The role of sirtuin 2 activation by nicotinamide phosphoribosyltransferase in the aberrant proliferation and survival of myeloid leukemia cells

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ABSTRACT

Background

Inhibitors of nicotinamide phosphoribosyltransferase have recently been validated as therapeutic targets in leukemia, but the mechanism of leukemogenic transformation downstream of this enzyme is unclear.

Design and Methods

Here, we evaluated whether nicotinamide phosphoribosyltransferase's effects on aberrant proliferation and survival of myeloid leukemic cells are dependent on sirtuin and delineated the downstream signaling pathways operating during this process.

Results

We identified significant upregulation of sirtuin 2 and nicotinamide phosphoribosyltransferase levels in primary acute myeloid leukemia blasts compared to in hematopoietic progenitor cells from healthy individuals. Importantly, specific inhibition of nicotinamide phosphoribosyltransferase or sirtuin 2 significantly reduced proliferation and induced apoptosis in human acute myeloid leukemia cell lines and primary blasts. Intriguingly, we found that protein kinase B/AKT could be deacetylated by nicotinamide phosphoribosyltransferase and sirtuin 2. The anti-leukemic effects of the inhibition of nicotinamide phosphoribosyltransferase or sirtuin 2 were accompanied by acetylation of protein kinase B/AKT with subsequent inhibition by dephosphorylation. This leads to activation of glycogen synthase kinase-3 β via diminished phosphorylation and, ultimately, inactivation of β-catenin by phosphorylation.

Conclusions

Our results provide strong evidence that nicotinamide phosphoribosyltransferase and sirtuin 2 participate in the aberrant proliferation and survival of leukemic cells, and suggest that the protein kinase B/AKT/ glycogen synthase kinase-3 β/β-catenin pathway is a target for inhibition of nicotinamide phosphoribosyltransferase or sirtuin 2 and, thereby, leukemia cell proliferation.

Key words: Nampt, SIRT2, Akt, GSK3, acute myeloid leukemia, acetylation.

Citation: Dan L, Klimenkova O, Klimiankou M, Klusman J-H, van den Heuvel-Eibrink MM, Reinhardt D, Welte K, and Skokowa J. The role of sirtuin 2 activation by nicotinamide phosphoribosyltransferase in the aberrant proliferation and survival of myeloid leukemia cells. Haematologica 2012;97(4):551-559. doi:10.3324/haematol.2011.055236

Acknowledgments: we thank A. Gigina, A. Müller Brechlin and M. Reuter for their excellent technical assistance.

Manuscript received on September 14, 2011. Revised version arrived on November 21, 2011. Manuscript accepted on December 19, 2011.

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The online version of this article has a Supplementary Appendix.

Introduction

Recently, we demonstrated that nicotinamide phosphoribosyltransferase (NAMPT) is an essential enzyme mediator of granulocyte colony-stimulating factor (G-CSF)-triggered granulopoiesis.[1] NAMPT is the rate-limiting enzyme in the biosynthesis of NAD^+ and supplies NAD^+ to sirtuins, which require NAD^+ to activate their protein deacetylase functions.[2] *In vitro* stimulation of $CD34^+$ cells with NAMPT leads to granulocytic differentiation via SIRT1-C/EBP (CCAAT/enhancer binding protein)-dependent activation of autocrine G-CSF synthesis and G-CSF receptor expression in myeloid cells.[1] In addition, a specific inhibitor of NAMPT has been validated as a therapeutic target in leukemia,[3] suggesting that different mechanisms operate downstream of NAMPT in "normal" and leukemogenic myeloid cells. However, the mechanisms downstream of NAMPT which are responsible for the aberrant proliferation and apoptosis of leukemic cells have remained elusive.

Sirtuins are members of the NAD^+-dependent class III histone deacetylase family; seven members (SIRT1-7) of this family have been described in humans. Sirtuins possess either histone or protein deacetylase activity, and play a particularly important role in the response to certain types of stress and toxicity. Sirtuins are involved in lifespan extension, age-related disorders, obesity, heart disease, neurological function and cancer.[4] SIRT1, one of the most extensively studied sirtuins, is known to deacetylate, and thereby inactivate, p53 and FOXO3a.[5] SIRT2, unlike SIRT1, is mostly found in the cytoplasm[2] and shows cell cycle-dependent intracellular localization, undergoing rapid nucleo-cytoplasmic shifts during G2/M cell-cycle progression. This observation together with the demonstration that overexpression of SIRT2 mediates a delay in cellular proliferation[6] suggest that SIRT2 may play a role in cell-cycle regulation. The importance of nucleo-cytoplasmic shuttling in SIRT2 function is highlighted by the observation that abnormal intracellular SIRT2 localization may lead to pathological downstream effects, such as abnormal cellular response of leukemic cells to DNA damage.[7] SIRT2 also deacetylates α-tubulin,[6,8] suggesting a function in cytoskeletal organization. In addition to targeting α-tubulin, SIRT2 is known to specifically target NF-κB,[9] FOXO transcription factors[10-12] and p53.[13-15]

The AKT pathway is frequently activated in acute myeloid leukemia (AML).[16-18] However, the mechanisms leading to AKT activation in AML are not completely clear. NAMPT (visfatin) has recently been shown to induce AKT phosphorylation in endothelial cells and in cardiac fibroblasts.[19,20] AKT phosphorylates and thereby inhibits a serine-threonine kinase glycogen synthase kinase 3β (GSK3β).[21] GSK3β is a well-known inhibitor of Wnt signaling. GSK3β targets the proto-oncogene β-catenin and promotes its ubiquitination and proteasome-mediated degradation.[22,23] Inactivation of GSK3β leads to β-catenin accumulation and redistribution to the nucleus.[22,23] Nuclear β-catenin interacts with LEF-1/TCF transcription factors, which regulate cell survival and proliferation by activation of target genes c-myc, survivin and cyclin D1.[24] β-catenin induces proliferation and survival, but inhibits differentiation of hematopoietic stem cells (HSC).[25-28] Hyperactivated β-catenin has been described in various hematologic malignancies, such as acute and chronic myeloid leukemia, chronic lymphocytic leukemia, B-cell neoplasia and multiple myeloma.[27-32]

Here, we aimed to evaluate the involvement of NAMPT and SIRT2 in the aberrant proliferation and survival of leukemic cells, and to ascertain whether the AKT/GSK3β/β-catenin signaling pathway plays a role in mediating this process.

