

Nicotinamide Blocks Proliferation and Induces Apoptosis of Chronic Lymphocytic Leukemia Cells through Activation of the p53/miR-34a/SIRT1 Tumor Suppressor Network

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Abstract

Because of its relatively indolent clinical course, chronic lymphocytic leukemia (CLL) offers a versatile model for testing novel therapeutic regimens and drug combinations. Nicotinamide is the main NAD⁺ precursor and a direct inhibitor of four classes of enzymes, including the sirtuins. SIRT1, the main member of the sirtuin family, inactivates p53 by deacetylating a critical lysine residue. In this study, we showed that CLL cells express high levels of functional SIRT1, which is inhibited by exogenous nicotinamide. This agent blocks proliferation and promotes apoptosis selectively in leukemic cells that express wild-type (wt) p53. Nicotinamide modulates the p53-dependent genes *p21*, *NOXA*, *BAX*, and *Mcl-1*, indicating an activation of the p53 pathway and of caspase-3. DNA-damaging chemotherapeutics, such as etoposide, activate a functional loop linking SIRT1 and p53 through the induction of miR-34a. When leukemic cells are simultaneously exposed to nicotinamide and etoposide, we observe a significant increase in miR-34a levels with a concomitant inhibition of SIRT1. Furthermore, p53 acetylation levels are higher than with either agent used alone. Overall, treatment with both nicotinamide and etoposide shows strongly synergistic effects in the induction of apoptosis. We therefore concluded that nicotinamide has the dual property of inhibiting SIRT1 through a noncompetitive enzymatic block (p53 independent) and at the same time through miR-34a induction (p53 dependent). These observations suggested the therapeutic potential of nicotinamide, a novel, safe, and inexpensive drug, to be used in addition to chemotherapy for CLL patients with wt p53. *Cancer Res*; 71(13); 4473–83. ©2011 AACR.

Introduction

NAD⁺, an essential cofactor in the oxidative phosphorylation chain (1), is a substrate for 4 classes of enzymes (2, 3), involved in genomic stability, apoptosis, cell signaling, stress tolerance, and metabolism (4–7). Among these enzymes, sirtuins catalyze the deacetylation of acetyl-lysine residues by cleaving NAD⁺ and generating *O*-acetyl ADP-ribose. When active, these enzymes consume NAD⁺ and release nicotinamide, in turn a direct inhibitor of enzyme activities and the

main precursor of NAD⁺ in most mammalian cells (8). Hence, substrates (NAD⁺), enzymes, and final products (nicotinamide) are linked in a dynamic balance, in which nicotinamide may be considered a master regulator (8, 9). SIRT1, the major mammalian member of the sirtuin family, deacetylates histones and nonhistone proteins, including p53, Ku70, and FOXO (10, 11). For these reasons, its functions are implicated in the regulation of aging, circadian rhythm, and endocrine signaling (12). SIRT1 is upregulated in several human tumor types, including breast and colon cancers (13), in which it may work as an oncogene by suppressing p53 functions. Indeed, p53 deacetylation mediated by SIRT1 leads to functional inactivation (14). The consequence is that an increase in SIRT1 expression and function is followed by a decrease in the active form of p53, leading to genome instability and resistance to apoptosis, among the other effects (15, 16). For these reasons, there is intense investigation in designing molecular tools that inhibit sirtuins. Besides nicotinamide, these include NAD⁺ analogs, direct kinase inhibitors, suramin, and compounds identified by using computational approaches (17, 18).

Because of a longstanding experience in the use of nicotinamide for the treatment of pellagra and the lack of reported side effects (19), this agent is in the pipeline of SIRT1 inhibitors to be tested in clinical settings. This work was designed to address the *in vitro* effects of nicotinamide on chronic

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lymphocytic leukemia (CLL) cells, selected as disease model. CLL is characterized by the progressive expansion of a population of mature monoclonal B lymphocytes expressing CD5 (20). These cells are intrinsically resistant to apoptosis, limiting the therapeutic efficacy of many drugs. Furthermore, CLL has been a playground to test regimens that combine chemotherapy with other drugs, mostly immunomodulatory agents, reaching previously unattained response rates (21). From the biological standpoint, the signaling pathways operative in CLL lymphocytes are well characterized, offering an accessible model for studying the effects of old and new drugs (22).

The results of this study confirm that CLL cells are characterized by increased expression and function of SIRT1, both directly inhibited by exposure to nicotinamide. Treatment of CLL cells with this agent leads to a block of proliferation and induction of apoptosis, which is dependent on the activation of the p53 pathway. These effects substantiate the existence of a tumor suppressor network, linking p53 and SIRT1 through miR-34a. It seems that nicotinamide may potentiate the effects of chemotherapeutics, which operate through a p53-mediated apoptosis, thus becoming a potential adjunct in the treatment of selected CLL patients.

Materials and Methods

Patients and cells

Following informed consent, peripheral blood samples were obtained from 65 patients fulfilling diagnostic and immunophenotypic criteria for CLL (Supplementary Table S1). Blood samples were from age- and sex-matched donors, whereas tonsils from children undergoing surgery at the Regina Margherita Hospital (Turin, Italy). Peripheral blood mononuclear cells (PBMC), obtained by Ficoll-Hypaque (GE Healthcare) centrifugation, were cultured in RPMI-1640 + 10% fetal calf serum (Sigma). B lymphocytes were purified by negative selection by using anti-CD3, -CD14, and -CD16 antibodies (produced and purified in-house) and Dynal magnetic beads (Invitrogen; ref. 23), with a purity of more than 95%.

Antibodies and reagents

Antibodies used were anti-SIRT1 (Upstate-Millipore Biotechnology), anti-p53, anti-acetylated-Lys382-p53, and anti-caspase-3 (both from Cell Signaling Technologies), anti-Mcl1, anti-BAX, anti-p21, and anti-actin horseradish peroxidase (HRP)-conjugated, (all from Santa Cruz Biotechnology) and anti-NOXA (Imgenex). Secondary reagents were goat anti-mouse IgG-HRP conjugated (G α MlgG-HRP; Perkin Elmer) and goat anti-rabbit HRP-conjugated (G α R-HRP; Santa Cruz Biotechnology).

Nicotinamide, recombinant human interleukin (IL)-2 (rh-IL-2, 100 IU/mL) and etoposide (50 μ mol/L) were from Sigma. CpG oligonucleotide (1 μ g/mL, InvivoGen) and carboxyfluorescein diacetate N-succinimidyl ester (CFSE, 10 μ mol/L; Invitrogen) were also used. p53 short-hairpin RNA (shRNA) lentiviral particles (Santa Cruz Biotechnology) were used to inhibit p53 expression.

Proliferation assays

Cell were labeled with CFSE (24) and incubated with CpG/IL-2. Nicotinamide (1.2–10 mmol/L dose range) was added at the beginning of culture. Proliferation was measured after 5 days by staining with anti-CD19PE (Aczon). Cells were analyzed by flow cytometry, by using a fluorescence-activated cell sorting CantoII equipment (BD Biosciences) and the Diva and WinMDI softwares. Cell proliferation was also measured by [³H]-thymidine (Amersham Bioscience Buckinghamshire) incorporation at day 5. The percentage of proliferating cells was calculated as [(number of proliferated cells/total number of cells) \times 100].

Apoptosis assays

Apoptosis was measured by using the Annexin-V fluorescein isothiocyanate (FITC) Apoptosis Kit (Invitrogen). Data are presented as density plots of Annexin-V FITC (AV, x-axis) and propidium iodide (PI, y-axis) stainings. The percentage of apoptotic cells was calculated by adding AV⁺/AV⁺PI⁺/PI⁺ cells.

