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

Due to 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 show 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 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 conclude that nicotinamide has the dual property of inhibiting SIRT1 through a non-competitive enzymatic block (p53-independent) and at the same time through miR-34a induction (p53-dependent). These observations suggest the therapeutic potential of nicotinamide, a novel, safe and inexpensive drug, to be used in addition to chemotherapy for CLL patients with wild-type p53.
Introduction

Nicotinamide adenine dinucleotide (NAD\textsuperscript{+}), an essential co-factor in the oxidative phosphorylation chain (1), is a substrate for four 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\textsuperscript{+} and generating O-acetyl ADP-ribose. When active, these enzymes consume NAD\textsuperscript{+} and release nicotinamide, in turn a direct inhibitor of enzyme activities and the main precursor of NAD\textsuperscript{+} in most mammalian cells (8). Hence, substrates (NAD\textsuperscript{+}), enzymes and final products (nicotinamide) are linked in a dynamic balance, where nicotinamide may be considered a master regulator (8, 9). SIRT1, the major mammalian member of the sirtuin family, deacetylates histones and non-histone proteins, including p53, Ku70 and FOXO (10, 11). For these reasons, its functions are implicated in the regulation of ageing, circadian rhythm and endocrine signaling (12). SIRT1 is up-regulated in several human tumor types, including breast and colon cancers (13), where 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\textsuperscript{+} analogs, direct kinase inhibitors, suramins and compounds identified using computational approaches (17, 18).

Because of a longstanding experience in the use of nicotinamide for the treatment of pellagra and because of 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 \textit{in vitro} effects of nicotinamide on chronic 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 appears 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 1). Blood samples were from age- and sex-matched donors, while tonsils from children undergoing surgery at the Regina Margherita Hospital (Turin, Italy). Peripheral blood mononuclear cells (PBMCs), obtained by Ficoll-Hypaque (GE Healthcare, Piscataway, NJ) centrifugation, were cultured in RPMI-1640 + 10% FCS (Sigma, Milan, Italy). B lymphocytes were purified by negative selection using anti-CD3, -CD14 and -CD16 antibodies (produced and purified in-house) and Dynal magnetic beads (Invitrogen, Milan, Italy) (23), with a purity >95%.

Antibodies and reagents

Antibodies used were anti-SIRT1 (Upstate-Millipore Biotechnology, Charlottesville, VA), anti-p53, anti-acetylated-Lys382-p53, and anti-caspase-3 (both from Cell Signaling Technologies, Danvers, MA), anti-Mcl1, anti-BAX, anti-p21 and anti-actin horseradish peroxidase (HRP)–conjugated, (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-NOXA (Imgenex, San Diego, CA, USA). Secondary reagents were goat anti-mouse IgG-HRP conjugated (GαMIgG-HRP; Perkin Elmer, Wellesley, MA) and goat anti-rabbit HRP-conjugated (GαR-HRP; Santa Cruz Biotechnology).

Nicotinamide, recombinant human IL-2 (rh-IL-2, 100 IU/ml) and etoposide (50 μM) were from Sigma. CpG oligonucleotide (1 μg/ml, InvivoGen, San Diego, CA) and carboxyfluorescein diacetate N-succinimidy1 ester (CFSE, 10 μM, Invitrogen) were also used. p53 short-hairpin RNA 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 mM dose range) was added at the beginning of culture. Proliferation was measured after 5 days by staining
with anti-CD19PE (Aczon, Bologna, Italy). Cells were analyzed by flow cytometry, using a FACS CantoII equipment (BD Biosciences, Milan, Italy) and the Diva and WinMDI softwares. Cell proliferation was also measured by $[^3]$H-thymidine (Amersham Bioscience Buckinghamshire, UK) incorporation at day 5. The percentage of proliferating cells was calculated as $\frac{(\text{number of proliferated cells} / \text{total number of cells}) \times 100}$.

**Apoptosis assays**

Apoptosis was measured using the annexin-V 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 using the Human Active Caspase-3 Immunoassay (R&D Systems Europe, Abingdon, UK). Optical density (OD) was detected using a microplate reader (Bio-Tek Instruments, Milan, Italy) 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 PVDF membranes (Upstate-Millipore). Blots were incubated with the indicated antibodies and developed using enhanced chemiluminescence (ECL, Perkin Elmer). The human Embryonic Kidney 293T cell line was used as positive control for SIRT1 expression. Densitometric analyses were performed on scanned films using the public domain ImageJ software.