Design and Methods

Patients and control subjects

Primary blasts from 11 patients with AML and $CD34^+$ bone marrow cells from six healthy individuals were isolated from bone marrow mononuclear cells by Ficoll-Hypaque gradient centrifugation and were subsequently sorted using MACS beads. We obtained approval for this study from Hannover Medical School's institutional review board. Informed consent was obtained from the study participants in accordance with the Declaration of Helsinki.

Cell lines and culture conditions

NB4 and HL60 AML cell lines were cultured in RPMI-1640 medium with 10% fetal calf serum and 1% penicillin/streptomycin. $CD34^+$ cells from healthy individuals and AML blasts were cultured for 4 days in 24-well tissue-culture plates (2×10^5 cells/well) in *X-vivo* medium supplemented with 1% heat-inactivated autologous human serum with 20 ng/mL of interleukin-3, 20 ng/mL of interleukin-6, 20 ng/mL of thrombopoietin, 50 ng/mL of stem cell factor and 50 ng/mL of Flt3 ligand.

Assessment of cell proliferation, cell cycle and apoptosis

Cells (2×10^4/100 μL) were plated in 96-well plates and incubated with different concentrations of FK866 or AC 93253 for the indicated times. We used the CellTiter-Glo Luminescence Cell Proliferation Assay to assess cell proliferation. To analyze apoptosis we used the Caspase-Glo 3/7 Activity Assay and counted viable cells using trypan blue dye exclusion. Both assays were performed according to the manufacturer's instructions (Promega). For cell cycle analysis we measured BrdU uptake (20 min) using a BrdU Flow Kit (Pharmingen).

Quantitative reverse transcriptase polymerase chain reaction analysis

We isolated RNA using a RNeasy Mini Kit, amplified cDNA with random hexamer primers and a cDNA synthesis kit (Qiagen). We measured mRNA expression using a SYBR green qPCR kit. Target gene mRNA expression was normalized to β-actin and was represented as arbitrary units (AU). Primer sequences are available on request.

Western blot analysis

We used mouse anti-β-actin, rabbit anti-AKT, rabbit anti-phospho-AKT (Ser473) (193H12), rabbit anti-GSK-3β (27C10), and rabbit anti-phospho-GSK-3β (Ser9) (5B3) (all from Cell Signaling Technology). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology) were used as appropriate. We obtained whole-cell lysates through direct disruption in Laemmli loading buffer. We separated proteins by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and probed the blots overnight at 4°C.

Detection of proteins and protein acetylation

The detection of proteins and protein acetylation by *in situ* proximity ligation assay (DUOLINK) is described in the *Online Supplementary Design and Methods*.

Determination of sirtuin 2 activity

SIRT2 activity was measured in cell lysates using the CycLex® SIRT2 Deacetylase Fluorometric Assay Kit (CycLEx, MBL).

Microarray analysis of pediatric acute myeloid leukemia samples

cDNA amplified from RNA samples from 33 patients with high-risk AML and from 18 with standard-risk AML were used. Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, USA) were further used for hybridization of cDNA samples according to the manufacturer's guidelines. Data were acquired using 'expresso' (Bioconductor package 'Affymetrix').

Statistical analysis

Statistical analysis was performed with the SPSS version 9.0 statistical package (SPSS Inc). A two-sided unpaired Student's *t* test was used to analyze differences in mean values between groups.

Results

Sirtuin 2 mRNA and protein levels are significantly upregulated in acute myeloid leukemia blasts

Recently, we found that G-CSF induces the synthesis of NAMPT in hematopoietic cells.[1] NAMPT subsequently converts nicotinic acid (NA) to NAD^+, leading to activation of the sirtuin family of NAD^+-dependent protein deacetylases, which bind and deacetylate myeloid-specific transcription factors and thereby regulate transcription (Figure 1A). As a first step towards the assessment of the role of NAMPT and sirtuins in AML, we measured the levels of NAMPT, SIRT1, and SIRT2 in blasts of AML patients. We found upregulation of NAMPT mRNA and protein levels in AML blasts (n = 11) compared to the levels in $CD34^+$ hematopoietic cells from healthy individuals (n = 6) (Figure 1B,E). We also detected significantly elevated levels of SIRT2 mRNA and protein expression in AML blasts (Figure 1C,F). In contrast, SIRT1 mRNA levels were unchanged or even slightly diminished in AML blasts compared to the levels in cells from healthy individuals (Figure 1D).

In a separate set of experiments using microarrays to analyze mRNA expression levels in patients, we identified significantly higher levels of SIRT2 mRNA in a high-risk group of AML patients (n = 33) compared to in a standard-risk group (n = 18) (Figure 1G, *Online Supplementary Table S1*). Interestingly, we found significantly diminished levels of SIRT2 in CBF-AML samples – with t(8;21) and inv(16) – in comparison to other AML types (P=0.0475) (Figure 1H). Moreover, SIRT2 expression was different between AML FAB subtypes: we observed the lowest SIRT2 levels in undifferentiated acute myeloblastic leukemia (AML M1), intermediate rates in acute myeloblastic leukemia with granulocytic maturation (AML M2) as well as in acute myelomonocytic leukemia (AML M4) and highest SIRT2 expression in acute monoblastic leukemia (AML M5) (Figure 1I).

