THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 19, pp. 12290–12299, May 8, 2015

Sirtuin Inhibition Induces Apoptosis-like Changes in Platelets and Thrombocytopenia*

Received for publication, October 2, 2014, and in revised form, March 24, 2015 Published, JBC Papers in Press, March 31, 2015, DOI 10.1074/jbc.M114.615948

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Background: The role of sirtuins in regulating platelet aging is largely unexplored.
Results: Sirtuin inhibitors induced apoptosis-like changes in blood platelets, associated with a rise in active Bax and a significant drop in platelet count.
Conclusion: Sirtuins act as a central player in the determination of platelet aging.
Significance: This study refocuses attention on the potential side effect of sirtuin inhibition in delimiting platelet life span and management of thrombosis.

Sirtuins are evolutionarily conserved NAD$^+$-dependent acetyl-lysine deacetylases that belong to class III type histone deacetylases. In humans, seven sirtuin isoforms (Sirt1 to Sirt7) have been identified. Sirtinol, a cell-permeable lactone ring derived from naphthol, is a dual Sirt1/Sirt2 inhibitor of low potency, whereas EX-527 is a potent and selective Sirt1 inhibitor. Here we demonstrate that Sirt1, Sirt2, and Sirt3 are expressed in enucleate platelets. Both sirtinol and EX-527 induced apoptosis-like changes in platelets, as revealed by enhanced annexin V binding, reactive oxygen species production, and drop in mitochondrial transmembrane potential. These changes were associated with increased phagocytic clearance of the platelets by macrophages. Expression of acetylated p53 and the conformationally active form of Bax were found to be significantly higher in both sirtinol- and EX-527-treated platelets, implicating the p53-Bax axis in apoptosis induced by sirtuin inhibitors. Administration of either sirtinol or EX-527 in mice led to a reduction in both platelet count and the number of reticulated platelets. Our results, for the first time, implicate sirtuins as a central player in the determination of platelet aging. Because sirtuin inhibitors are being evaluated for their antitumor activity, this study refocuses attention on the potential side effect of sirtuin inhibition in delimiting platelet life span and management of thrombosis.

Histone deacetylases (HDACs)[3] are classified in four classes depending on sequence identity and domain organization (1). Sirtuins are evolutionarily conserved NAD$^+$-dependent acetyl-lysine deacetylases that belong to the class III type of HDACs. Sirtuins are involved in the regulation of metabolism and life span (2). They are also implicated in determining the balance between apoptosis, cell survival, and cell proliferation. In humans, seven sirtuin isoforms (Sirt1 to Sirt7) have been identified, which localize either in the nucleus, cytoplasm, or mitochondria (3).

A number of studies have demonstrated that Sirt1 plays an important role in the regulation of cell fate and stress response in mammalian cells. Sirt1 promotes cell survival by inhibiting apoptosis or cellular senescence induced by stresses such as DNA damage and oxidative stress. Like Sirt1, Sirt2 is a ubiquitous, nuclear, and cytoplasmic protein deacetylase (4). Sirt2 is implicated in tumorigenesis and cell cycle regulation. An increasing number of proteins have been identified as substrates of Sirt1 and Sirt2, including p53, FoxO, and peroxisome proliferator-activated receptor-γ (5). Sirtinol is a cell-permeable six-membered lactone ring derived from naphthol and is a dual Sirt1/Sirt2 inhibitor of low potency (6). Sirtinol has been reported to induce senescence-like growth arrest in human breast cancer cells as well as in H1299 and leukemic cells (7–9). EX-527 is a potent and selective Sirt1 inhibitor (10).

Platelets, the enucleate blood cells derived from megakaryocytes, are discoid in shape, with size ranging between 2–4 μm. In response to vascular injury, platelets tether, adhere, aggregate, and finally form platelet plugs in injured vessel walls to arrest bleeding from blood vessels (11). HDAC inhibition has been reported previously to affect platelet function (12, 13). Inhibition of sirtuins with sirtinol attenuated the activation phenotype of platelets, which included agonist-induced platelet aggregation, a rise in intracellular Ca^{2+}, and the generation of thromboxane B2 (6). However, it is not yet clear whether sirtuins have any role in platelet survival, as demonstrated for other cells (7–9). Earlier studies, including ours, have shown that delimitation of platelet life span involves balancing interactions between Bcl-X_L, Bax/Bak, and the proteasome system (14, 15). Here we asked whether sirtuins have a regulatory role in apoptosis-like events in platelets. In this study, we evaluated the effect of three different pharmacological inhibitors of Sirt1/Sirt2 on human and mouse platelets both under *in vitro* and *in vivo* conditions. We demonstrated that Sirt1, Sirt2, and Sirt3

* This work was supported by grants from the Department of Biotechnology and the Department of Science and Technology, Government of India; the DST-FIST Program; the Indian Council of Medical Research; and the Council of Scientific and Industrial Research, and a Tata innovation fellowship grant from the DBT (to D. D.).
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[3] The abbreviations used are: HDAC, histone deacetylase; ROS, reactive oxygen species; DMSO, dimethyl sulfoxide; TMRM, tetramethylrhodamine methyl ester; PE, phycoerythrin.

are all expressed in enucleate platelets. We found that inhibitors of sirtuin deacetylases, sirtinol, EX-527, and AGK2, markedly stimulated apoptosis-like changes in platelets in a dose-dependent manner, as revealed by enhanced annexin V binding to the platelet surface, generation of reactive oxygen species (ROS), and disruption in mitochondrial transmembrane potential ($\Delta\Psi_m$). Apoptosis-like changes in platelets were associated with enhanced phagocytic clearance of cells by macrophages. The apoptosis-like phenotype in platelets induced by sirtuin inhibitors was attributable to p53-mediated transcription-independent induction of proapoptotic Bax and was calpain-dependent. Administration of either sirtinol or EX-527 in mice resulted in a decrease in both platelet count as well as in the number of reticulated platelets.

