections of bone marrow (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 Biotechnology; ref. 31). IHC was performed using the Ventana BenchMark XT automated immunostainer. Tissue sections were deparaffinized and rehydrated. After antigen
samples were evaluated by the unpaired Student t test. Differences in (GEO) datasets (accession numbers GSE12417 for AML; 7q22.3 locus (where NAMPT maps) in tumor cell lines was collected in Oncomine portals at http://www.oncomine.org in human cancer cell lines were obtained from datasets data (4, 7, 12, 17, 33), an analysis of the Cancer Cell Line Encyclopedia database (34) revealed higher NAMPT expression in hematologic malignancies (including lymphomas, leukemias, and multiple myeloma) compared with cancer cell lines of epithelial origin (Fig. 1A). An array-based comparative genomics analysis database (http://www. broadinstitute.org/ccle). Next, data were analyzed using the Integrative Genomics Viewer (IGV) analysis software. Expression levels of NAMPT in different hematologic 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 the unpaired Student 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 hematologic 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 database (34) revealed higher NAMPT expression levels in hematologic malignancies (including lymphomas, leukemias, and multiple myeloma) compared with cancer cell lines of epithelial origin (Fig. 1A). An array-based comparative genomics hybridization 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 Fig. S1A and S1B). These findings, supported by the strong NAMPT expression in leukemia cell lines (Fig. 1B) and by our previous study of NAMPT's role in multiple myeloma (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 AML and B-CLL patients exhibit significantly increased NAMPT levels than samples obtained from healthy donors (Fig. 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 Fig. 1D, high NAMPT expression in hematologic malignancies was significantly associated with poor overall survival (OS). 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 antitumor 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 Fig. 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) (38–40). 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 Fig. S2), 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 (41–43). Thus, we reasoned that Pgp inhibition may be the mechanism underlying CsA-mediated enhancement of APO866 antileukemic 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 MEC1–B-CLL; Fig. 2B and C) and in primary leukemia cells from a cohort of patient that included cases of AML (n = 6) and B-CLL (n = 19; Fig. 2C and Tables 1–3).

Consistent with the hypothesis that APO866 may be a Pgp substrate, using HPLC/MS, we were able to show that both CsA and PGP-4008 increase APO866 intracellular levels in primary B-CLL cells (Fig. 2D and Supplementary Fig. S3). Finally, additional evidence in support of the notion that APO866 antileukemic 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 2 days after transfection (Fig. 3A, upper insets). As shown in Fig. 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 pharmacologic Pgp inhibitors. Subsequently, Doxo40 (44) and LAMA84+ (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

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bitors or their combination induced cell death in these cells, PBMC and PBPC, too. However, neither APO866 nor Pgp inhibition can be with Pgp inhibitors, including CsA. 

with the hypothesis that APO866 is a substrate of Pgp and that APO866 activity can be with Pgp inhibitors, including CsA. 

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 (Fig. 4A and B and Supplementary Fig. S6). 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 ΔΨm 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 ΔΨm dissipation, an

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 Fig. S5A and S5B). 

CsA enhances NAD⁺ shortage, ΔΨm 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). Using 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 (Fig. 4A and B and Supplementary Fig. S6). 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 ΔΨm 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 ΔΨm dissipation, an

Figure 1.

NAMPT expression and its prognostic relevance in hematologic malignancies. A, NAMPT mRNA expression in solid tumors and hematologic cancers across lines included in the Cancer Cell Line Encyclopedia (Oncomine at http://www.oncomine.org: Barretina Cell Line dataset). In the box plots, the middle bars represent the median value, the boxes encompass the values from the lower to the upper quartile (25th–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 hematoxylin and eosin (H&E); magnification, ×60. D, Kaplan–Meier plots showing prognostic relevance of NAMPT expression on OS of AML (n = 79; GSE12417; ref. 56), chronic lymphocytic leukemia (n = 148; GSE22762; ref. 57), and diffuse large B-cell lymphoma (n = 296; GSE4475; ref. 58) patients. Medians ± SD are presented. The group of patients with higher SIRT6 expression (red line) had shorter OS than patients with lower SIRT6 expression (blue line; log-rank test).