Caspase-3 activity was measured by using the human active caspase-3 immunoassay (R&D Systems Europe). Optical density (OD) was detected by using a microplate reader (Bio-Tek Instruments) set to 450 nm (wavelength correction set to 540 nm). Enzymatic activities were expressed as arbitrary OD units.

Western blot analysis

Cells were lysed (25), resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Upstate-Millipore). Blots were incubated with the indicated antibodies and developed by using enhanced chemiluminescence (Perkin Elmer). The human embryonic kidney 293T cell line was used as positive control for SIRT1 expression. Densitometric analyses were done on scanned films by using the public domain ImageJ software.

Nuclear extracts

Nuclear extraction was done as previously described (26).

SIRT1 enzymatic activity assay

SIRT1 activity was determined by using the SIRT1 Fluorimetric Kit (Enzo Life Sciences UK) and expressed as arbitrary fluorescent units (AFU).

RNA extraction and quantitative real-time PCR

RNA was extracted with TRI Reagent (Sigma) and converted to cDNA by using the reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was conducted by using the 7900 HT Fast Real-Time PCR System (SDS2.3 software), using commercially available primers (all from Applied Biosystems). Reactions were done in triplicate from the same cDNA reaction (technical replicates). Comparative CT methods was used to calculate the relative expression of the gene under analysis.

Statistics

Data are expressed as the means \pm SD. Student's *t* test was used to evaluate statistical significance.

Results

SIRT1 levels are significantly higher in CLL than in normal B lymphocytes

SIRT1 is a NAD⁺-dependent nuclear deacetylase, often overexpressed in solid tumors (27–29). qRT-PCR data showed that CLL cells ($n = 15$) express significantly higher levels of SIRT1 when compared with a pool of normal B cells purified from the PB of adult age- and sex-matched donors ($n = 6$) and from tonsil B cell preparations ($n = 4$), obtained from children ($P < 0.0001$; Fig. 1A). The levels of SIRT1 mRNA in normal B cells were constantly lower than in CLL cells, independently of the age of the donor or the activation status of the cell. Analyses at the protein level confirm that CLL cells express more SIRT1 protein than normal B lymphocytes ($P = 0.002$; Fig. 1A), providing evidence that SIRT1 overexpression is a hallmark of tumor transformation also in the CLL context. In line with the increased expression, nuclear extracts from CLL cells ($n = 15$) contained significantly more SIRT1 activity when compared with normal B cells ($n = 5$, $P < 0.0001$; Fig. 1B).

SIRT1 functions are inhibited by nicotinamide in CLL cells

We then asked whether nicotinamide, the main precursor of NAD⁺ and a noncompetitive inhibitor of SIRT1 (30, 31), is active in leukemic cells. The *in vitro* inhibitory effects of nicotinamide on recombinant human SIRT1 were confirmed by showing that with 5 and 10 mmol/L doses SIRT1 deacetylation activity was completely inhibited (Fig. 1C). Culture of CLL lymphocytes in the presence of the same doses of nicotinamide was followed by a marked inhibition of the deacetylation activity of endogenous SIRT1, as present in nuclear extracts ($P = 0.005$; Fig. 1C). The effect was evident after 20 minutes exposure to nicotinamide (Fig. 1C) and was maintained when cells were cultured for 60 minutes (not shown). SIRT1 inhibition in CLL cells was independent of negative prognostic markers, such as CD38 expression, the mutational status of the IgVH genes, and deletions/mutations of p53 (del/mut p53; ref. Fig. 1D). This inhibition was not apparent in similar experiments on normal B lymphocytes ($P = 0.94$; Fig. 1C).

Nicotinamide treatment blocks proliferation of CLL cells in a p53-dependent manner

We then investigated the effects of nicotinamide on CLL cells by analyzing changes in proliferation and apoptosis, 2 biological processes in which SIRT1 is involved, through the modulation of the p53 pathway.

CFSE-labeled purified CLL cells from 20 patients were activated by using an agonist specific for toll-like receptor 9 (TLR9) and IL-2 (32; ref. Fig. 2A). Proliferation was significantly inhibited in the presence of nicotinamide ($P < 0.0001$; Fig. 2A and B), in a dose-dependent way (Fig. 2B and Supplementary Fig. S1). Proliferation of normal B lymphocytes was only partially affected by nicotinamide ($P = 0.050$; Fig. 2A and B), implying that normal B cells are less sensitive than CLL cells, in line with their low constitutive levels of SIRT1.

These inhibitory effects were apparently independent of negative prognostic markers, including surface CD38 expression ($P < 0.0001$ for CD38⁺ and $P = 0.001$ for CD38⁻ patients; Fig. 2C) and IgVH mutational status ($P = 0.007$ for IgVH UM and $P = 0.001$ for IgVH MUT patients; Fig. 2C). A relevant exception was represented by patients with p53 inactivation, resistant to the effects of nicotinamide ($P = 0.0007$ for wild-type (wt) p53 and $P = 0.42$ for del/mut p53 patients; Fig. 2C; ref. 33).

Nicotinamide treatment induces apoptosis of CLL cells in a p53-dependent manner

Attention was then focused on apoptosis, constitutively high in CLL cells cultured *in vitro*. CLL cells ($n = 20$) and control nonneoplastic B lymphocytes ($n = 6$) were incubated with 3 different concentrations of nicotinamide (5, 10, and 50 mmol/L) for 72 hours and then labeled with Annexin-V-FITC (AV) and PI (Fig. 3A). Even in the presence of the lowest concentration tested (5 mmol/L; Fig. 3A and B), CLL cell viability was significantly reduced at day 3 ($P = 0.002$; Fig. 3B). The effect was dose and time dependent, starting after only 6 hours exposure and peaking at day 3 (Fig. 3C). In contrast, normal B cells entered apoptosis only in the presence of 50 mmol/L nicotinamide (Fig. 3B). As with the proliferation experiments, CLL cells behaved in a homogeneous manner, when the cohort was divided on the basis of CD38 expression ($P = 0.05$ for CD38⁺ and $P = 0.04$ for CD38⁻ patients; Fig. 3D) or IgVH mutational status ($P = 0.05$ for IgVH UM and $P = 0.03$ for IgVH MUT patients; Fig. 3D). On the contrary, patients with p53 inactivation were resistant to apoptosis when compared with the rest of the cohort ($P = 0.008$ for wt p53 and $P = 0.11$ for del/mut p53 patients; Fig. 3D).

Nicotinamide treatment activates the p53 pathway

In line with the hypothesis that the activities of SIRT1 rely on the presence of a functional p53 protein, treatment of wt p53 CLL cells with nicotinamide induced p53 protein expression in a time- and dose-dependent manner ($P = 0.02$ with 5 mmol/L and $P = 0.004$ with 10 mmol/L; Fig. 4A and B and Supplementary Fig. S2). p53 controls the cell cycle by modulating p21, in turn inducing G₁-phase arrest by cyclin-dependent kinase-2 inhibition (34). p21 activation was confirmed in wt p53 CLL samples treated with nicotinamide ($P = 0.04$ with 5 mmol/L and $P = 0.001$ with 10 mmol/L; Fig. 4A and B), in line with previous results showing that increased p53 acetylation at lysines 373/382 (K382 is deacetylated by SIRT1) induces p21 expression (16, 35).