EXPERIMENTAL PROCEDURES

ABT-737 was purchased from Selleck Chemicals. Annexin V-FITC was from BD Biosciences. Rabbit polyclonal anti-p53, acetyl-p53, Sirt1, and Sirt3 antibodies were procured from Cell Signaling Technology. *N*-hydroxysuccinimidobiotin, phycoerythrin (PE)-streptavidin, JC-1, thiazole orange, carbonyl cyanide *p*-chlorophenylhydrazone, 6-carboxy-2′,7′-dichlorodihydrofluorescein, apyrase, EGTA, EDTA, sodium orthovanadate, acetylsalicylic acid, bovine serum albumin (fraction V), rabbit polyclonal anti-actin, Triton X-100, thrombin, protease inhibitors, DMSO, sirtinol, EX527, AGK2, tetramethylrhodamine methyl ester (TMRM), acetyl-DEVD-7-amido-4-methyl coumarin, rabbit polyclonal Sirt2, and mouse monoclonal anti-Bax (6A7) antibody were purchased from Sigma. Calcein-AM, TRIzol, and *t*-butoxy carbonyl-Leu-Met-chloromethyl coumarin were from Invitrogen. RPMI 1640 medium and Hysep were purchased from HiMedia. Diethylpyrocarbonate was bought from Amresco. The high-capacity cDNA reverse transcription kit was from Applied Biosystems. Sets of forward and reverse primers were purchased from Eurofins and Operon. 2× SYBR Green Supermix was procured from Bio-Rad. Reagents for electrophoresis were products of Merck. PVDF membranes and Immobilon Western chemiluminescent HRP substrate were from Millipore. HRP-labeled secondary antibody was purchased from Bangalore Genei. All other reagents were of analytical grade. Milli-Q-grade type 1 deionized water (Millipore) was used for preparation of solutions.

Platelet Preparation

Platelets were isolated from fresh human blood by differential centrifugation as described previously (16). The final cell count was adjusted to $0.5–0.8 \times 10^9$/ml with a cell counter (Beckman Coulter Multisizer 4). All steps were carried out under sterile conditions, and precautions were taken to maintain the cells in a resting condition. Approval for the animal experiment was given by the university ethics review board, and all human participants gave written informed consent. The study was conducted according to the Declaration of Helsinki.

Cytofluorimetric Analysis of the Mitochondrial Transmembrane Potential

$\Delta\Psi_m$ was evaluated using the potential-sensitive fluorochrome JC-1, which selectively moves across a polarized mitochondrial membrane and forms aggregates (red). As the membrane potential collapses, the color changes from red to green because of the release of monomeric dye (17). To study $\Delta\Psi_m$, platelets were pretreated with either sirtinol (50 or 100 μM), carbonyl cyanide *p*-chlorophenylhydrazone (100 μM), or DMSO (vehicle) for 30 min; AGK2 (100 μM); or EX-527 (10 or 50 μM); followed by incubation with 2 μM JC-1 for 15 min at 37 °C in the dark. Cells were washed in PBS, and JC-1 fluorescence was analyzed in the FL1 and FL2 channels of a flow cytometer (FACSCalibur, BD Biosciences) for detection of the dye monomer and aggregates, respectively. Forward and side scatter voltages were set at E00 and 273, respectively, with a threshold of 52 V. An amorphous region (gate) was drawn to encompass the platelets to differentiate from noise and multiplatelet particles. The ratio of red to green (FL2/FL1) fluorescence reflected the mitochondrial transmembrane potential. TMRM, another potential-specific dye that is sequestered by active mitochondria, was also employed to study the effect of sirtuin inhibitors. For evaluating the loss of mitochondrial transmembrane potential, platelets were incubated with 1 μg/ml TMRM for 15 min prior to treatment with reagents (carbonyl cyanide *p*-chlorophenylhydrazone, 100 μM; sirtinol, 100 μM). TMRM-stained platelets were analyzed in the FL2 channel of the flow cytometer.

Flow Cytometric Measurement of ROS

Platelets pretreated with either sirtinol (50 and 100 μM), AGK2 (100 μM), EX-527 (10 or 50 μM), or vehicle (DMSO) were washed with PBS and incubated with 6-carboxy-2′,7′-dichlorodihydrofluorescein (1 μM) for 30 min at 37 °C in dark. For a positive control, platelets were treated with H_2O_2 (1%) for 10 min. Cells were again washed, and fluorescence was analyzed in the FL1 channel of the flow cytometer. Sirtinol itself is fluorescent, and values for sirtinol were deducted from the observed values in the experiments.

Measurement of Annexin V Binding by Flow Cytometry

Platelets (1×10^8 cells in 100 μl) were incubated at 37 °C for 30 min in the presence of sirtinol (50 and 100 μM), AGK2 (100 μM), EX-527 (10 or 50 μM), or vehicle (DMSO). For a positive control, platelets were treated with thrombin (1 unit/ml) for 10 min without stirring and resuspended in annexin V-binding buffer. Samples were incubated with 5 μl of FITC-labeled annexin V at room temperature for 30 min in the dark and analyzed on the flow cytometer. Data from 10,000 CD61-positive events were collected for each sample.