Inhibition of NAMPT (by FK866) or SIRT2 (by AC93253) in acute myeloid leukemia leads to elevated apoptosis and reduced proliferation

It has recently been shown that specific inhibition of NAMPT by treatment with FK866 leads to elevated apoptosis and reduces proliferation of AML cells.[33] Inhibition of SIRT2 by the specific inhibitor AC93253 induces cell death in a variety of solid cancer cell lines.[34] We compared the effects of NAMPT inhibition with FK866 and SIRT2 inhibition with AC93253 on the proliferation and apoptosis of the leukemia cell lines (NB4 and HL60) and primary AML blasts (AML M2) (n = 3). To validate the specific effects of these inhibitors, we first measured SIRT2 activity in HL-60 cells treated with SIRT2 or NAMPT inhibitors and indeed found diminished SIRT2 activity after exposure to FK866 or AC93253 compared to the activity in control cells treated with DMSO (Figure 2A,B). We also demonstrated that FK866 treatment or incubation with the SIRT2 inhibitor AC93253 both resulted in dose- and time- dependent increases of apoptosis of NB4 and HL60 cells and primary AML M2 blasts compared to vehicle (DMSO)-treated control cells (Figure 2C,E). Moreover, we found that inhibition of NAMPT or SIRT2 led to diminished proliferation of NB4, HL60 cells and of primary AML M2 blasts in a dose- and time- dependent manner (Online Supplementary Figure 1A,B). The most consistent effect on cell proliferation and survival was achieved with 10 nM of FK866 after 48 h of treatment and 100 nM of AC93253 after 96 h of treatment. Low doses of FK866 (1 nM) and AC93253 (10 nM) induced slight inhibition of proliferation and elevated apoptosis. Since we could not exclude toxic effects of treatment with high doses of FK866 (100 nM) and AC93253 (1000 nM), we used 10 nM of FK866 and 100 nM of AC93253 for futher analyses.

The anti-proliferative effects of AC93253 treatment were due to cell-cycle arrest, as evidenced by a significant reduction in the number of HL60 and NB4 cells in S phase and complete loss of cells in G2/M phase compared to DMSO-treated control samples (Figure 3A). Moreover, we found 2- and 3-fold reductions in the proportion of $BrdU^+$ proliferating NB4 and HL60 cells, respectively, after treatment with AC93253, as compared to control DMSO treated cells (Figure 3B). In line with previously published reports,[33,34] we found that neither NAMPT nor SIRT2 inhibitors diminished the proliferation (Figure 2D,F) or induced apoptosis (*Online Supplementary Figure S1C,D*) of $CD34^+$ hematopoietic cells.

Inhibition of NAMPT or sirtuin 2 inactivates AKT and activates GSK-3β

We next analyzed the mechanism underlying the anti-proliferative and pro-apoptotic effects of FK866 and AC93253 in the HL60 cell line. The phosphoinositol 3-kinase (PI3K)/AKT pathway is frequently activated in AML,[16-18] and it is known that NAMPT induces AKT phosphorylation.[19,20] We did not detect any changes in the levels of phospho-PI3K p85 (Tyr 458) or expression of total PI3K p85 protein in HL-60 cells treated with FK866 or AC93253 compared to DMSO-treated control cells (*Online Supplementary Figure S2*). However, after inhibition of NAMPT or SIRT2 with FK866 or AC93253 we did find reduced phospho-AKT levels (Figure 4A). SIRT2 is a protein deacetylase. Therefore, we next analyzed whether NAMPT or SIRT2 inhibition may lead to reduced AKT phosphorylation by acetylation of AKT protein. Indeed, we found significantly elevated levels of acetylated AKT after inhibition of NAMPT or SIRT2, in comparison to the levels in control DMSO-treated cells (Figure 4B). Because AKT phosphorylates GSK3β on Ser 9 and inhibits GSK3β activity (Figure 4C), we assessed the levels of phospho-GSK3β in HL60 cells treated with

FK866 or AC93253. We detected a dramatic reduction in the levels of phospho-GSK3β, but not of total GSK3β proteins, in cells exposed to FK866 or AC93253 compared to DMSO-treated controls (Figure 4D). An *in situ* proximity ligation assay also revealed severely reduced levels of phospho-GSK3β in HL60 cells after inhibition of NAMPT or SIRT2 (Figure 4E).

Inhibition of NAMPT or sirtuin 2 is associated with β-catenin inhibition

GSK3β is a potent inhibitor of β-catenin. The dephosphorylated (active) form of GSK3β promotes β-catenin degradation by phosphorylating β-catenin on Ser33/37.[22,23] Consistent with the known activation of β-catenin in AML blasts,[27,32] we found that the levels of nuclear β-catenin were elevated in blasts of a selected AML patient (Figure 5A). We, therefore, analyzed whether NAMPT or SIRT2 inhibition resulted in increased phosphorylation of β-catenin. Indeed, we found elevated levels of inactivated phospho-β-catenin in HL60 cells treated with FK866 or AC93253, and in primary blasts of three AML M2 patients treated with AC93253 compared to the levels in DMSO-treated control cells (Figure 5B,C). Moreover, mRNA expression levels of the β-catenin target genes, LEF-1, survivin, cyclin D1 and c-Myc, were also significantly reduced in AML blasts treated with either FK866 or AC93253 compared to in DMSO-treated control cells (Figure 5D). Taken together, these results indicate that inhibition of NAMPT or SIRT2 results in the deactivation