Moreover, nicotinamide treatment increased expression of NOXA ($P = 0.001$ at both 5 and 10 mmol/L; Fig. 4A and B) and BAX ($P = 0.001$ with 5 mmol/L and $P = 0.007$ with 10 mmol/L; Fig. 4A and B) with a concomitant decrease of the prosurvival Mcl-1 protein ($P = 0.02$ with 5 mmol/L and $P = 0.0006$ with 10 mmol/L; Fig. 4A and B). Protein modulation was measurable after 6 hours, and was highly reproducible in the 5 patients studied. The end effect was the dose-dependent activation of caspase-3, with the onset of apoptosis ($P = 0.02$ with 5 mmol/L and $P = 0.003$ with 10 mmol/L, Supplementary Fig. S3A and B). Results showed a reduction in total caspase-3 protein levels

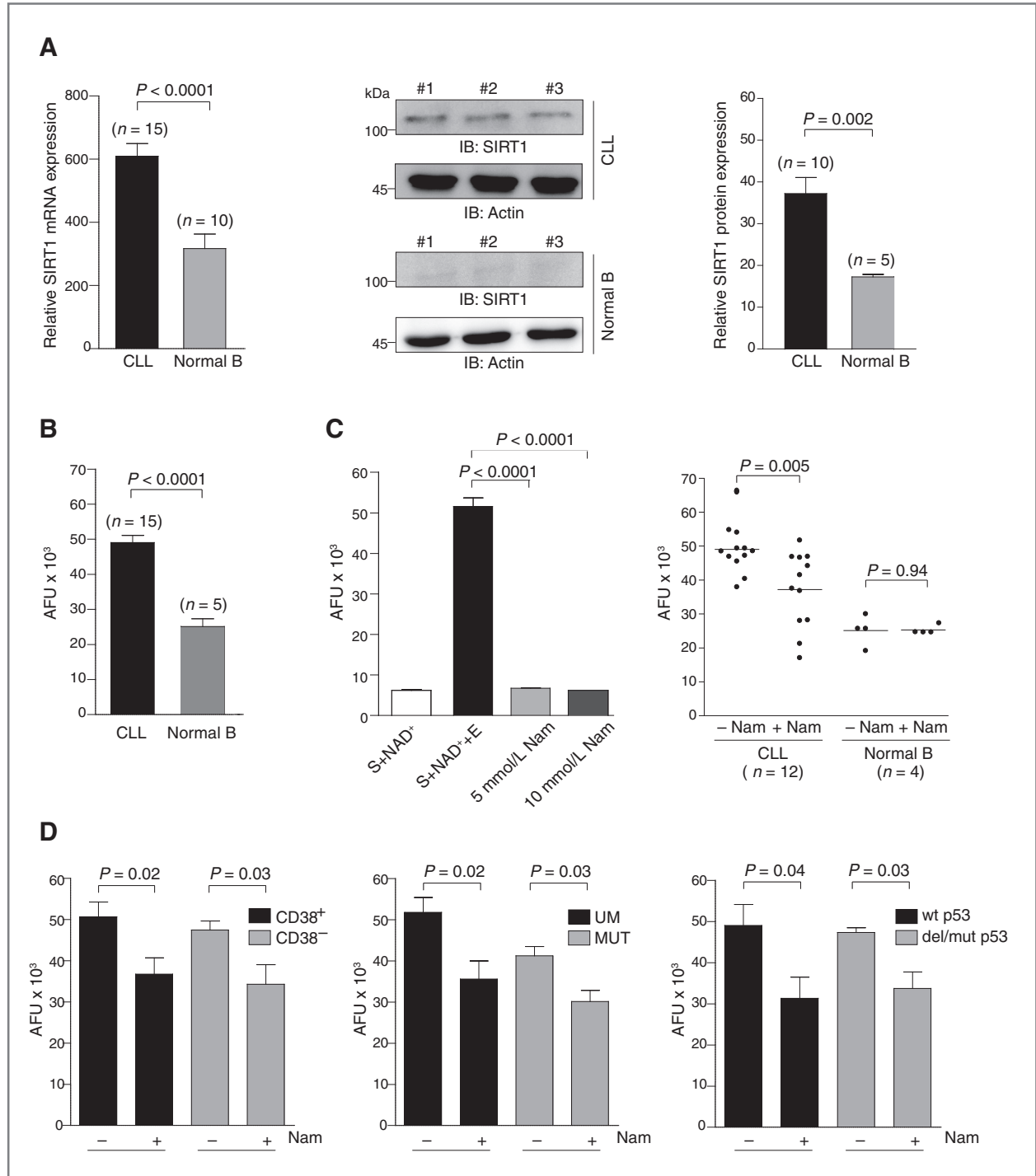


Figure 1. Functional SIRT1 is overexpressed in CLL cells and is inhibited by exogenous nicotinamide. **A**, left, expression levels of SIRT1 mRNA in B lymphocytes from CLL patients ($n = 15$) or nonleukemic donors ($n = 10$) were measured by qRT-PCR and normalized to GADPH. Mean values \pm SD are shown. Middle, Western blot analysis of basal SIRT1 protein expression in CLL ($n = 10$) or normal B ($n = 5$) cells. Three representative experiments are shown. Right, cumulative data showing relative SIRT1 expression. Mean values \pm SD are shown. **B**, nuclear extracts of B lymphocytes from CLL patients ($n = 15$) and nonleukemic donors ($n = 5$) were incubated with recombinant SIRT1, NAD⁺, and the substrate Fluor de Lys. SIRT1 activity was expressed as AFU. The recombinant activity of SIRT1 was subtracted from the total activity to provide the endogenous activity of SIRT1 in nuclear extracts. Mean values \pm SD are shown. **C**, left, the effects of nicotinamide on the activity of recombinant human SIRT1 were determined after incubation of the enzyme with nicotinamide (5 and 10 mmol/L). Mean values \pm SD are shown. Right, B lymphocytes from CLL patients ($n = 12$) and nonleukemic donors ($n = 4$) were treated with nicotinamide (5 mmol/L, 20 min, 37°C), before nuclear extraction and fluorimetric assay. **D**, effects of exogenous nicotinamide administration on SIRT1 enzymatic activity in CLL patients divided on the basis of CD38 expression, mutational status of the IgVH genes, or p53 status. Mean values \pm SD are shown. n indicates the number of samples analyzed. Nam, nicotinamide.

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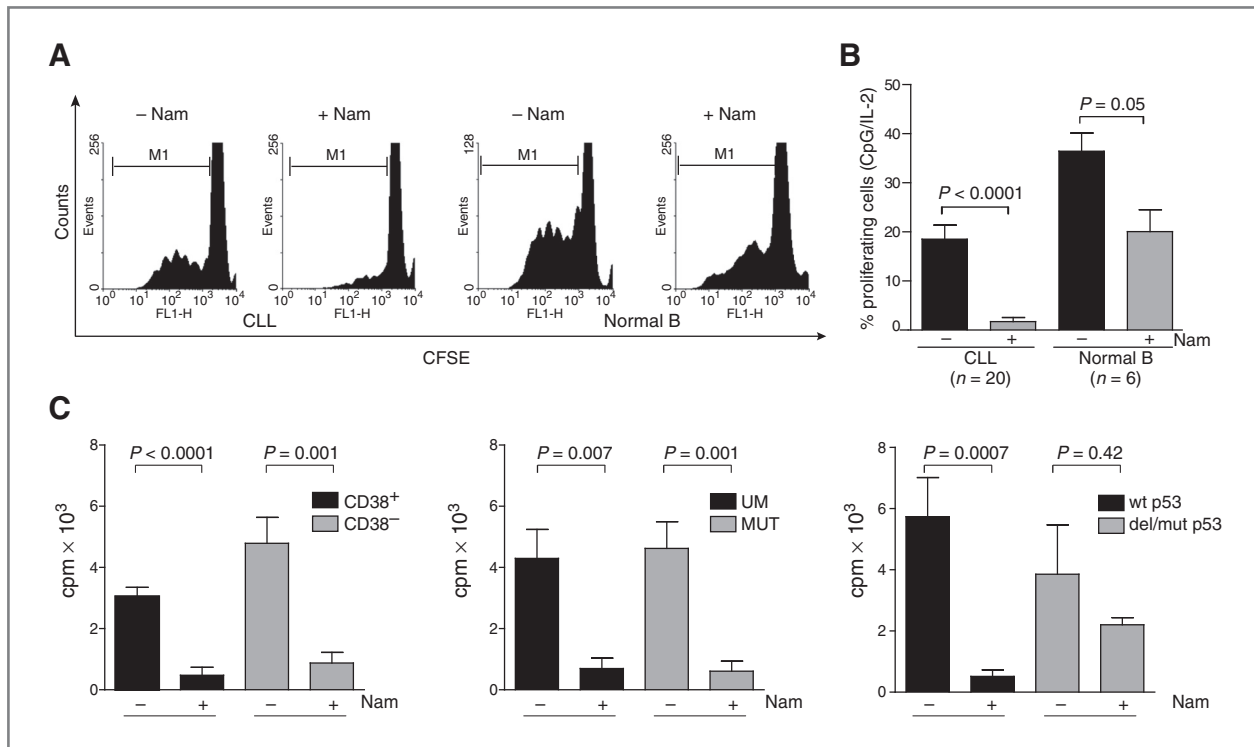