Monocyte Isolation, Culture, and Phagocytic Recognition of Platelets

Human monocytes were isolated and cultured as described previously (18–19). Briefly, blood from healthy donors was collected in citrate, and peripheral blood mononuclear cells were isolated using Hysep according to the instructions of the manufacturer. Monocytes were further isolated by plating the peripheral blood mononuclear cells on polystyrene-coated tissue culture flasks for 4 h at 37 °C, followed by three washes with PBS to remove non-adherent lymphocytes. Monocytes (250,000 in a 500-μl volume) were then plated on 6-well plates

in RPMI 1640 medium supplemented with 10% fetal bovine serum and cultured for 7 days to obtain monocyte-derived macrophages. Control and sirtinol (100 μM)-treated platelets labeled with calcein-AM were incubated with a monolayer of autologous monocyte-derived adherent macrophages for 45 min. Following the incubation period, the phagocyte monolayer was washed to remove non-interacting platelets, and adherent macrophages were removed by treatment with trypsin at 37 °C for 5 min, followed by 5 mM EDTA at 4 °C. monocyte-derived macrophages were recovered by trypsin/EDTA treatment for 15 min at 37 °C and subjected to flow cytometric and epifluorescense microscopic analysis.

Western Blotting

Proteins were separated by 10% SDS-PAGE and electrophoretically transferred onto a PVDV membrane for 2 h at 0.8 mA/cm^2 in a semidry blotter (TE 77 PWR, GE Healthcare). To block residual protein binding sites, blots were incubated for 1 h with Tris-buffered saline containing 0.1% Tween 20 (TBST) supplemented either with 5% (w/v) BSA (for Bax and Sirt2) or with 5% skimmed milk (for acetyl-p53, p53, Sirt1, and Sirt3). Membranes were incubated overnight with antibodies against Bax (conformationally changed, clone 6A7, 1:500), acetyl-p53 (1:1000), p53 (1:1000), Sirt1 (1:1000), Sirt2 (2 μg/ml), and Sirt3 (1:1000). To detect binding of the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, diluted 1:10,000 in TBST for Bax and 1:2500 in skimmed milk for acetyl-p53, p53, Sirt1, Sirt2, and Sirt3, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were exposed to photographic films, and the optical density was estimated using scanning densitometry. For the protein loading control, membranes containing whole cell lysates were reprobed with the anti-β-actin antibody.

Caspase-3 Activity Assay—To determine cytosolic caspase-3 activity, samples were pretreated with either sirtinol (50 and 100 μM), EX-527 (10 and 50 μM), or vehicle (DMSO) and lysed with equal amounts of 2× radioimmune precipitation assay buffer. After a 10-min incubation in ice, an equal volume of 2× substrate buffer (20 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 10 μM caspase substrate acetyl-DEVD-7-amido-4-methyl coumarin) was added to each lysate and further incubated for 30 min at 37 °C (17). Caspase-3 activity was determined from the extent of cleavage of fluorogenic substrate measured at 460 nm emission (excitation, 360 nm).

Calpain Activity Assay—Intracellular calpain activity was measured as described previously (20). Washed human platelets in 96-well plates were exposed to either DMSO or sirtinol (50 and 100 μM) or EX-527 (10 and 50 μM) for 30 min and then loaded with *t*-butoxycarbonyl-Leu-Met-chloromethylcoumarin (10 μM). After 30 min of incubation, cellular fluorescence was quantified with a fluorescence microplate reader (BioTek FLx800) at 37 °C (excitation, 351 nm; emission, 430 nm).

Quantitative RT Real-time PCR

RNA Extraction—Platelets were isolated from human blood as described above. Precaution was taken to prevent leukocyte contamination. Cells were counted in a Beckman Coulter Multisizer 4. Total RNA was extracted from platelets (2.5–2.8 × 10^8 cells/ml) using TRIzol reagent according to the protocol of the manufacturer and suspended in diethylpyrocarbonate-treated water.

Reverse Transcription—Platelet RNA (1 μg) was transcribed to cDNA using a high-capacity cDNA reverse-transcription kit (Applied Biosystems) according to the instructions of the manufacturer. Samples were amplified in a PTC-150 thermal cycler (MJ Research) by using the following program: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min.

Quantitative Real-time PCR—Primers were designed using the latest version of Primer3 Input software. The primers for target gene (p53) were AGAGGAAGAG-AATCTCCGCA (forward) and GTTTCTTCTT-TGGCTGGGGA (reverse). GAPDH was used as the reference gene, and the primer sequences for GAPDH were GAAGGTG-AAGGTCGGAGTC (forward) and GAAGAT-GGTGATGGGATTTC (reverse). We performed real-time PCR employing SYBR Green SuperMix in a CFX-96 real-time PCR system (Bio-Rad). Thermal cycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles consisting of 10 s of denaturation at 95 °C, 10 s of annealing (at 56 °C in the case of GAPDH and 59 °C in the case of p53 genes), and extension at 72 °C. A melt peak analysis of amplicons was carried to rule out nonspecific amplifications.