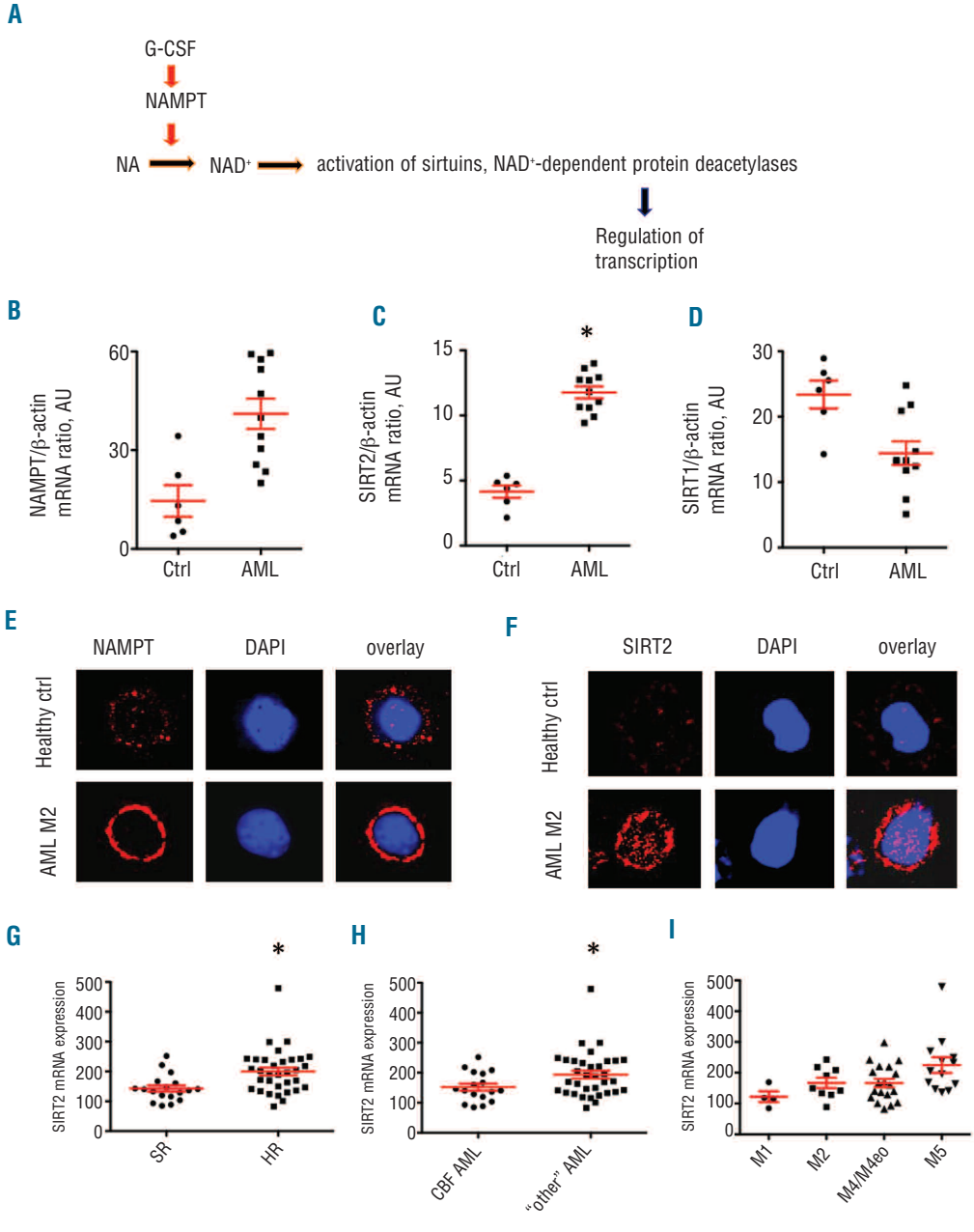


Figure 1. (A) Model of the effects of NAMPT. NAMPT is the rate-limiting enzyme for converting nicotinamide (NA) into NAD+, which, in turn, activates sirtuins, protein deacetylases, with further transcriptional regulation by deacetylation. (B-D) NAMPT (B), SIRT2 (C) and SIRT1 (D) mRNA expression levels in CD34+ cells of healthy individuals (n = 6) and AML patients (n = 11) were measured by quantitative reverse transcriptase polymerase chain reaction, normalized to β-actin levels and reported as arbitrary units (AU). Data represent means ± SD of triplicate measurements (*P<0.05); (E,F) representative DUOLINK images of NAMPT (E) and SIRT2 (F) protein expression in AML blasts (n = 6) and in CD34+ cells from healthy individual (n = 6); (G-I) intensity of mRNA expression levels of SIRT2 in AML patients, measured by Affymetrix Human Genome U133 Plus 2.0 Arrays (*P<0.05): (G) 33 high-risk (HR) AML patients and 18 standard-risk (SR) AML patients; (H) 17 AML patients with CBF AML – t(8;21) and inv(16) – and 35 patients with other types of AML; (I) four AML M1 patients, nine AML M2 patients, 19 AML M4 patients including 11 with AML M4eo, and 13 AML M5 patients.

of AKT and activation of GSK-3β via dephosphorylation, leading to inactivation of β-catenin through phosphorylation.