Figure 2. Nicotinamide inhibits proliferation of CLL cells with a wt p53. A, CFSE-labeled PBMC from CLL patients and nonleukemic donors were cultured for 5 days with CpG/IL-2 ± nicotinamide (10 mmol/L). Samples were acquired by gating on CD19⁺ cells. M1 labels proliferating cells. B, cumulative data of proliferation experiments after CpG/IL-2 activation by using CLL cells ($n = 20$) and normal B cells ($n = 6$). Mean values ± SD are shown. C, effects of exogenous nicotinamide administration on the proliferative response to CpG/IL-2 in CLL patients divided on the basis of CD38 expression, mutational status of the IgVH genes, or p53 status. Proliferation was measured by [³H]-thymidine incorporation. Mean values ± SD are shown. n indicates the number of samples analyzed. Nam, nicotinamide.

and a concomitant increase in caspase-3 activity, measured in 10 CLL patients after 24 hours exposure to 10 mmol/L nicotinamide ($P = 0.03$; Supplementary Fig. S3C). Altogether, these changes suggested that nicotinamide activates a p53-dependent pathway, leading to mitochondrial apoptosis via upregulation of NOXA and BAX and downmodulation of the Mcl-1 protein (36), directly induced by NOXA (37). They also strongly suggested that nicotinamide is able to interfere with the proliferative response and apoptotic program of CLL cells, by activating the p53 pathway.

SIRT1 is part of a p53/miR-34a tumor suppressor network

The next issue was whether nicotinamide might synergize with DNA-damaging chemotherapeutics, known to activate a p53-regulated loop which involves miR-34a (38, 39), in turn targeting SIRT1 (40).

To this purpose, wt p53 CLL cells ($n = 10$) were treated with 50 μmol/L etoposide (a DNA-damaging agent that activates the p53 pathway in a time- and dose-dependent manner; ref. 16) alone or in combination with nicotinamide (6 hours, 37°C) and checked for miR-34a levels. Etoposide treatment increased miR-34a levels and the same effect was observed by using nicotinamide, although the difference was not significant in either case. The combination of etoposide and nicotinamide resulted in a marked induction of miR-34a, as

compared with basal levels ($P = 0.009$) or to either agent used alone ($P = 0.03$, in both instances), in all cases studied (Fig. 5A). A cohort of CLL patients ($n = 5$) del/mut p53 was used as the control. These patients displayed constitutively lower miR-34a levels than p53 wt CLL patients (139.1 ± 73 vs. 43.8 ± 31 ; $P = 0.04$), not significantly modulated under any experimental condition (Fig. 5A). This finding was in line with recent studies showing that CLL patients featuring low levels of miR-34a lack p53 or express a dysfunctional protein (41, 42). Normal B lymphocytes ($n = 5$) were more resistant to etoposide-mediated apoptosis (not shown), and consistently failed to modulate the miR-34a pathway (Fig. 5A). These findings indicated that (i) the increase in miR-34a requires p53 activation, (ii) miR-34a is directly involved in the p53-dependent response to DNA damage in CLL cells, and (iii) nicotinamide synergizes with etoposide in the activation of the pathway.

Translational repression of the target SIRT1 mRNA following nicotinamide/etoposide exposure was verified by simultaneously measuring miR-34a and SIRT1 mRNA levels in the different treatment conditions. Nicotinamide/etoposide exposure selectively resulted in the inverse correlation between the miR and its target, that is, high levels of miR-34a were paralleled by low levels of SIRT1 mRNA ($r = -0.75$; $P = 0.005$; Fig. 5B), further supporting the notion of a functional cooperation between these 2 agents in the regulation of the network.