[Image 102x412 to 540x723]
apoptotic cell phenotype (AnnexinV⁺/PI⁻ or AnnexinV⁺/PI⁺), as well as a strong increase in hypodyploid (apoptotic) cell nuclei in primary B-CLL cells (Fig. 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 Δψm loss and apoptotic phenotype. Because 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 not 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 endoplasmic reticulum (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, because ER stress is a main trigger for apoptotic responses, we assessed its potential relevance in the antileukemic effects of APO866, Pgp inhibitors and their combination (13, 47–51). ER stress inhibition with the chemical chaperone 4-phenyl butyric acid (4-BPA; ref. 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 (Fig. 6A). An analysis of ER stress-related markers was also performed. As predicted, in cells that were cotreated 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 (Fig. 6B and Supplementary Fig. S7). A marked increase in the

Figure 2. NAMPT inhibition triggers synergistic antileukemia effect with CsA. A, 2.5 × 10⁵ primary B-CLL cells were incubated in 96-well plates in the presence or absence of 3 nmol/L APO866 and different antileukemia drugs (0.5 μmol/L melphalan, 0.3 μmol/L doxorubicin, 5 μmol/L fludarabine, 0.01 μmol/L velcade, and 1 μmol/L CsA) or their combination. Cell death was assessed 96 hours later by PI staining and flow cytometry. Results are means of triplicates ± SD; ns, not significant; ***, P = 0.01; ****, P = 0.002; *****, P < 0.0001. B, 2.5 × 10⁵ OCI/AML3 (left) or Mec.1 (right) cells were plated in 96-well plates and treated with or without increasing doses of APO866 (3 nmol/L) for 48 hours, and then vehicle or Pgp modulators (10 μmol/L verapamil, 1 μmol/L CsA, and 10 μmol/L PGP-4008) were added for further 48 hours. Viability was assessed using PI staining and FACS analysis. Data, means of triplicate ± SD (n = 3; ***, P = 0.02, ****, P = 0.001 < P < 0.003; *****, P < 0.0001). C, primary leukemic cells from 25 patients (19 B-CLL and 6 AML) were plated in 96-well plates and incubated with 3 nmol/L APO866 and various Pgp modulators (verapamil, Nilotinib, or PGP-4008). Cell death was assessed 96 hours later by PI staining and flow cytometry. Results are means of triplicates ± SD. CI values < 1, = 1 and > 1 mean indicate synergistic, additive or intra-additive effect, respectively. D, 2 × 10⁵ primary B-CLL cells per well were plated in 6-well plates and treated for 24 hours with 3 nmol/L APO866 in presence or absence of CsA 1 μmol/L. 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.
molecular chaperone BIP was also detected in primary B-CLL cells treated with APO866 and CsA (Fig. 6C). Finally, a considerable accumulation of misfolded proteins, detected as a smear of high-molecular weight adducts (Fig. 6B) in response to combined CsA and APO866 was also documented, whereas a weaker smear, previously identified as made of polyubiquitinated 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 antileukemic activity of APO866 and of its combination with Pgp inhibitors.