Discussion

Sirtuins represent a complex family of proteins (SIRT1-7) that show homology to the yeast class III NAD⁺-dependent protein deacetylase SIR2. Sirtuins have attracted considerable interest in recent years as regulators of diverse cellular processes, including cell growth, aging, apoptosis, metabolism and tumorigenesis.[4-6,34,35] SIRT1, the best-characterized nuclear sirtuin, has been shown to interact with a number of transcription factors (e.g. p53, FOXO) in the regulation of cellular processes that determine longevity.[5] In contrast, SIRT2 is a predominantly cytoplasmic protein[2] that deacetylates a number of cytoplasmic proteins, including α-tubulin.[6] However, SIRT2 has also been observed in the nucleus, where it has been implicated in the deacetylation of histones and several transcription factors, including p53 and FOXO proteins.[11,36,37] Recently, Zhao *et al.* showed that SIRT2 is the main deacetylase of FOXO1 and that the dissociation of FOXO1 from SIRT2 leads to acetylation of FOXO1 and, ultimately, to autophagy.[12] SIRT2 therefore antagonizes the tumor-suppressive and cell death-promoting functions of FOXO1 and is considered to be crucial in cellular defenses to stress following drug treatment of malignant diseases.[35]

In our study, we demonstrated that SIRT2 expression was significantly upregulated in leukemic cells from patients suffering from AML, whereas SIRT1 levels were unchanged or even diminished. Moreover, we found significantly elevated levels of SIRT2 in patients with high-risk AML, in comparison to a standard-risk group, which demonstrates the involvement of SIRT2 in the proliferation of leukemic blasts and in the resistance against chemotherapy. Because the effects of SIRT2 inhibition were similar to those of NAMPT inhibition, we hypothesize that overexpression of SIRT2 in AML is caused by its NAMPT-dependent activation. Recently, we demonstrated that G-CSF, a cytokine that is also known to stimulate myeloid leukemic cells, is a potent inducer of NAMPT expression. We further showed that NAMPT subsequently leads to substantial activation of SIRT1.[1] The data showing dramatically elevated levels of NAMPT in AML

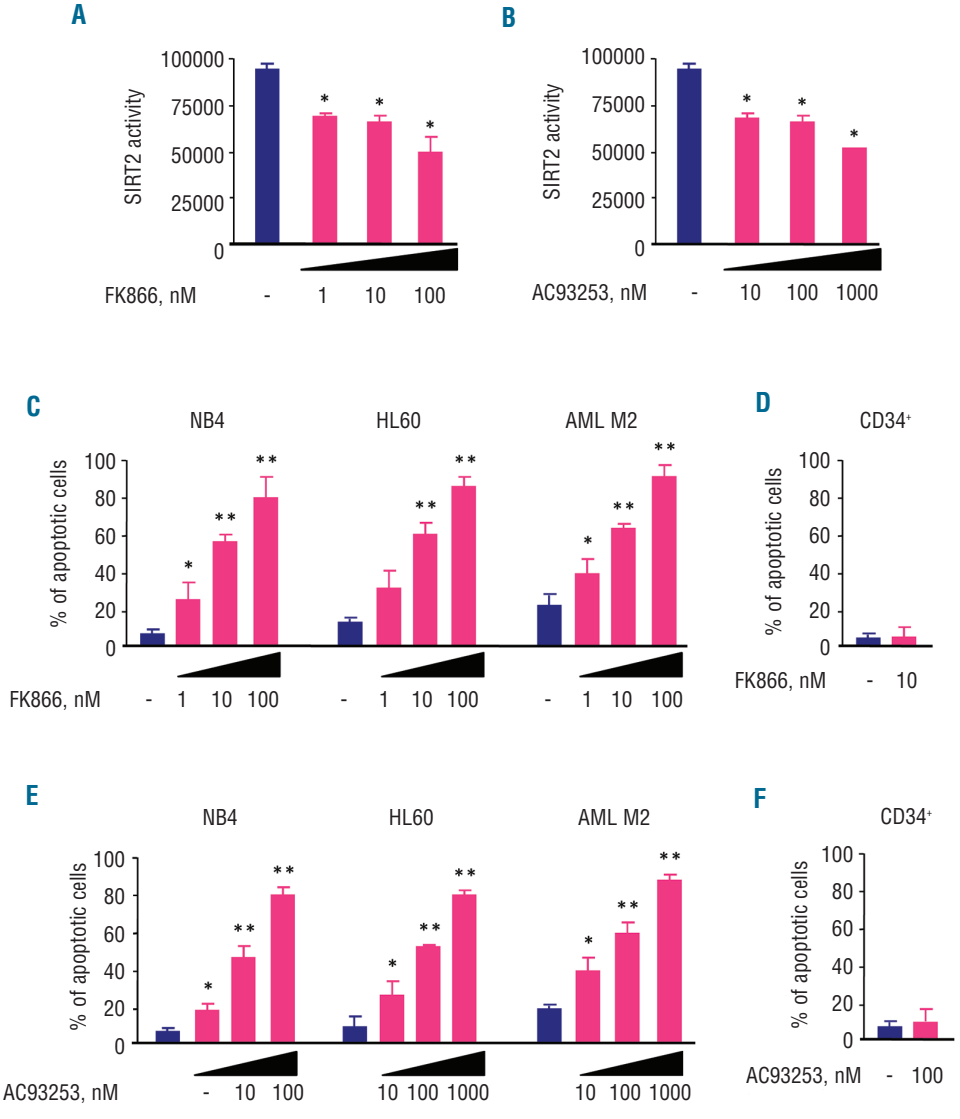


Figure 2. (A, B) HL60 cells were cultured with 1 nM, 10 nM, or 100 nM of FK866 for 96 h or 10 nM, 100 nM or 1000nM of AC93253 for 48 h, or with DMSO as a control. SIRT2 activity was measured using a CycLex® SIRT2 Deacetylase Fluorometric Assay Kit; data represent means ± SD of triplicate measurements derived from three experiments (*P<0.05); (C,E) HL60 cells, NB4 cells, or primary blasts of three AML patients were cultured with 1 nM, 10 nM, or 100 nM of FK866 for 96 h (C) or 10 nM, 100 nM or 1000 nM of AC93253 for 48 h (E), or with DMSO for the respective times as a control; apoptosis was assessed by the Caspase-GLO 3/7 Assay; the ratio of apoptotic to viable cells is shown; data represent means ± SD of triplicate measurements and are derived from three experiments (*P<0.05; **P<0.01); (D,F) bone marrow CD34⁺ cells were cultured in *ex-vivo* supplemented medium in the presence of 10 nM of FK866 for 96 h, or 100 nM of AC93253 for 48 h or with DMSO for the respective times; appoptosis was assessed as described above.