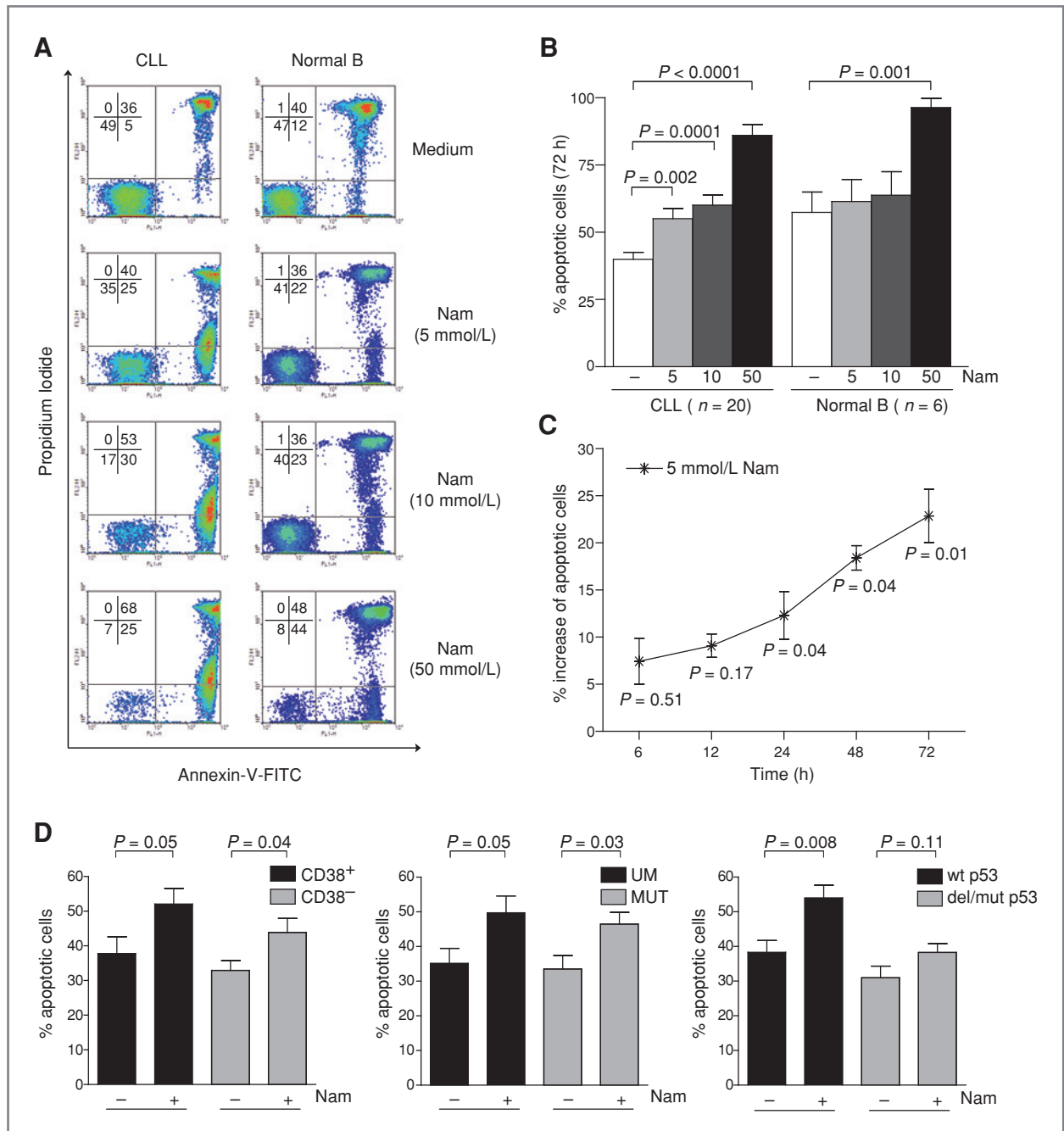


Figure 3. Nicotinamide induces apoptosis in CLL cells with a wt p53. A, B lymphocytes from CLL patients ($n = 20$) and nonleukemic donors ($n = 6$) were treated with nicotinamide (5, 10, and 50 mmol/L, 72 h). Cells were then labeled with AV and PI and analyzed by flow cytometry. B, cumulative data of apoptosis experiments. Mean values \pm SD are shown. C, time-dependent kinetic (6–72 hours) of CLL apoptosis induction following nicotinamide (5 mmol/L) exposure. Mean values \pm SD are shown. At least in 4 different samples/time point were analyzed. D, effects of exogenous nicotinamide administration on spontaneous apoptosis in CLL patients divided on the basis of CD38 expression, mutational status of the IgVH genes, or p53 status. Mean values \pm SD are shown. n indicates the number of samples analyzed. Nam, nicotinamide.

Nicotinamide potentiates the apoptotic effects of etoposide in a p53-dependent manner

Next, a direct effect on SIRT1 protein levels was shown by Western blot, which indicated that nicotinamide/etoposide led to a more pronounced inhibition of SIRT1 protein expres-

sion than either agent used alone ($P = 0.02$ compared with nicotinamide and $P = 0.003$ compared with etoposide alone; Fig. 6A). This effect was only evident in patients with a wt p53 pathway, as cells with a del/mut p53 did not modulate SIRT1 expression following nicotinamide exposure (Supplementary

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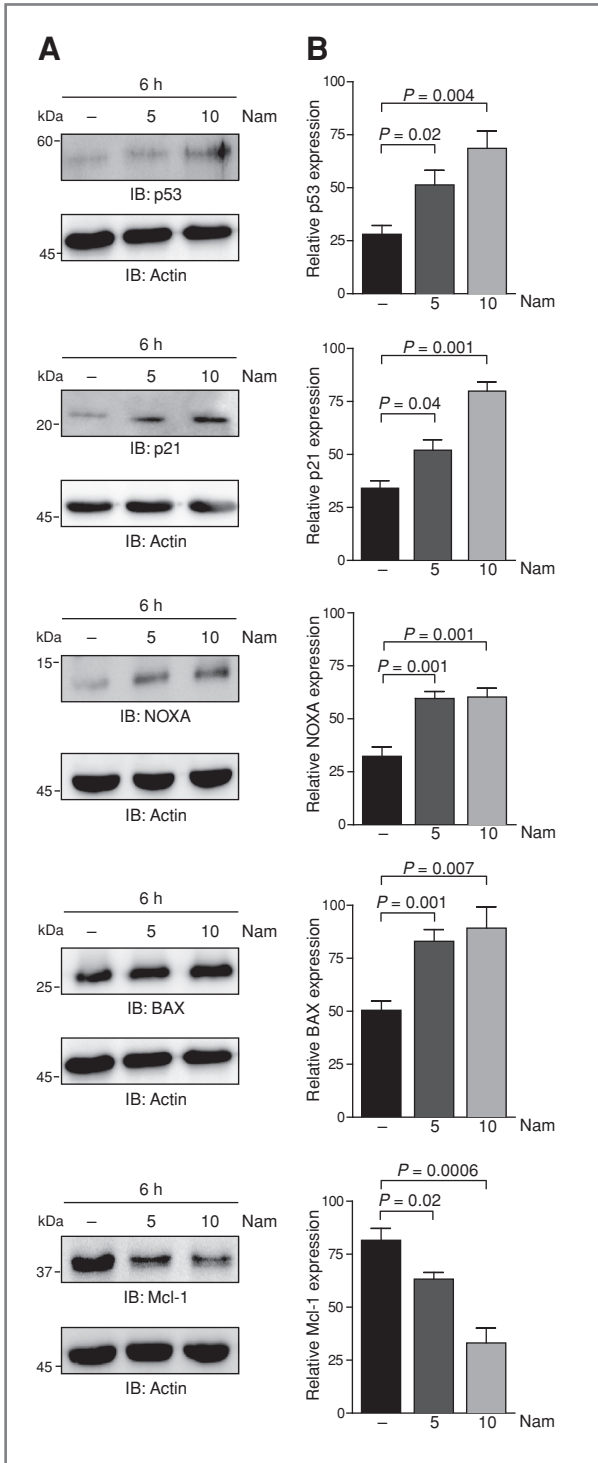


Figure 4. Nicotinamide induces expression of p53-dependent genes. CLL cells ($n = 5$) were treated with nicotinamide (5 mmol/L and 10 mmol/L, 6 hours), lysed and analyzed by Western blot for the expression of total p53 protein, p21, NOXA, BAX, and Mcl-1. β -Actin was used as the reference loading control. Panel (A) shows a representative experiment for each protein, while the graphs in (B) represent mean values \pm SD. Nam, nicotinamide.

Fig. S4). Consistent with the activation of p53, the combination of nicotinamide and etoposide induced the highest levels of acetylated p53 at lysine 382 ($P < 0.0001$ vs. basal, $P < 0.0001$ vs. nicotinamide alone, and $P = 0.007$ vs. etoposide; Fig. 6A), an event directly regulated by SIRT1 and influencing activation of the molecule (39). Patients with del/mut p53 and did not express detectable p53 protein and failed to modulate it in response to either treatment, independently or in combination (not shown). Normal B lymphocytes revealed no activation of this network with no detectable modification of p53 levels (not shown).

These data indicated that the p53/miR-34a/SIRT1 network is operative in CLL cells with a wt p53 and that nicotinamide enhances this pathway by negatively regulating SIRT1. This was substantiated also by a strong synergism between etoposide and nicotinamide in the induction of apoptosis, selectively in cells with a wt p53 protein ($P < 0.0001$; Fig. 6B and C). Patients with del/mut p53 were significantly less responsive to etoposide treatment, as expected, and a limited functional synergy was observed when adding nicotinamide ($P = 0.01$; Fig. 6B and C). The formal proof linking nicotinamide effects to the activation of the p53 tumor suppressor network was obtained by silencing p53 in wt CLL patients. Treatment with lentiviral particles carrying shRNA specific for p53 was followed by $\sim 30\%$ reduction of expression of the target mRNA (not shown) and by a decreased sensitivity to nicotinamide-induced apoptosis, as compared with the same cells treated with a control virus (Fig. 6D). The combination of the 2 drugs led to a limited functional synergy, when compared with the apoptosis induced by etoposide alone ($P = 0.2$). This effect was lower than what scored by p53 wt CLL cells ($P = 0.0004$), and by CLL cells treated with a control virus ($P = 0.005$). This behavior was comparable with what observed in the naturally occurring del/mut p53 subset, confirming that the integrity of the p53 pathway is critical for nicotinamide actions (Fig. 6B–D).