**Nuclear extracts**

Nuclear extraction was performed as previously described (26).

**SIRT1 enzymatic activity assay**

SIRT1 activity was determined using the SIRT1 Fluorimetric Kit (Enzo Life Sciences UK, Exeter, UK) and expressed as arbitrary fluorescent units (AFU).
RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was extracted with TRI Reagent (Sigma) and converted to cDNA using the Reverse Transcription kit (Applied Biosystems, Monza, Italy). qRT-PCR was performed using the 7900 HT Fast Real Time PCR System (SDS2.3 software), using commercially available primers (all from Applied Biosystems). Reactions were performed 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 ± S.D. 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 over-expressed in solid tumors (27-29). qRT-PCR data showed that CLL cells (n=15) express significantly higher levels of SIRT1 when compared to 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, Figure 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, Figure 1A), providing evidence that SIRT1 over-expression 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 to normal B cells (n=5, p<0.0001, Figure 1B).

SIRT1 functions are inhibited by nicotinamide in CLL cells

We then asked whether nicotinamide, the main precursor of NAD⁺ and a non-competitive 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 mM doses SIRT1 deacetylation activity was completely inhibited (Figure 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 Figure 1C). The effect was evident after 20 min exposure to nicotinamide (Figure 1C) and was maintained when cells were cultured for 60 min (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 mutations/deletions of p53 (del/mut p53) (Figure 1D). This inhibition was not apparent in similar experiments on normal B lymphocytes (p=0.94 Figure 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, two biological processes where SIRT1 is involved, through the modulation of the p53 pathway.

CFSE-labeled purified CLL cells from 20 patients were activated using an agonist specific for toll-like receptor 9 (TLR9) and IL-2 (32) (Figure 2A). Proliferation was significantly inhibited in the presence of nicotinamide (p<0.0001, Figure 2A-B), in a dose-dependent way (Figure 2B and Supplementary Figure 1). Proliferation of normal B lymphocytes was only partially affected by nicotinamide (p=0.050, Figure 2A-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, Figure 2C) and IgVH mutational status (p=0.007 for IgVH UM and p=0.001 for IgVH MUT patients, Figure 2C). A relevant exception was represented by patients with p53 inactivation, resistant to the effects of nicotinamide (p=0.0007 for wt p53 and p=0.42 for del/mut p53 patients, Figure 2C). (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 non-neoplastic B lymphocytes (n=6) were incubated with 3 different concentrations of nicotinamide (5, 10 and 50 mM) for 72 hours and then labeled with Annexin-V-FITC (AV) and Propidium Iodide (PI, Figure 3A). Even in the presence of the lowest concentration tested (5 mM, Figure 3A-B), CLL cell viability was significantly reduced at day 3 (p=0.002, Figure 3B). The effect was dose- and time-dependent, starting after only 6 hours exposure and peaking at day 3 (Figure 3C). In contrast, normal B cells entered apoptosis only in the presence of 50 mM nicotinamide (Figure 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 Figure 3D) or IgVH mutational status (p=0.05 for IgVH UM and p=0.03 for IgVH MUT patients, Figure 3D). On the contrary, patients with p53 inactivation were resistant to apoptosis when compared to the rest of the cohort (p=0.008 for wt p53 and p=0.11 for del/mut p53 patients, Figure 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 mM and p=0.004 with 10 mM, Figure 4A-B and Supplementary Figure 2). p53 controls the cell cycle by modulating p21, in turn inducing G1-phase arrest by cyclin-dependent kinase-2 (Cdk2) inhibition (34). p21 activation was confirmed in wt p53 CLL samples treated with nicotinamide (p=0.04 with 5 mM and p=0.001 with 10 mM, Figure 4A-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 mM, Figure 4A-B) and BAX (p=0.001 with 5 mM and p=0.007 with 10 mM, Figure 4A-B) with a concomitant decrease of the pro-survival Mcl-1 protein (p=0.02 with 5 mM and p=0.0006 with 10 mM, Figure 4A-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 5mM and p=0.003 with 10 mM, Supplementary Figure 3A-B).