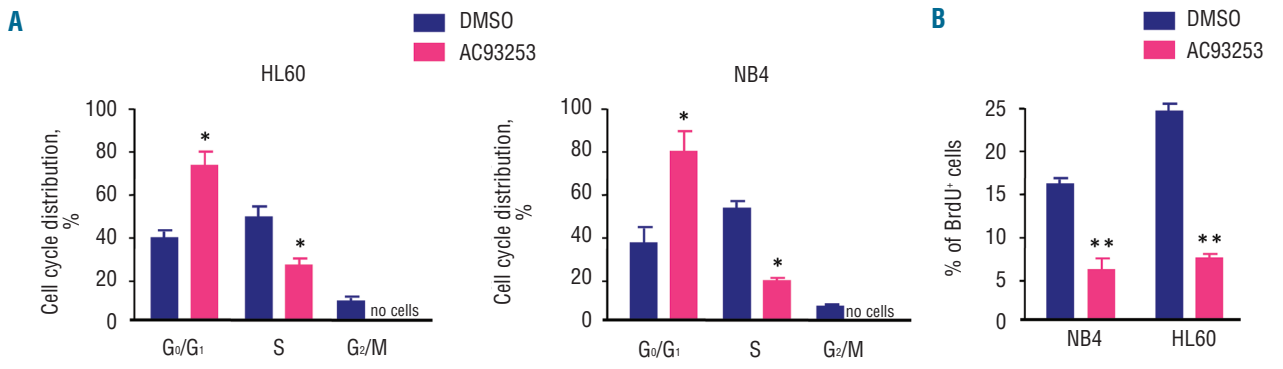
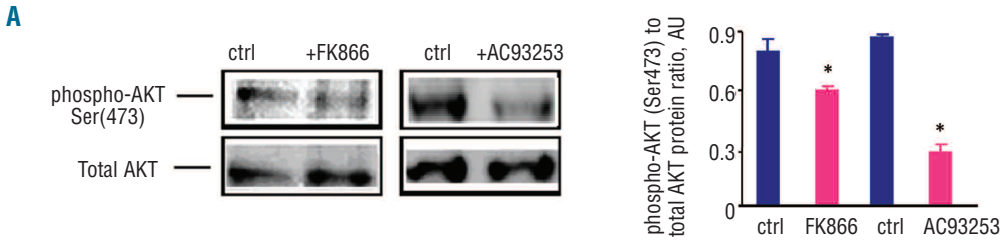
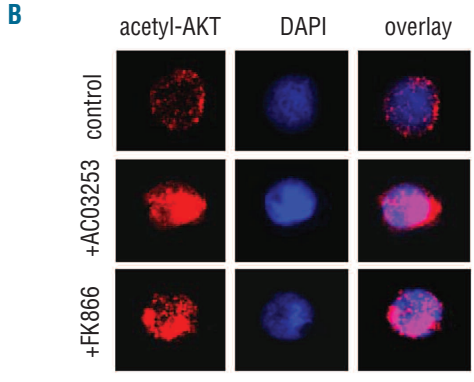


Figure 3. (A, B) HL60 and NB4 cells were labeled with BrdU for 20 min, cell cycle profile (A) and percentage of BrdU+ cells (B) were assessed by the BrdU Flow Kit and FACS analysis; data are means ± SD of duplicate measurements and are derived from three experiments (*P<0.05).





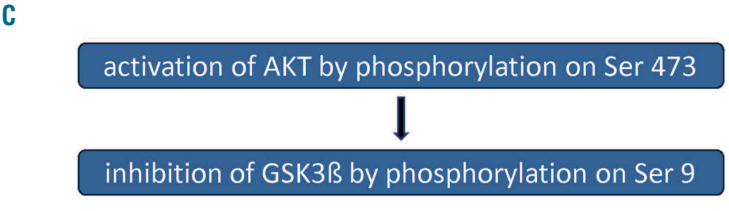
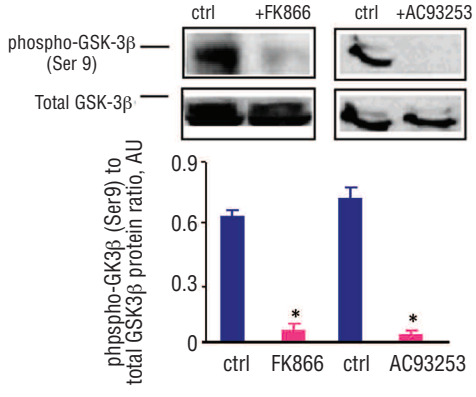
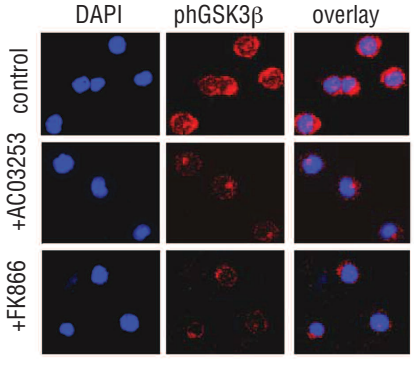