Discussion

SIRT1 is a class III nuclear deacetylase that can activate or repress genetic programs by modifying histones and transcription factors. The first finding of this work is that the enzyme is overexpressed and overfunctional in CLL cells, taking normal B lymphocytes as a comparison. This is in keeping with independent observations linking increased expression of the enzyme to tumor transformation (10). The translational inference is that targeting SIRT1 could lead to modulation of pathways regulating tumor growth and progression. Nicotinamide, a known feedback inhibitor of SIRT1, was selected in virtue of its experienced use in the therapy of pellagra and of the lack of toxicity, even in the presence of high levels of the drug (43). The results of this work confirm that exposure to nicotinamide is followed by a significant drop in the enzymatic activity of SIRT1. The effects are specific for CLL cells, whereas normal B lymphocytes are not influenced. These findings represent the basis for proposing nicotinamide as a powerful inhibitor for this deacetylase, at doses reported to be achievable *in vivo* (43).

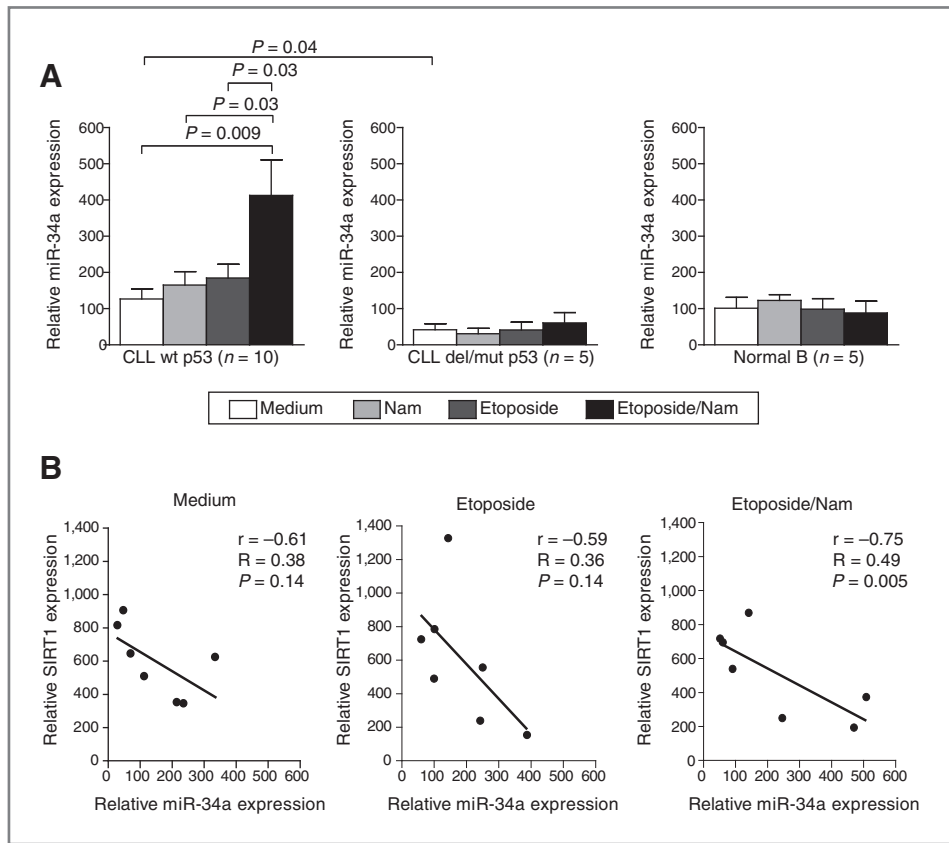


Figure 5. Combined treatment with nicotinamide/etoposide induces activation of the miR-34a/SIRT1 axis in wt p53 CLL cells. A, CLL cells [wt p53 (n = 10) or del/mutp53 (n = 5)] and normal B cells (n = 5) were treated with nicotinamide, etoposide, or their combination. miR-34a levels were measured by qRT-PCR and normalized to U6. Mean values ± SD are shown. n indicates the number samples analyzed. Expression of SIRT1 mRNA was quantified in the same samples (B) and normalized to GAPDH. The relative expression of miR-34a (x axis) was plotted against that of SIRT1 (y axis) after exposure to medium, etoposide, and a combination of the two. r indicates the Pearson coefficient and R-squared the coefficient of determination. Nam, nicotinamide.

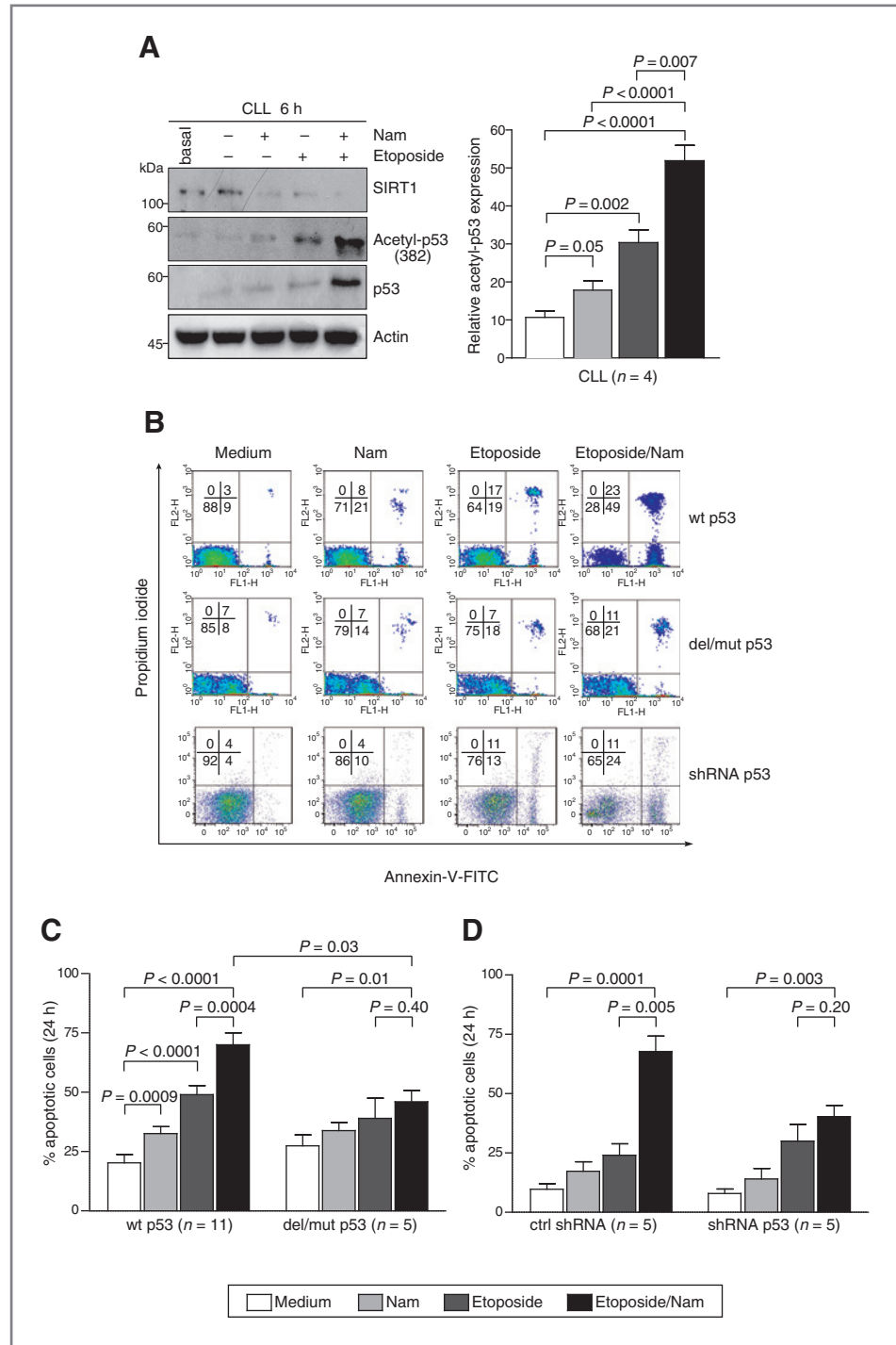
SIRT1 inhibition by nicotinamide leads to a block of proliferation and to the activation of apoptosis. These effects are comparatively more apparent in leukemic lymphocytes than in normal mature B cells. Furthermore, they seem to be strictly dependent on the presence of a functional p53 protein: indeed, the same effects are not detectable in cells from patients with a mutated or deleted p53. Because SIRT1 is reported to inactivate p53 by deacetylating a critical lysine residue, it is reasonable to hypothesize that a block of SIRT1 may lead to the activation of the p53 pathway. Confirms were obtained by showing that nicotinamide exposure induces activation of the p53-dependent genes *p21*, *NOXA*, and *BAX*, which may contribute at various degrees to the proliferation block and to the onset of apoptosis. Moreover, nicotinamide exposure down-regulates Mcl-1, a direct target of NOXA, as confirmed also in primary CLL cells (37). A link between SIRT1 and p53 in the induction of apoptosis has been previously shown by showing that SIRT1 inactivation is followed by reduced survival and increased expression of p53 targets (15). Other supporting data come from *Drosophila* models, in which a decrease in nicotinamide levels, obtained by overexpressing the enzyme nicotinamidase, prolongs fly life span (44). Similarly, reduction of nicotinamide levels in human neuronal cells induces protection from apoptosis and from oxidative stress (44). In both instances, the effects were attributed to a functional modulation of SIRT1, inhibited by nicotinamide.

