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


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 (∆Ψm), 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⁺ precursor cells and peripheral blood mononuclear cells. Combining Pgp inhibitors with APO866 led to increased intracellular APO866 levels, compounded NAD⁺ and ATP shortage, and induced ∆Ψm 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.

Cancer Res; 1–12. ©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).
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), 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.

Translational Relevance
The rate-limiting enzyme in nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) biosynthesis from nicotinamide, NAMPT (nicotinamide phosphoribosyltransferase), regulates growth and metastatic potential of tumor cells. Leukemic cells show a higher NAD\textsuperscript{+} turnover rate than normal cells, suggesting that NAD\textsuperscript{+} 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 \( \mu \)mol/L L-glutamine, 100 U/mL penicillin, and 100 \( \mu \)g/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 \( \times \) 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 \( \times \) 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\textsuperscript{+} 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\textsuperscript{+} cells were purified using the CD34 MicroBead Kit from Miltenyi Biotec (Bergisch Gladbach) according to the manufacturer’s instructions. Using this method, CD34\textsuperscript{+} 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 \( \times \) 10\(^5\) cells per well (primary leukemia cells, PBMCs and PBPCs) or 5 \( \times \) 10\(^4\) cells per well (OCI/AML3 and MEC.1) were plated in 96-well plates in a final volume of 200 \( \mu \)L in the presence or absence of the indicated stimuli. Dead cells were quantified by incubation with PI for 10 minutes. Cells were evaluated by flow cytometry using the FCS Express software version 5.0 for Macintosh (DeNovo Software, Los Angeles).
Table 1. Clinical and laboratory features of B-CLL patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age, y</th>
<th>Cytogenetics</th>
<th>RAI stage</th>
<th>IgVH</th>
<th>Zap70</th>
<th>CD38</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>M</td>
<td>71</td>
<td>Nor</td>
<td>RAI 0</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#2</td>
<td>F</td>
<td>67</td>
<td>13q-</td>
<td>RAI IV</td>
<td>Mutated</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>#3</td>
<td>F</td>
<td>68</td>
<td>Nor</td>
<td>RAI IV</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#4</td>
<td>M</td>
<td>74</td>
<td>17p-</td>
<td>RAI 0</td>
<td>Unmutated</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>#5</td>
<td>M</td>
<td>76</td>
<td>Nor</td>
<td>RAI II</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#6</td>
<td>M</td>
<td>65</td>
<td>ND</td>
<td>RAI IV</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#7</td>
<td>M</td>
<td>66</td>
<td>17p-</td>
<td>RAI II</td>
<td>Mutated</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>#8</td>
<td>M</td>
<td>58</td>
<td>Nor</td>
<td>RAI II</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#9</td>
<td>M</td>
<td>79</td>
<td>13q-17p-</td>
<td>RAI 0</td>
<td>Unmutated</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>#10</td>
<td>M</td>
<td>72</td>
<td>Nor</td>
<td>RAI IV</td>
<td>Unmutated</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>#11</td>
<td>F</td>
<td>67</td>
<td>ND</td>
<td>RAI 0</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#12</td>
<td>M</td>
<td>56</td>
<td>17p-</td>
<td>RAI 0</td>
<td>Mutated</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>#13</td>
<td>M</td>
<td>77</td>
<td>12+</td>
<td>RAI II</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#14</td>
<td>M</td>
<td>75</td>
<td>ND</td>
<td>RAI III</td>
<td>Unmutated</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>#15</td>
<td>M</td>
<td>73</td>
<td>13q-,11q</td>
<td>RAI IV</td>
<td>Unmutated</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>#16</td>
<td>M</td>
<td>67</td>
<td>Nor</td>
<td>RAI 0</td>
<td>Mutated</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>#17</td>
<td>M</td>
<td>65</td>
<td>ND</td>
<td>RAI II</td>
<td>Mutated</td>
<td>Poss</td>
<td>Pos</td>
</tr>
<tr>
<td>#18</td>
<td>M</td>
<td>77</td>
<td>12+</td>
<td>RAI IV</td>
<td>Mutated</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>#19</td>
<td>F</td>
<td>75</td>
<td>ND</td>
<td>RAI II</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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

Table 2. Clinical and laboratory features of AML patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age</th>
<th>Sample source</th>
<th>FAB Stage</th>
<th>% Blasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>#20</td>
<td>M</td>
<td>53</td>
<td>BM</td>
<td>M4</td>
<td>95</td>
</tr>
<tr>
<td>#21</td>
<td>F</td>
<td>50</td>
<td>PB</td>
<td>M1</td>
<td>78</td>
</tr>
<tr>
<td>#22</td>
<td>M</td>
<td>80</td>
<td>BM/MI</td>
<td>M0/M1</td>
<td>90</td>
</tr>
<tr>
<td>#23</td>
<td>M</td>
<td>78</td>
<td>PB</td>
<td>Secondary</td>
<td>80</td>
</tr>
<tr>
<td>#24</td>
<td>M</td>
<td>79</td>
<td>PB</td>
<td>Secondary</td>
<td>70</td>
</tr>
<tr>
<td>#25</td>
<td>F</td>
<td>79</td>
<td>PB</td>
<td>M4</td>
<td>80</td>
</tr>
</tbody>
</table>

Abbreviations: BM, bone marrow; PB, peripheral blood.