Platelet Clearance Analysis

N-hydroxysuccinimidobiotin (600 mg) was injected into tail vein of mice, followed by either DMSO (control) or sirtinol (15 mg/kg) (21). At various time points (0, 12, 36, 60, and 84 h), 25 μl of retro-orbital blood was drawn from each mouse in the control as well as treated groups, mixed with 200 μl of buffered saline glucose-citrate buffer (116 mM NaCl, 13.6 mM trisodium citrate, 8.6 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 0.9 mM EDTA, and 11.1 mM glucose (pH 7.3)), followed by 1 ml of balanced salt solution (149 mM NaCl, 3.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 7.4 mM HEPES, 1.2 mM KH_2PO_4, 0.8 mM K_2HPO_4, and 3% bovine calf serum). Cells were pelleted at 1400 × *g* for 10 min and resuspended in 300 μl of sheath fluid. They were stained with FITC-conjugated rat anti-CD41, which labels only platelets, followed by PE-streptavidin for 1 h on ice. They were then washed in balanced salt solution and analyzed by flow cytometry to determine the fraction of the platelet population labeled with PE (14).

Labeling of Reticulated Platelets

Mice were injected intravenously with either DMSO or sirtinol (15 mg/kg), AGK2 (15 mg/kg), or EX-527 (20 mg/kg). Blood was collected from the retro-orbital plexus of mice at different time points (0, 12, 36, 60, and 84 h). Staining for reticulated platelets was carried out by incubation of 5 μl of blood with 50 μl of thiazole orange (0.1 mg/ml in PBS) and 1 μl of PE-conjugated CD41 antibody for 15 min at room temperature in the dark, followed by fixation with 1 ml of paraformaldehyde (1%) in PBS (14). Cells were washed with PBS, resuspended in 300 μl of sheath fluid, and analyzed by flow cytometry. After appropriate compensation, fluorescence data were collected using four-quadrant logarithmic amplification. The number of thiazole orange-positive platelets provided an estimate of new platelet production. A platelet count was carried out using a cell coun-

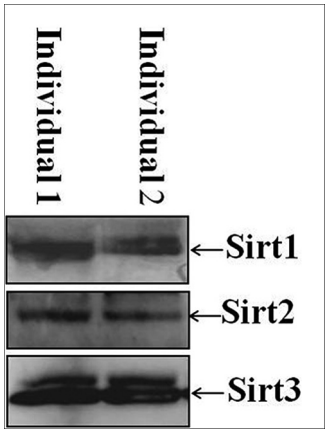

FIGURE 1. **Sirt1, Sirt2, and Sirt3 are expressed in human platelets.** Platelet proteins from two healthy individuals were resolved by SDS/PAGE and immunoblotted with antibodies directed against Sirt1, Sirt2, and Sirt3.

ter (Beckman Coulter Multisizer 4) at different time points (0, 12, 36, 60, and 84 h). After the experiment, the mice were sacrificed with an intraperitoneal injection of 2,2,2-tribromoethanol (500 mg/kg).

Statistical Methods

Standard statistical methods were used. Parametric methods (Student's *t* test) were used for evaluation, and tests were considered significant at $p < 0.05$ (two-tailed tests). All statistical tests were carried out using Sigma Plot version 11 statistics software. Data are presented as means ± S.D. of at least three individual experiments from different blood donors.

RESULTS

Human Platelets Express Sirt1, Sirt2, and Sirt3—Because sirtinol is known to inhibit multiple sirtuin isoforms, we checked for the presence of Sirt1, Sirt2, and Sirt3 in human platelets by Western blot analysis. An earlier study has already reported the expression of Sirt1 in human platelets (22). As presented in Fig. 1, all three sirtuin isoforms were found to be expressed in human platelets, which underscored the functional relevance of these sirtuins in platelets.

Sirtuin Inhibition Induces Apoptosis-like Signaling in Human Platelets in Vitro—Sirtinol is known to possess antitumor activity and to induce apoptosis in several cancer cell lines (7–9). To examine the effect of sirtuin inhibitors on platelets, we studied $\Delta\psi_m$, an indicator of intrinsic cell death, in both sirtinol- as well as EX-527-treated platelets. The lipophilic cation JC-1 was used to detect the drop in $\Delta\Psi_m$ by flow cytometry. Either of the inhibitors evoked progressive dissipation of platelet $\Delta\Psi_m$ with increasing concentrations (sirtinol, 50 and 100 μM; EX-527, 10 and 50 μM; Fig. 2*A*). Carbonyl cyanide *p*-chlorophenylhydrazone-treated platelets were employed as a positive control. Similar results were observed when we substituted JC-1 with TMRM, a pharmacologically distinct mitochondrial membrane potential probe (data not shown).

Phosphatidylserine redistribution from the inner to the outer leaflet of the surface membrane is an early and widespread event during apoptosis (23). Annexin V-FITC has a strong Ca^{2+}-dependent affinity for phosphatidylserine and, therefore, is used as a probe for the detection of apoptosis. Pretreatment of platelets with increasing concentrations of either sirtinol (50 and 100 μM) or EX-527 (10 and 50 μM) resulted in a dramatic increase in annexin V binding (by 42.0 ± 5% and 80.7 ± 5%, respectively, for sirtinol and 23 ± 5% and 120 ± 5%, respectively, for EX-527) compared with the untreated (control) cells, indicative of induction of apoptosis-like events upon inhibition of sirtuin (Fig. 2*B*). Thrombin-treated platelets were employed as a positive control.

ROS play an important role in the initiation and execution of apoptosis (24). We determined the effect of sirtuin inhibition on the level of cytosolic ROS. The dye 6-carboxy-2′,7′-dichlorodihydrofluorescein was used for measurement of ROS, which was oxidized to dichlorofluorescein by reactive oxygen species. The results revealed a dose-dependent increase in ROS in platelets treated with either sirtinol or EX-527 (Fig. 2*C*). Hydrogen peroxide-treated platelets were employed as a positive control. Similar results were obtained when we substituted sirtinol with AGK2, a pharmacologically distinct inhibitor of Sirt1/Sirt2 (data not shown).