Considered together, these findings led to the hypothesis that nicotinamide may be useful in potentiating p53 activation

driven by chemotherapeutic agents. By inducing DNA damage, these drugs activate a p53-regulated loop, which involves the miR-34 family (38). miR-34a, the most studied component of the family, mediates some of the functional consequences, including apoptosis (45). SIRT1 displays the dual characteristic of being a target of miR-34a and, simultaneously, an inhibitor of p53, suggesting that nicotinamide may work synergistically with chemotherapeutics in inducing apoptosis. The administration of a combination of nicotinamide and etoposide (selected as a prototype chemotherapeutic) to CLL cells significantly enhanced miR-34a expression, SIRT1 inhibition and p53 activity, as compared with either agent used alone (Fig. 7). The same treatment also led to a net increase in whole p53 levels. The picture taking shape in the CLL cell is that these 3 players are linked in a positive feedback loop, in which DNA damage induces p53, which in turn induces miR-34a, which represses SIRT1 (40). Thus, nicotinamide inhibits SIRT1 through 2 clearly distinct mechanisms, 1 linked to a noncompetitive enzymatic block (p53-independent), whereas the second is mediated via miR-34a (p53-dependent; Fig. 7). The increase in the acetylated form of p53 obtained by nicotinamide treatment may also be instrumental in preventing ubiquitination of key lysine residues by the MDM2 enzyme and subsequent proteasomal degradation (46). A direct interplay between the MDM2 enzyme and p53 in tumor models, including CLL, influences not only tumorigenesis but also treatment outcome (47, 48). The upshot is that a treatment with nicotinamide combined with etoposide reduces CLL cell

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Figure 6. A combination of etoposide and nicotinamide induces maximal decrease of SIRT1, activation of p53, and induction of apoptosis. A, CLL cells ($n = 4$) with wt p53 were treated with nicotinamide, etoposide, or their combination (6 hours, 37°C). SIRT1, acetylated p53, total p53, and β -actin were measured by immunoblotting in whole cell extracts. Left panel shows a representative experiment, while cumulative data from 4 independent patients with the relative expression of acetylated p53 are on the right. Graph represents mean values \pm SD. B, AV and PI staining of CLL cells with wt p53, del/mut p53, or silenced p53 (shRNA p53), treated for 24 hours with nicotinamide, etoposide, or their combination. Graph represents cumulative data by using patients with wt p53 and del/mut p53 (C) or with ctrl shRNA and shRNA p53 (D). Mean values \pm SD are shown. n indicates the number of samples analyzed. Nam, nicotinamide.



viability at levels significantly higher than when either agent used alone. The levels of miR-34a are decreased in leukemic clones with a mutated or deleted p53. Here nicotinamide/etoposide treatment fails to modulate p53 expression. Supporting evidence comes from (i) CLL cases characterized by low expression of miR-34a, which lack p53 or else express a

mutated form and (ii) by silencing p53 expression in wt p53 patients. The latter treatment is followed by the acquisition of resistance to apoptosis mediated by nicotinamide alone or in combination with etoposide. From the clinical point of view, this set of patients is characterized by a more aggressive form of leukemia and by resistance to chemotherapy (33, 41, 42).

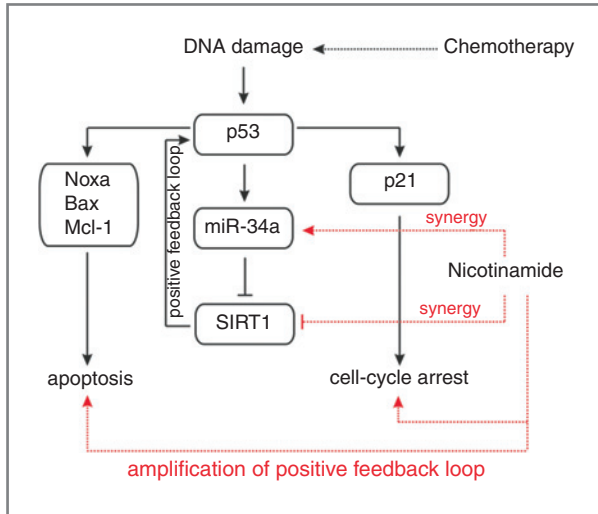


Figure 7. Schematic representation of the effects of nicotinamide on the p53/miR-34a/SIRT1 network in CLL cells. Activation of p53 following DNA damage increases miR-34a transcription, which in turn suppresses SIRT1 translation. The outcome is an increase in the acetylated functional form of p53, as part of a positive feedback loop. Addition of nicotinamide provides independent inhibition of SIRT1 and further upregulation of miR-34a (red pathway), with a resulting modulation of p53-dependent genes including *NOXA*, *BAX*, *Mcl-1*, and *p21*. The final outcome for the CLL cell is a block of the cell cycle and a strong induction of apoptosis, potentiating the effects of chemotherapy. Nam, nicotinamide.