Results showed a reduction in total caspase-3 protein levels and a concomitant increase in caspase-3 activity, measured in 10 CLL patients after 24 hours exposure to 10 mM nicotinamide (p=0.03 Supplementary Figure 3C). Altogether, these changes suggest that nicotinamide activates a p53-dependent pathway, leading to mitochondrial apoptosis via up-regulation of NOXA and BAX.
and down-modulation of the Mcl-1 protein (36), directly induced by NOXA (37). They also strongly suggest 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 the purpose, wt p53 CLL cells (n=10) were treated with 50 µM etoposide [a DNA-damaging agent that activates the p53 pathway in a time- and dose-dependent manner (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 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 to basal levels (p=0.009) or to either agent used alone (p=0.03, in both instances), in all cases studied (Figure 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 (Figure 5A). This finding is 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 (Figure 5A). These findings indicate that i) the increase in miR-34a requires p53 activation, that ii) miR-34a is directly involved in the p53-dependent response to DNA damage in CLL cells and that 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, i.e. high levels of miR-34a were paralleled by low levels of SIRT1 mRNA ($r=-0.75$, $p=0.005$, Figure 5B), further supporting the notion of a functional cooperation between these two agents in the regulation of the network.

**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 expression than either agent used alone ($p=0.02$ compared to nicotinamide and $p=0.003$ compared to etoposide alone, Figure 6A). This effect was only evident in patients with a wt p53 pathway, as cells with a mutated/deleted p53 did not modulate SIRT1 expression following nicotinamide exposure (Supplementary Figure 4). 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 Figure 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 indicate 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 is substantiated also by a strong synergism between etoposide and nicotinamide in the induction of apoptosis, selectively in cells with a wt p53 protein ($p<0.0001$, Figure 6B-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, Figure 6B-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 ~ 30% reduction of expression of the target mRNA (not shown) and by a decreased sensitivity to nicotinamide-induced apoptosis, as compared to the same cells treated with a control virus (Figure 6D). The combination of the two drugs led to a limited functional synergy, when compared to the apoptosis induced by etoposide alone (p=0.2). This effect was lower than what scored by p53 wt CLL cells (p=0.0004), as well as by CLL cells treated with a control virus (p=0.005). This behavior was comparable to what observed in the naturally occurring del/mut p53 subset, confirming that the integrity of the p53 pathway is critical for nicotinamide actions (Figure 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 over-expressed and over-functional 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, while 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).

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 appear 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 demonstrated by
showing that SIRT1 inactivation is followed by reduced survival and increased expression of p53 targets (15). Other supporting data come from *Drosophila* models, where a decrease in nicotinamide levels, obtained by over-expressing 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 to either agent used alone (Figure 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 three players are linked in a positive feedback loop, where DNA damage induces p53, which in turn induces miR-34a, which represses SIRT1 (40). Thus, nicotinamide inhibits SIRT1 through two clearly distinct mechanisms, one linked to a non-competitive enzymatic block (p53-independent), while the second is mediated via miR-34a (p53-dependent) (Figure 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.
The upshot is that a treatment with nicotinamide combined with etoposide reduces CLL cell 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).

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 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

None to disclose.

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References


Figure legends

Figure 1. Functional SIRT1 is over-expressed in CLL cells and is inhibited by exogenous nicotinamide. (A) Left panel: Expression levels of SIRT1 mRNA in B lymphocytes from CLL patients (n=15) or non-leukemic donors (n=10) were measured by qRT-PCR and normalized to GADPH. Mean values ± SD are shown. Middle panel: Western blot analysis of basal SIRT1 protein expression in CLL (n=10) or normal B (n=5) cells. 3 representative experiments are shown. Right panel: Cumulative data showing relative SIRT1 expression. Mean values ± SD are shown. (B) Nuclear extracts of B lymphocytes from CLL patients (n=15) and non-leukemic donors (n=5) were incubated with recombinant SIRT1, NAD⁺ and the substrate Fluor de Lys. SIRT1 activity was expressed as arbitrary fluorescence units (AFU). The recombinant activity of SIRT1 was subtracted from the total activity to provide the endogenous activity of SIRT1 in nuclear extracts. Mean values ± SD are shown. (C) Left panel: The effects of nicotinamide on the activity of recombinant human SIRT1 were determined after incubation of the enzyme with nicotinamide (5 and 10 mM). Mean values ± SD are shown. Right panel: B lymphocytes from CLL patients (n=12) and non-leukemic donors (n=4) were treated with nicotinamide (5 mM, 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 ± SD are shown. n indicates the number of samples analyzed. Nam=nicotinamide.