Phagocytic Uptake of Sirtinol-pretreated Platelets by Macrophages—Platelets undergoing apoptotic changes are removed by the reticuloendothelial system through the process of phagocytosis, which eventually leads to the deletion of senescent platelets (25). We evaluated the macrophage-assisted clearance of platelets following Sirt1/Sirt2 inhibition. Calcein-stained platelets pretreated with sirtinol (100 μM) or DMSO were incubated with a monolayer of autologous monocyte-derived adherent macrophages for 45 min. The macrophage layer was then washed to remove non-interacting platelets. Macrophages were recovered by trypsin/EDTA treatment and subjected to flow cytometry as well as fluorescence microscopic analysis to examine the phagocytic uptake of platelets. For flow cytometry, macrophages were gated, and calcein fluorescence (FL1) within the "gate" was evaluated. Significantly higher fluorescence was found to be associated with macrophages incubated with sirtinol-treated platelets than with those with untreated cells (Fig. 3*A*). This observation was further supported by fluorescence microscopy, where significantly higher fluorescence was recorded in macrophages incubated with sirtinol-pretreated platelets in contrast to the control samples (Fig. 3*B*).

Apoptosis-like Changes Induced by Sirtuin Inhibitors Are Mediated through Activation of the Proapoptotic Proteins Bax and p53 and Are Calpain-dependent—Bax is a component of the Bcl-2 gene family and is known to be expressed in platelets (15). Although located in the cytoplasm, it undergoes conformational change and mitochondrial translocation upon induction of apoptosis (26). We evaluated the effect of both sirtinol as well as EX-527 on the expression of active Bax in human platelets using an antibody specific for the conformationally active form of the protein (clone 6A7). As expected, the BH3-mimetic ABT-737 induced considerable activation of Bax in platelets. Both sirtinol and EX-527 treatment led to significant increments in conformationally changed Bax in a dose-dependent manner (Fig. 4*A*), suggestive of a critical role of Bax in sirtuin-mediated changes in human platelets.

Acetyl-p53 is known to regulate the transcription-independent pathway of apoptosis (27). The best characterized proapoptotic function of p53 involves its translocation to mitochon-

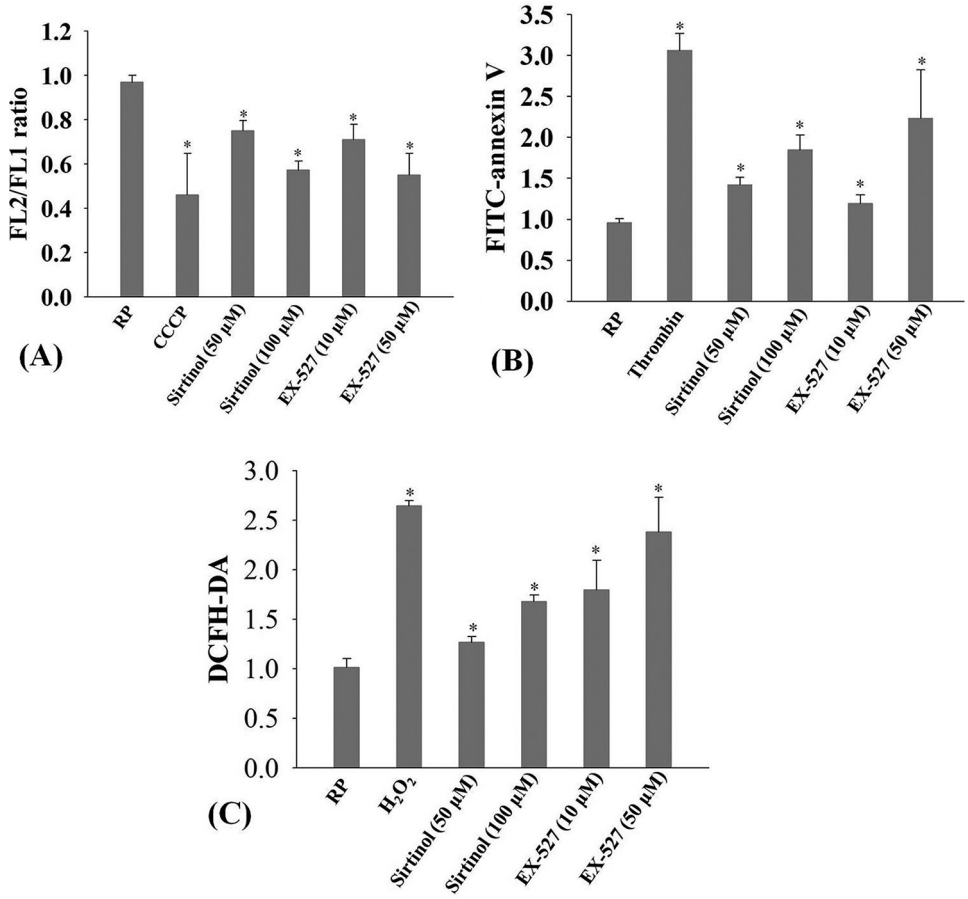

FIGURE 2. **Study of apoptosis-like features in platelets treated either with sirtinol or EX-527.** Mitochondrial transmembrane potential (*FL2/FL1 ratio*, *A*), phosphatidylserine exposure (*FITC-annexin V binding*, *B*), and ROS generation (*C*) were studied in control resting platelets (*RP*) as well as in cells pretreated either with sirtinol or EX-527 as indicated. Data are mean ± S.D. of five different experiments. *, $p < 0.05$ compared with DMSO-pretreated resting platelets. *CCCP*, carbonyl cyanide p-chlorophenylhydrazone.