Figure 4. (A, B) HL60 and NB4 cells were cultured with or 10 nM of FK866 for 96 h or 100 nM of AC93253 for 48 h, or with DMSO as a control, (A) representative western blot images of total- and phospho-AKT (Ser473) (left part) as well as densitometric analysis of phospho- to total-AKT (Ser473) protein levels of three experiments (right part); data represent means ± SD (*P<0.05); (B) representative images of acetylated-AKT protein, as measured by the DUOLINK technique; left panel, Cy3 dots of acetylated-AKT staining; middle panel, DAPI stained nuclei; right panel, DAPI/Cy3 overlay; (C) effects of AKT on GSK3β: activated (phosphorylated) AKT phosphorylates and by this inhibits GSK3β; (D) HL60 cells were cultured with 10 nM of FK866 for 96 h or 100 nM of AC93253 for 48 h, or with DMSO as a control. Representative western blot images of total- and phospho-GSK3β (Ser9) (left part) as well as densitometric analysis of phospho- to total- GSK3β (Ser9) proteins levels (right part) of three experiments; data represent means ± SD (*P<0.05); (E) representative DUOLINK images of phospho-GSK3β (Ser9) protein expression in HL60 cells treated with 10 nM of FK866 for 96 h or 100 nM of AC93253 for 48 h, or with DMSO as a control.





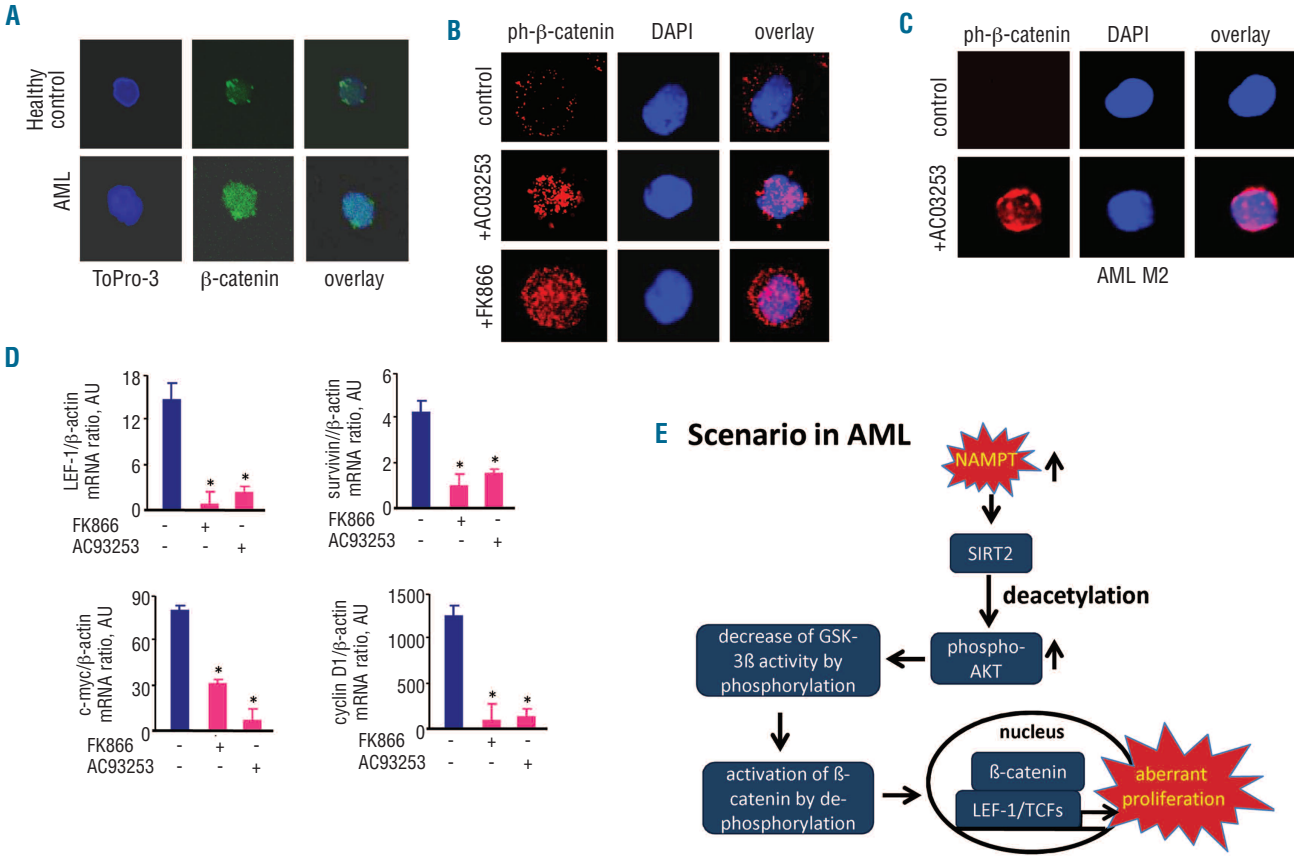


Figure 5. (A) Nuclear localization of β-catenin protein in AML blasts, in comparison to that in CD34⁺ cells from healthy individuals. Representative images of immunofluorescence staining with β-catenin antibody and ToPro-3 (nuclei); (B) representative images of inactive phospho-β-catenin (Ser33/37) protein expression in HL60 cells treated with 10 nM of FK866, 100 nM of AC93253 or DMSO as a control; (C) representative images of inactive phospho-β-catenin (Ser33/37) protein expression in AML blasts treated or not with 100 nM of AC03253, or DMSO as control; (D) downregulation of mRNA levels of Wnt/β-catenin target genes in AML blasts after treatment with 10 nM of FK866 or 100 nM of AC93253 or DMSO as control; assessed by quantitative reverse transcriptase polymerase chain reaction analysis, data represent mean ± SD of triplicate measurements (*P<0.05) and are derived from three experiments; (E) elevated levels of NAMPT are involved in aberrant proliferation of AML blasts by sequential activation of SIRT2, further elevated by deacetylation, phosphorylation and activation of AKT with subsequent phosphorylation/deactivation of GSK3β. This leads to activation and nuclear accumulation of the proto-oncogene, β-catenin. Nuclear β-catenin binds its co-factors LEF-1/TCF transcription factors and by this activates target genes (e.g. c-myc, cyclin D1 and survivin) involved in cell proliferation as well as in the regulation of cell cycle and apoptosis, which may contribute to aberrant proliferation of AML blasts.