Figure 2. Nicotinamide inhibits proliferation of CLL cells with a wt p53. (A) CFSE-labeled PBMC from CLL patients and non-leukemic donors were cultured for 5 days with CpG/IL-2 ± nicotinamide (10 mM). Samples were acquired by gating on CD19⁺ cells. M1 labels proliferating cells. (B) Cumulative data of proliferation experiments after CpG/IL-2 activation 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 $[^3]H$-thymidine incorporation. Mean values ± SD are shown. n indicates the number of samples analyzed. Nam=nicotinamide.

**Figure 3. Nicotinamide induces apoptosis in CLL cells with a wt p53.** (A) B lymphocytes from CLL patients (n=20) and non-leukemic donors (n=6) were treated with nicotinamide (5, 10 and 50 mM, 72h). Cells were then labeled with AV and PI and analyzed by flow cytometry. (B) Cumulative data of apoptosis experiments. Mean values ± SD are shown. (C) Time-dependent kinetic (6-72 hours) of CLL apoptosis induction following nicotinamide (5 mM) exposure. Mean values ± 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 ± SD are shown. n indicates the number of samples analyzed. Nam=nicotinamide.

**Figure 4. Nicotinamide induces expression of p53-dependent genes.** CLL cells (n=5) were treated with nicotinamide (5 mM and 10 mM, 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 ± SD. Nam=nicotinamide.

**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 GADPH. 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

**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 ± 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 using patients with wt p53 and del/mut p53 (C) or with ctrl shRNA and shRNA p53 (D). Mean values ± SD are shown. n indicates the number of samples analyzed. Nam=nicotinamide

**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 up-regulation 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.
FIGURE 1

A

Relative SIRT1 mRNA expression

- CLL (n=15)
- Normal B (n=10)

B

AFU x 10^3

- CLL (n=15)
- Normal B (n=5)

C

AFU x 10^3

- S-NAD^+ + Nam
- S+NAD^+ + Nam
- 5 mM Nam
- 10 mM Nam

D

AFU x 10^3

- CD38^+ vs CD38^-
- UM vs MUT
- wt p53 vs del/mut p53

P values:
- P < .0001
- P = .002
- P = .005
- P = .94
- P = .02
- P = .03
- P = .04
- P = .03
- P = .002
**Figure 2**

(A) Flow cytometry histograms showing the distribution of M1 counts in CLL and Normal B cells with and without Nam treatment. The Y-axis represents the number of events, and the X-axis represents FL1-H. The M1 region is highlighted.

(B) Bar graph showing the percentage of proliferating cells (CpG/IL-2) in CLL (n=20) and Normal B (n=6) cells with and without Nam treatment. The P-values are indicated above the bars.

(C) Graphs showing the cpm x 10^3 for CD38+ and CD38- cells with and without Nam treatment. The P-values are indicated above the bars. Additional graphs show cpm x 10^3 for UM and MUT with and without Nam treatment, with P-values indicated above the bars.

Legend:
- CLL
- Normal B
- Nam
- Nam + Nam
- Nam - Nam
- CD38-
- CD38+
- UM
- MUT
- wt p53
- del/mut p53

Statistical significance:
- P < 0.0001
- P = 0.007
- P = 0.001
- P = 0.0007
- P = 0.42
- P = 0.05
FIGURE 4

A

IB: Actin

IB: p53

IB: p21

IB: NOXA

IB: BAX

IB: Mcl-1

B

Relative NOXA expression

P = .001

Relative Mcl-1 expression

P = .001

Relative BAX expression

P = .001

Relative p21 expression

P = .001

Relative p53 expression

P = .001
FIGURE 5

A

Relative miR-34a expression

- CLL wt p53 (n=10)
- CLL del/mut p53 (n=5)
- Normal B (n=5)

Legend:
- Medium
- Nam
- Etoposide
- Etoposide/Nam

P = .009
P = .03
P = .03

B

Medium

Etoposide

Etoposide/Nam

P = .14
P = .03
P = .04

P = .14
P = .04
P = .005
FIGURE 7

DNA damage $\rightarrow$ Chemotherapy

- p53
  - Noxa
  - Bax
  - Mcl-1
  - positive feedback loop
- miR-34a
- SIRT1
  - synergy with Nicotinamide
  - cell cycle arrest
  - amplification of positive feedback loop
- p21
  - synergy with Nicotinamide
  - apoptosis
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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