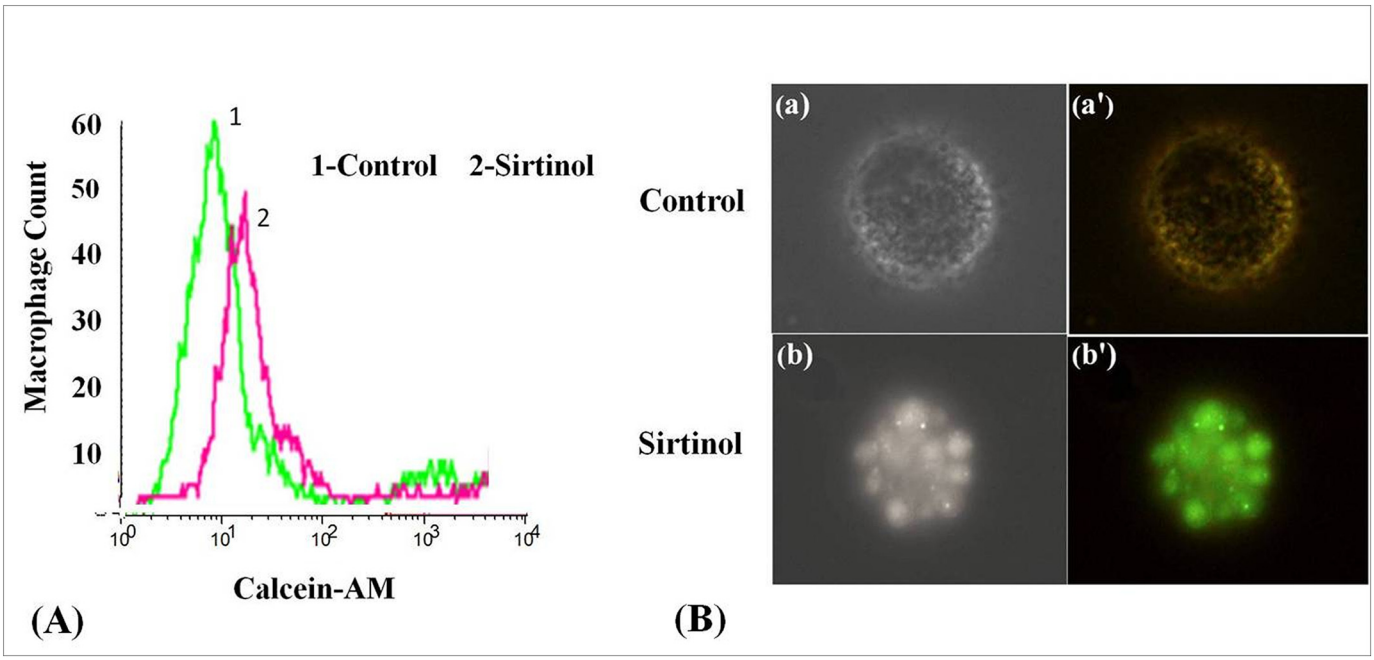

FIGURE 3. **Phagocytic uptake of platelets by autologous macrophages.** Shown are flow cytometry (*A*) and epifluorescence microscopy (*B*) of macrophages coincubated with calcein-labeled platelets pretreated either with sirtinol (100 μM) or DMSO (control). *a* and *b* represent phase-contrast micrographs, whereas *a'* and *b'* represent corresponding fluorescent images. Data are representative of five different experiments.