Nicotinamide and nicotinic acid completely abolish activity of cotreatment 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, 55). On the basis of 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 antileukemic activity of APO866, both as a single agent and in combination with CsA (Supplementary Fig. S8A and S8B), confirming the role of NAD$^+$ depletion in the activity of these antileukemic 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 antileukemic 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 antileukemic activity, we discovered CsA as a Pgp modulator treatment combined with APO866 enhances NAD\(^+\) and ATP depletion produced by APO866. A, 2 \times 10^6 primary leukemic (AML and B-CLL) cells per well were plated in 6-well plates and incubated with 3 nmol/L APO866, 1 \mu mol/L CsA, and 6 \mu mol/L Verapamil alone or in combination. After 24 hours (left) or 48 hours (right) later, cells were harvested and NAD\(^+\) or ATP levels were determined in cell extracts. B, 1 \times 10^5 Mec.1 cells per well were plated in 6-well plates and incubated with or without 3 nmol/L APO866 and Pgp modulators (10 \mu mol/L Verapamil, 1 \mu mol/L CsA). After 24 hours (left) or 48 hours (right) later, cells were harvested and intracellular NAD\(^+\) and ATP levels were evaluated and compared with those in control cells. A and B, means \pm SD of 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.

**Table 3.** Synergistic interactions between APO866 and efflux pump inhibitors in primary leukemia cell

<table>
<thead>
<tr>
<th>Patient nr.</th>
<th>CSa (1 \mu mol/L)</th>
<th>Verapamil (6 \mu mol/L)</th>
<th>APO866 (10 nmol/L)</th>
<th>CSa (\times) APO866</th>
<th>Verapamil (\times) APO866</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>2.7</td>
<td>ND</td>
<td>5</td>
<td>67.56 (0.3)</td>
<td>ND</td>
</tr>
<tr>
<td>#2</td>
<td>1.8</td>
<td>ND</td>
<td>43.5</td>
<td>94.24 (0.4)</td>
<td>ND</td>
</tr>
<tr>
<td>#3</td>
<td>5.58</td>
<td>ND</td>
<td>1.13</td>
<td>51.32 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>#4</td>
<td>3.42</td>
<td>ND</td>
<td>1.41</td>
<td>44.44 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>#5</td>
<td>6.13</td>
<td>8.8</td>
<td>46.43</td>
<td>75.51 (0.7)</td>
<td>67.35 (0.8)</td>
</tr>
<tr>
<td>#6</td>
<td>17.55</td>
<td>2.19</td>
<td>43.11</td>
<td>86.77 (0.7)</td>
<td>73.73 (0.6)</td>
</tr>
<tr>
<td>#7</td>
<td>0.9</td>
<td>26</td>
<td>24</td>
<td>84 (0.3)</td>
<td>84 (0.6)</td>
</tr>
<tr>
<td>#8</td>
<td>9</td>
<td>ND</td>
<td>5.6</td>
<td>35.7 (0.4)</td>
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</tr>
<tr>
<td>#9</td>
<td>21.1</td>
<td>ND</td>
<td>4.7</td>
<td>89.8 (0.3)</td>
<td>ND</td>
</tr>
<tr>
<td>#10</td>
<td>14.6</td>
<td>ND</td>
<td>33.3</td>
<td>99.35 (0.5)</td>
<td>ND</td>
</tr>
<tr>
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<td>35.8</td>
<td>9.5</td>
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<td>83.78 (0.7)</td>
<td>76.5 (0.5)</td>
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<tr>
<td>#12</td>
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<td>ND</td>
</tr>
<tr>
<td>#13</td>
<td>27.6</td>
<td>32.7</td>
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<td>59.2 (0.9)</td>
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<tr>
<td>#14</td>
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<td>10.9</td>
<td>30.3</td>
<td>80 (0.8)</td>
<td>55.6 (0.7)</td>
</tr>
<tr>
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<td>75.9 (0.7)</td>
<td>69.23 (0.8)</td>
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<tr>
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<td>6</td>
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<td>95.4 (0.5)</td>
<td>95.1 (0.5)</td>
</tr>
<tr>
<td>#17</td>
<td>20.4</td>
<td>13.9</td>
<td>54.9</td>
<td>84.8 (0.8)</td>
<td>85.05 (0.8)</td>
</tr>
<tr>
<td>#18</td>
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<td>42.44 (0.2)</td>
<td>ND</td>
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<tr>
<td>#19</td>
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<td>13.9</td>
<td>54</td>
<td>84.81 (0.8)</td>
<td>85.05 (0.79)</td>
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<tr>
<td>#20</td>
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<td>ND</td>
<td>37.6</td>
<td>95.1 (0.5)</td>
<td>ND</td>
</tr>
<tr>
<td>#21</td>
<td>40.5</td>
<td>ND</td>
<td>24.7</td>
<td>73.5 (0.8)</td>
<td>ND</td>
</tr>
<tr>
<td>#22</td>
<td>15</td>
<td>ND</td>
<td>7.46</td>
<td>30.3 (0.8)</td>
<td>ND</td>
</tr>
<tr>
<td>#23</td>
<td>23.4</td>
<td>27.6</td>
<td>37.8</td>
<td>68.63 (0.8)</td>
<td>66.45 (0.9)</td>
</tr>
<tr>
<td>#24</td>
<td>21</td>
<td>ND</td>
<td>34</td>
<td>87.5 (0.6)</td>
<td>ND</td>
</tr>
<tr>
<td>#25</td>
<td>43.4</td>
<td>7.6</td>
<td>37.8</td>
<td>84.8 (0.5)</td>
<td>81.9 (0.5)</td>
</tr>
</tbody>
</table>