blasts with subsequent hyperactivation of SIRT2 support our theory about dose-dependent effects of NAMPT in hematopoiesis: NAMPT is essential for proper myeloid differentiation, but hyperactivation of NAMPT may lead to leukemogenic transformation. Indeed, we previously showed that NAMPT and SIRT1 induce myeloid differentiation,[1] but it remains to be investigated whether SIRT2 could also be involved in myeloid differentiation, redundantly to SIRT1. Our demonstration that both FK866, a specific inhibitor of NAMPT, and AC93253, a specific SIRT2 inhibitor, decreased proliferation of *de novo* AML cells and AML cell lines (HL60 and NB4), inducing apoptosis of these cells, supports our previous observation that NAMPT is responsible, at least in part, for the increase in SIRT2 levels. Moreover, inhibition of NAMPT led to diminished SIRT2 activity. These data confirm our hypothesis that NAMPT induces aberrant proliferation of leukemic cells through activation of SIRT2 and that NAMPT and SIRT2 play crucial roles in the increased and sustained proliferation of myeloid leukemic cells. It has been shown that AC93253 also inhibits SIRT1, albeit at significantly higher concentrations, as we used in our study.[34] Therefore, from the presented set of experiments we could not exclude at least partial effects of SIRT1 inhibition on cell proliferation and survival of AML cells.

SIRT2 plays a key role in cell-cycle progression. During the G2/M phase, it rapidly translocates to the nucleus, binds chromatin and decreases histone acetylation.[36] SIRT2 might function as a mitotic checkpoint protein to prevent chromosomal instability and inhibit uncontrolled cell division: SIRT2 overexpression prolonged the M phase and delayed mitotic exit.[7] Tumors with high levels of SIRT2 are refractory to chemotherapy.[38] In our study, SIRT2 was localized in both the cytoplasm and nucleus. Moreover, inhibition of SIRT2 induced cell-cycle arrest, leaving no cells in the G2/M phase with a significant reduction of cells in S phase. Consistent with the aforementioned data, abnormal intracellular localization and activity of SIRT2 may be involved in leukemogenesis. Interestingly, SIRT2 expression was significantly lower in CBF AML cells than

in cells from other types of AML. It would be interesting to investigate the effects of core-binding factors (e.g. RUNX1/AML1) on SIRT2 expression and activity in leukemogenic transformation and "normal" hematopoiesis.

In our search for a pathomechanism of action of NAMPT and SIRT2, we found that treatment of leukemic cells with the respective inhibitors FK866 and AC93253 led to dephosphorylation (i.e., inactivation) of AKT kinase, which is known to be hyperactivated in AML. Several studies have demonstrated that NAMPT promotes phosphorylation of AKT.[19] Furthermore, it has been shown that both, AKT and SIRT1, are activated by common pathways. Thus, resveratrol increased longevity through its effects on SIRT1 and AKT in an *in vivo* myocardial stress model, suggesting the common activation mechanisms for AKT and SIRT1.[39] However, prior to our study, there were no data documenting the specific interplay between SIRT2 and AKT. We found that inhibition of NAMPT or SIRT2 induces strong acetylation of AKT, which is a new and exciting finding. Moreover, we demonstrated that NAMPT- and SIRT2- dependent acetylation of AKT diminished its phosphorylation and activation, describing a new post-transcriptional regulation of AKT functions by acetylation with following de-phosphorylation. During the preparation of our manuscript, Nagalingam *et al.* described SIRT1-dependent acetylation of AKT, which is in line with our observations.[40] However, it remains to be investigated, whether SIRT1 induces acetylation of AKT in AML cells.

One of the downstream effects of AKT is phosphorylation and inhibition of the kinase GSK3β.[21-22] GSK3β is a member of the β-catenin degradation complex (by phosphorylation of β-catenin) consisting of axin, adenomatous polyposis coli (APC) and GSK3β.[22,23] In the absence of active GSK3β, β-catenin is activated and migrates into the nucleus, where it induces transcription of target genes by associating with LEF-1/TCF transcription factors.[24] Active nuclear β-catenin has been detected in blasts of myeloid leukemia patients as well as in patients with other hematologic malignancies.[27-33] Interestingly, we demonstrated that inhibition of SIRT2 or NAMPT resulted in dephosphorylation/activation of GSK3β and subsequent phosphorylation/deactivation of β-catenin, which is the first step in β-catenin degradation. Because inhibition of either SIRT2 or NAMPT induced dephosphorylation/inactivation of AKT, this process is most likely AKT-dependent.

On the basis of our findings, we propose that elevated levels of NAMPT are involved in aberrant proliferation of myeloid leukemic cells via activation of SIRT2 and the proto-oncogene, β-catenin (Figure 5E). The fact that inhibition of NAMPT or SIRT2 had no effect on the proliferation of $CD34^+$ hematopoietic progenitor cells from healthy individuals establishes these proteins as promising therapeutic targets for the treatment of leukemia.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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