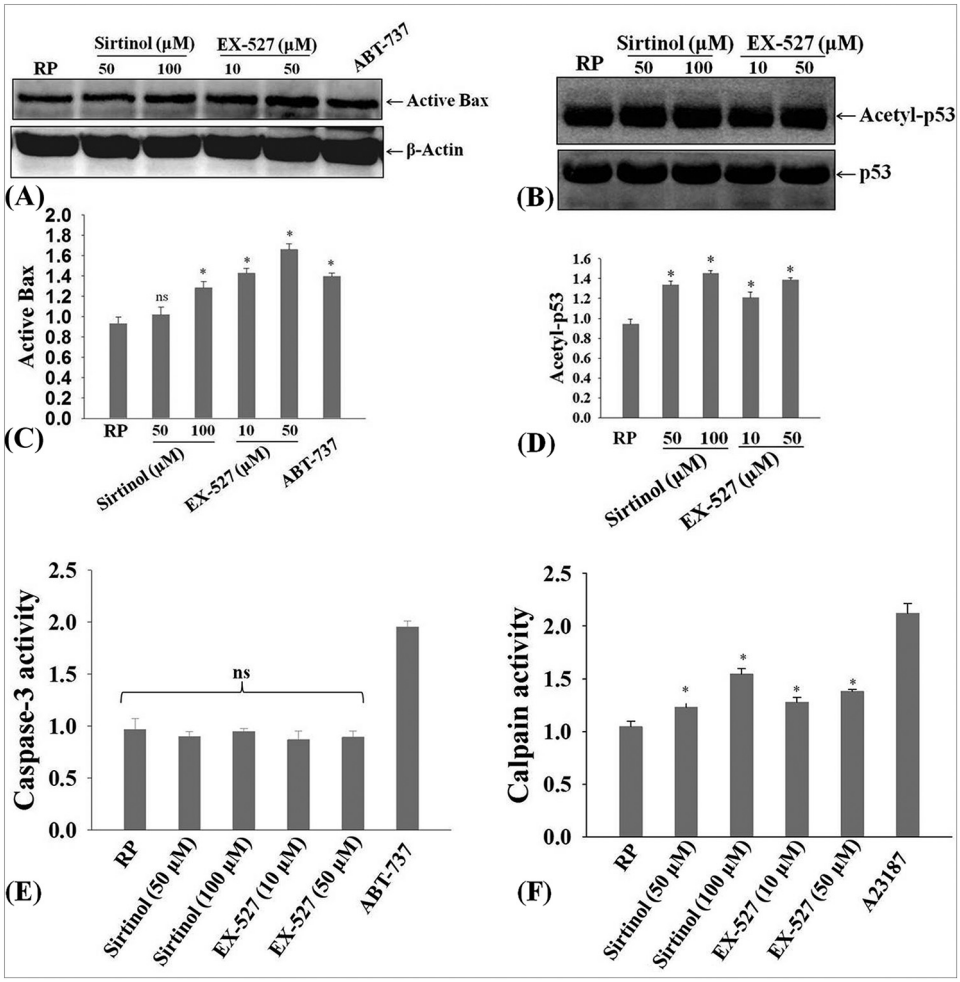

FIGURE 4. **Sirtinol-induced apoptosis-like changes in platelets are mediated through Bax, p53, and calpain.** *A*, Western blots representing expression of active Bax (*top panel*) and β-actin (loading control, *bottom panel*) in platelets pretreated with either DMSO (*RP*), sirtinol, EX-527, or ABT-737. *B*, Western blots representing expression of acetyl-p53 (*top panel*) and p53 (loading control, *bottom panel*) in platelets pretreated with either DMSO, sirtinol, or EX-527. *C* and *D*, corresponding densitometric analyses representative of at least five independent immunoblots each for active Bax and acetyl-p53 normalized against β actin or p53, respectively. *E*, caspase-3 activity determined from the extent of cleavage of the fluorogenic substrate acetyl-DEVD-7-amido-4-methyl coumarin in platelets pretreated with DMSO, sirtinol, EX-527, or ABT-737. *F*, calpain activity determined from the extent of cleavage of the fluorogenic substrate *t*-butoxy-carbonyl-Leu-Met-chloromethylcoumarin in platelets pretreated with DMSO, sirtinol, EX-527, or A23187. Data are mean ± S.D. of five different experiments. *, $p < 0.05$ compared with DMSO-pretreated resting platelets; ns, not significant.

dria in stressed cells, where it interacts with Bcl-2 family members and induces mitochondrial outer membrane permeabilization through Bax activation. Acetylation prevents ubiquitination of p53 and, therefore, promotes its stability by protecting it from proteasomal degradation (27). To understand the molecular underpinnings of sirtinol- or EX-527-induced changes, we studied the expression of acetyl-p53 in platelets treated with the inhibitors. Progressive increments in acetylated p53 were observed when cells were exposed to either sirtinol or EX-527 in increasing concentrations (Fig. 4*B*), therefore implicating p53 and Bax in sirtuin inhibition-induced apoptosis-like signaling in platelets. Because the total p53 level was unchanged in the presence of inhibitors, we checked for the presence of p53 mRNA in platelets by real-time quantitative PCR. The critical quantity values for p53 as well as GAPDH (endogenous control) were found to be 30.7 and 23.35, respectively (data not shown).These data strongly support a significant presence of p53 mRNA in platelets that could replenish protein loss because of proteasomal cleavage. A melt peak analysis was performed to rule out nonspecific amplification. Amplicons of GAPDH and p53 exhibited single sharp peaks at 83.5 °C and 85 °C, respectively, suggestive of a lack of nonspecific amplification. To the best of our knowledge, this is the first report of expression of p53 mRNA in human platelets.

Bax and other proapoptotic proteins induce the release of mitochondrial cytochrome *c* into the cytosol, which eventually leads to caspase-3 activation (28). However, growing evidence suggests that not all forms of programmed cell death are caspase-mediated. Platelets, specifically, are known to undergo caspase-independent cell death associated with the activation of calpains (15, 20, 29–31). To determine whether caspase-3 is involved in either sirtinol- or EX-527-mediated apoptosis-like changes in platelets, we measured its activity from cleavage of the fluorogenic substrate DEVD-AMC in platelets pretreated with the inhibitor or DMSO (vehicle). No significant increase in caspase-3 activity was detected in either sirtinol- or EX-527-treated platelets compared with control cells (Fig. 4*E*), consis-