NOTE: Primary B-CLL (#1–17) or AML (#18–25) cells were plated in 96-well plates and stimulated with 1 \mu mol/L CsA, 6 \mu mol/L verapamil, and 3 nmol/L APO866 alone or their combinations. Specific cell death was detected four days later by PI staining and flow cytometry. CIs are indicated in parentheses. Abbreviation: ND, not determined.
A highly effective potentiator. Notably, the combination of CsA with APO866, whereas highly active in different types of leukemias, was found to spare healthy PBMCs and PBPC, suggesting that this novel regimen should have a favorable 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 Fig. S8C). 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/PI staining and by the occurrence of hypodiploid 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).

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

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: A. Cagnetta, M. Gobbi, A. Nencioni, M. Cea

Development of methodology: A. Cagnetta, C. Acharya, D. Soncini, P. Acharya, M. Cea

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Caffa, C. Acharya, D. Soncini, P. Acharya, I. Pierri, M. Bergamaschi, G. Fraternali, L. Mastracci, C. Zucal, A. Salis, A. Nencioni, M. Cea

**Figure 5.**

The antileukemic effect of APO866 plus CsA occurs via apoptosis. A, $3 \times 10^5$ primary B-CLL cells per well were plated in 6-well plates and incubated for 72 hours with 3 nmol/L APO866 and different Pgp modulators alone or their combination. ΔΨm was monitored at the indicated time points by TMRE staining and flow cytometry. B and C, $1 \times 10^5$ primary B-CLL cells per well were plated in 6-well plates and treated for 48 hours with or without 3 nmol/L APO866 and Pgp modulators (1 μmol/L CsA, 10 μmol/L Verapamil, 10 nmol/L Nilotinib, or 10 nmol/L 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.
Mechanism of Synergy between APO866 and Cyclosporin-A

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Cagnetta, C. Acharya, D. Soncini, P. Acharya, G. Fraternali, G. Damonte, S. Bruzzone, A. Nencioni, M. Cea Writing, review, and/or revision of the manuscript: A. Cagnetta, S. Ademia, A. Provenzani, G. Damonte, F. Montecucco, F. Patrone, A. Nencioni, M. Cea Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Garuti, M. Cea Study supervision: F. Patrone, A. Ballestrero, M. Gobbi, A. Nencioni, M. Cea Other (technical support in performing and evaluation of immunohisto-chemical reactions): L. Mastracci Other (accepted the final version of the article): F. Montecucco

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References

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APO866 Increases Antitumor Activity of Cyclosporin-A by Inducing Mitochondrial and Endoplasmic Reticulum Stress in Leukemia Cells

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