The above results may provide a preliminary rationale for the design of a clinical trial to test the effects of oral administration of nicotinamide in combination with DNA-damaging chemotherapeutics in CLL patients with wt p53. A

References

- Maiese K, Chong ZZ, Hou J, Shang YC. The vitamin nicotinamide: translating nutrition into clinical care. *Molecules* 2009;14:3446–85.
- Koch-Nolte F, Haag F, Guse AH, Lund F, Ziegler M. Emerging roles of NAD⁺ and its metabolites in cell signaling. *Sci Signal* 2009;2:mr1.
- Houtkooper RH, Canto C, Wanders RJ, Auwerx J. The secret life of NAD⁺: an old metabolite controlling new metabolic signaling pathways. *Endocrine Rev* 2009.
- Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, et al. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev* 2008;88:841–86.
- Di Girolamo M, Dani N, Stilla A, Corda D. Physiological relevance of the endogenous mono(ADP-ribosyl)ation of cellular proteins. *FEBS J* 2005;272:4565–75.
- Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. *Nat Rev Mol Cell Biol* 2006;7:517–28.
- Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Ann Rev Pathol* 2010;5:253–95.
- Sauve AA. NAD⁺ and vitamin B3: from metabolism to therapies. *J Pharmacol Exp Ther* 2008;324:883–93.
- Sauve AA, Wolberger C, Schramm VL, Boeke JD. The biochemistry of sirtuins. *Ann Rev Biochem* 2006;75:435–65.
- Brooks CL, Gu W. How does SIRT1 affect metabolism, senescence and cancer? *Nat Rev Cancer* 2009;9:123–8.
- Finkel T, Deng CX, Mostoslavsky R. Recent progress in the biology and physiology of sirtuins. *Nature* 2009;460:587–91.
- Hursting SD, Berger NA. Energy balance, host-related factors, and cancer progression. *J Clin Oncol* 2010;28:4058–65.
- Liu T, Liu PY, Marshall GM. The critical role of the class III histone deacetylase SIRT1 in cancer. *Cancer Res* 2009;69:1702–5.
- Yi J, Luo J. SIRT1 and p53, effect on cancer, senescence and beyond. *Biochim Biophys Acta* 2010;1804:1684–9.
- Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, et al. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 2001;107:149–59.
- Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, et al. Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 2001;107:137–48.
- Marks PA, Xu WS. Histone deacetylase inhibitors: potential in cancer therapy. *J Cell Biochem* 2009;107:600–8.
- Schemies J, Uciechowska U, Sippl W, Jung M. NAD(+)-dependent histone deacetylases (sirtuins) as novel therapeutic targets. *Med Res Rev* 2010;30:861–89.
- Harris LJ. Nicotinamide and pellagra; a review of the development of knowledge and of the present position. *Practitioner* 1952;168:57–9.
- Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med* 2005;352:804–15.
- Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 2008;111:5446–56.
- Zenz T, Mertens D, Kuppers R, Dohner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat Rev Cancer* 2010;10:37–50.
- Deaglio S, Vaisitti T, Bergui L, Bonello L, Horenstein AL, Tamagnone L, et al. CD38 and CD100 lead a network of surface receptors relaying positive signals for B-CLL growth and survival. *Blood* 2005;105:3042–50.

clinical trial combining nicotinamide and etoposide in combination with the histone deacetylase inhibitor vorinostat is currently recruiting lymphoma patients (<http://clinicaltrials.gov/ct2/show/NCT00691210>). Future studies will confirm whether nicotinamide has potential as a novel, safe, and inexpensive drug to be used in combination with chemotherapy also for CLL patients with a wt p53 protein.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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24. Hawkins ED, Hommel M, Turner ML, Battye FL, Markham JF, Hodgkin PD. Measuring lymphocyte proliferation, survival and differentiation using CFSE time-series data. *Nat Protoc* 2007;2:2057–67.
25. Deaglio S, Vaisitti T, Aydin S, Bergui L, D'Arena G, Bonello L, et al. CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. *Blood* 2007;110:4012–21.
26. Saborit-Villarroya I, Vaisitti T, Rossi D, D'Arena G, Gaidano G, Malavasi F, et al. E2A is a transcriptional regulator of CD38 expression in chronic lymphocytic leukemia. *Leukemia* 2011;25:479–88.
27. Lee H, Kim KR, Noh SJ, Park HS, Kwon KS, Park BH, et al. Expression of DBC1 and SIRT1 is associated with poor prognosis for breast carcinoma. *Hum Pathol* 2011;42:204–13.
28. Jang KY, Kim KS, Hwang SH, Kwon KS, Kim KR, Park HS, et al. Expression and prognostic significance of SIRT1 in ovarian epithelial tumours. *Pathology* 2009;41:366–71.
29. Jung-Hynes B, Nihal M, Zhong W, Ahmad N. Role of sirtuin histone deacetylase SIRT1 in prostate cancer. A target for prostate cancer management via its inhibition? *J Biol Chem* 2009;284:3823–32.
30. Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J Biol Chem* 2002;277:45099–107.
31. Avalos JL, Bever KM, Wolberger C. Mechanism of sirtuin inhibition by nicotinamide: altering the NAD(+) cosubstrate specificity of a Sir2 enzyme. *Mol Cell* 2005;17:855–68.
32. Decker T, Schneller F, Sparwasser T, Tretter T, Lipford GB, Wagner H, et al. Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells. *Blood* 2000;95:999–1006.
33. Zenz T, Habe S, Denzel T, Mohr J, Winkler D, Buhler A, et al. Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. *Blood* 2009;114:2589–97.
34. He G, Siddik ZH, Huang Z, Wang R, Koomen J, Kobayashi R, et al. Induction of p21 by p53 following DNA damage inhibits both Cdk4 and Cdk2 activities. *Oncogene* 2005;24:2929–43.
35. Zhao Y, Lu S, Wu L, Chai G, Wang H, Chen Y, et al. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). *Mol Cell Biol* 2006;26:2782–90.
36. Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Commun* 2005;331:851–8.
37. Hallaert DY, Spijker R, Jak M, Derks IA, Alves NL, Wensveen FM, et al. Crosstalk among Bcl-2 family members in B-CLL: seliciclib acts via the Mcl-1/Noxa axis and gradual exhaustion of Bcl-2 protection. *Cell Death Differ* 2007;14:1958–67.
38. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. *Nature* 2007;447:1130–4.
39. Yamakuchi M, Lowenstein CJ. miR-34, SIRT1 and p53: the feedback loop. *Cell Cycle* 2009;8:712–5.
40. Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci U S A* 2008;105:13421–6.
41. Dijkstra MK, van Lom K, Tielmans D, Elstrodt F, Langerak AW, van 't Veer MB, et al. 17p13/TP53 deletion in B-CLL patients is associated with microRNA-34a downregulation. *Leukemia* 2009;23:625–7.
42. Asslaber D, Pinon JD, Seyfried I, Desch P, Stocher M, Tinhofer I, et al. MicroRNA-34a expression correlates with MDM2 SNP309 polymorphism and treatment-free survival in chronic lymphocytic leukemia. *Blood* 2010;115:4191–7.
43. Knip M, Douek IF, Moore WP, Gillmor HA, McLean AE, Bingley PJ, et al. Safety of high-dose nicotinamide: a review. *Diabetologia* 2000;43:1337–45.
44. Sahar S, Sassone-Corsi P. Metabolism and cancer: the circadian clock connection. *Nat Rev Cancer* 2009;9:886–96.
45. Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 2007;26:731–43.
46. Li M, Luo J, Brooks CL, Gu W. Acetylation of p53 inhibits its ubiquitination by Mdm2. *J Biol Chem* 2002;277:50607–11.
47. Bond GL, Hu W, Bond EE, Robins H, Lutzker SG, Arva NC, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* 2004;119:591–602.
48. Gryshchenko I, Hofbauer S, Stoecher M, Daniel PT, Steurer M, Gaiger A, et al. MDM2 SNP309 is associated with poor outcome in B-cell chronic lymphocytic leukemia. *J Clin Oncol* 2008;26:2252–7.