96 hours later by PI staining (2 μg/mL) and flow cytometry (FACS Calibur; Becton Dickinson). Specific death was calculated as follows: [(% experimental death − % spontaneous death) / (100 − % spontaneous death)] × 100. For Annexin-V/PI staining 3 × 10^6 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. Annexin-V-FITC and PI staining were 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 (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 min 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: anti-NAMPT (Bethyl Laboratories, Inc.), anti-BIP, -IRE1α, -ubiquitin, -CHOP, -MDR1/ABCB1 (Cell Signaling Technology), anti-γ-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) Quantitation 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 dC18 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 AOI 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
RNAi interference

RNAi was performed with an ON-TARGET PLUS SMART pool targeting ABCB1 (GE Dharmaco). A nontargeting scrambled negative control siRNA was used as negative control (GE Dharmaco). Briefly, OCI/AML3 and MEC-1 cells were transiently transfected with MDR-1 siRNA with the Amaxa technology (V-solution with X-001 or LI-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 the 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 datasets collected in Oncomine portals at http://www.oncomine.org (Barretina Cell Line dataset). 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). 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 (GEO) datasets (accession numbers GSE12417 for AML; GSE4475 for DLBCL and GSE22762 for CLL). Differences in expression levels of NAMPT in AML and MEC-1 cells transfected with MDR-1 siRNA with the Amaxa technology (V-solution with X-001 or LI-013 program, respectively).

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 genomic 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 retrosexively 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 MEC-1—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-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.
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 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 $\Delta \psi_m$ 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 $\Delta \psi_m$ dissipation, an (Fig. 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 Fig. S5A and S5B).

CsA enhances NAD\(^+\) shortage, $\Delta \psi_m$ 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 $\Delta \psi_m$ 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 $\Delta \psi_m$ 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 (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 Δcm 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...
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 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 antancer 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, consequent-

ly, 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 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. Golbi, 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, I. Mastracci, C. Zucal, A. Salis, A. Nencioni, M. Cea

Figure 5.

The antileukemic effect of APO866 plus CsA occurs via apoptosis. A, 3 × 10⁵ 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 × 10⁵ 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.
CsA plus APO866 antileukemic effect is mediated by ER stress–dependent UPR signaling. A, $1 \times 10^5$ OCI/AML3, Mec.1 or primary B-CLL cells per well were plated in 96-well plates and preincubated for 2 hours with or without 4-phenyl butyric acid (4-BPA). Thereafter, leukemia cells were treated with APO866 (3 nmol/L) for 48 hours; CsA (1 µmol/L) was then added for additional 48 hours followed by cell death analysis using PI staining and FACS analysis. Data, mean ± SD of triplicate samples (*, 0.05 < P < 0.01; **, 0.008 < 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 (3 nmol/L) for 24 hours, and then CsA (1 µmol/L) was added for additional 24 hours. 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 nmol/L), CsA (0.3–1 µmol/L), or combined therapy for 48 hours. Cell lysates were subjected to Western blot analysis, using anti-BIP and anti-tubulin Abs. Blots shown are representative of three independent experiments. Relative expression was calculated by taking the ratio of the densitometry signal for BIP to tubulin in each sample using the ImageJ software (1.37v; NIH, http://rsb.info.nih.gov/ij/). Bottom.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Cagnetta, C. Acharya, D. Sconcini, P. Acharya, G. Fraternali, G. Damonte, S. Bruzzone, A. Nencioni, M. Cea

Writing, review, and/or revision of the manuscript: A. Cagnetta, S. Adamia, 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. Garutti, M. Cea

Study supervision: F. Patrone, A. Ballestero, M. Gobbi, A. Nencioni, M. Cea

Other (technical support in performing and evaluation of immunohistochemical reactions): L. Mastracci

Other (accepted the final version of the article): F. Montecucco

Acknowledgments

The authors thank the NIMH Chemical Synthesis and Drug Supply Program for generously providing APO866 for this study.

References


2. Hasmann M, Schemainda I. FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel...


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

Antonia Cagnetta, Irene Caffa, Chirag Acharya, et al.

Clin Cancer Res  Published OnlineFirst May 11, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-3023

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/05/12/1078-0432.CCR-14-3023.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.