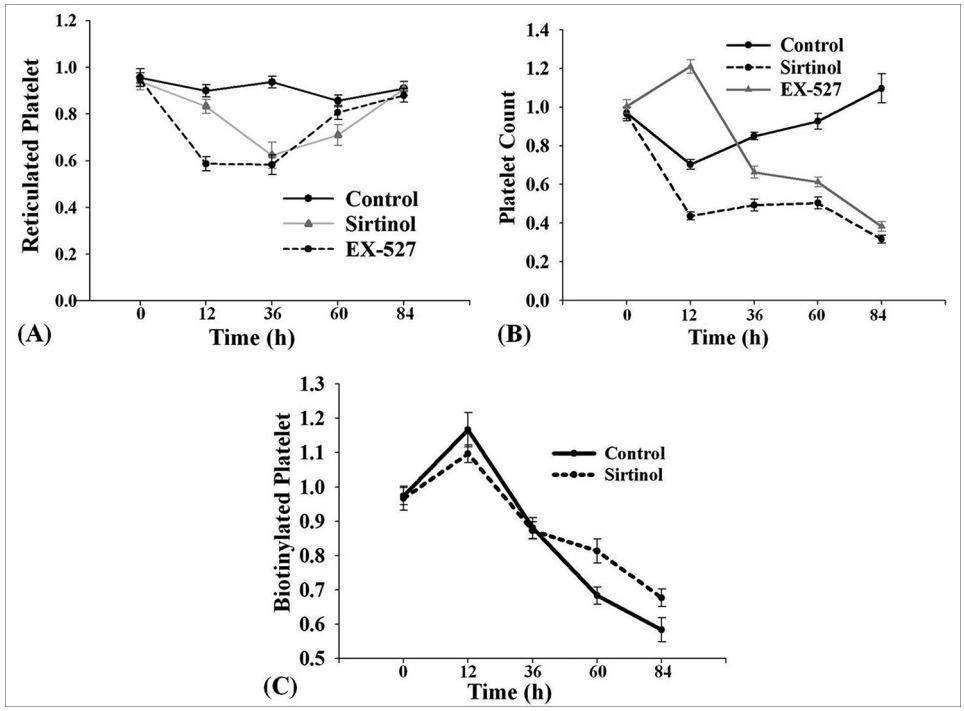

FIGURE 5. **Sirt1 inhibition leads to a reduction in reticulated platelets and platelet count.** *A*, relative number of reticulated platelets in a peripheral blood sample drawn from mice pretreated with DMSO (vehicle), sirtinol (15 mg/kg), or EX-527 (20 mg/kg) at intervals of 0, 12, 36, 60, and 84 h. *B*, relative platelet count in control-, sirtinol-, or EX-527-treated mice at different time points. *C*, relative number of biotinylated platelets in a peripheral blood sample drawn from mice pretreated with DMSO (vehicle) or sirtinol (15 mg/kg) at intervals of 0, 12, 36, 60, and 84 h. Data are mean ± S.D. of five different experiments. *, $p < 0.05$ compared with DMSO-pretreated resting platelets.

tent with a lack of involvement of caspase-3 in changes mediated by Sirt1 inhibition.

Next we looked for calpain activity in platelets undergoing either sirtinol- or EX-527-induced apoptosis-like changes. Remarkably, platelets pretreated with sirtinol (50 and 100 μM) were found to possess a significantly higher proteolytic activity of calpain (by 21.5 ± 5% and 49 ± 5%, respectively) than their vehicle-treated counterparts (Fig. 4*F*), therefore implicating calpain in sirtinol-mediated platelet cell death. Similar results were observed in EX-527-treated platelets.

Sirtuin Inhibition Leads to a Reduction in the Number of Reticulated Platelets and Thrombocytopenia—Apoptosis has now been established as an important regulator of platelet life span (14, 15, 29, 30) that determines the number of circulating platelets. Because sirtuin inhibition transforms platelets into the apoptotic phenotype, we investigated the impact of sirtinol on platelet count and life span under *in vivo* condition. Reticulated platelets are an RNA-containing younger cell population, which were stained with thiazole orange (12). Administration of either sirtinol (15 mg/kg) or EX-527 (20 mg/kg) into mice resulted in a decrease in the number of reticulated platelets (by 36.5% and 40.5%, respectively, after 36 h) (Fig. 5*A*) as well as a significant reduction in platelet count (by 59.94% in 12 h in mice administered sirtinol and by 44% in 36 h in mice administered EX-527) (Fig. 5*B*). After 36 h of sirtinol or EX-527 administration, the number of reticulated platelets eventually normalized (Fig. 5*A*). However, our experiments do not rule out platelet damage as a cause of sirtinol-induced thrombocytopenia in mice. The serum concentration of sirtinol achievable after the administered dose may not be sufficient or competent enough to induce strong apoptosis-like events in platelets leading to platelet clearance compared with the *in vitro* situation using human platelets, whereas the same serum concentration was sufficient to suppress platelet production and cause thrombocytopenia. Secondly, the possibility of impaired clearance of mouse platelets in the presence of sirtinol cannot be ruled out. A similar result was observed in mice administered AGK-2 (15 mg/kg, data not shown).

Next, we analyzed the platelet life span in mice administered sirtinol. Mice were injected with intravenous *N*-hydroxysuccinimidobiotin, allowing biotinylation of circulating platelets. The decrease in labeled platelets over time in sirtinol-treated mice was found to be similar to that in vehicle-treated counterparts (Fig. 5*C*). These data indicate that changes in platelet life span are unlikely to explain sirtinol-induced thrombocytopenia and is suggestive of decreased production of platelets, as described in mice treated with HDAC1/2-specific inhibitors (12).

DISCUSSION

Acetylation of proteins is a posttranslational modification catalyzed by acetyltransferases and deacetylases. Because of its reversible character, acetyl transfer regulates several signaling processes (32). Proteomics studies have identified thousands of acetylated mammalian proteins (32). In platelets, aspirin acts as an acetylating agent that transfers an acetyl group to a serine residue in the active site of the cyclooxygenase. Recent studies have revealed widespread abundance of protein lysine-acetylation as a vital regulation mechanism in different cells (33, 34). Sirtuin is a NAD^+-dependent acetyl-lysine deacetylase that

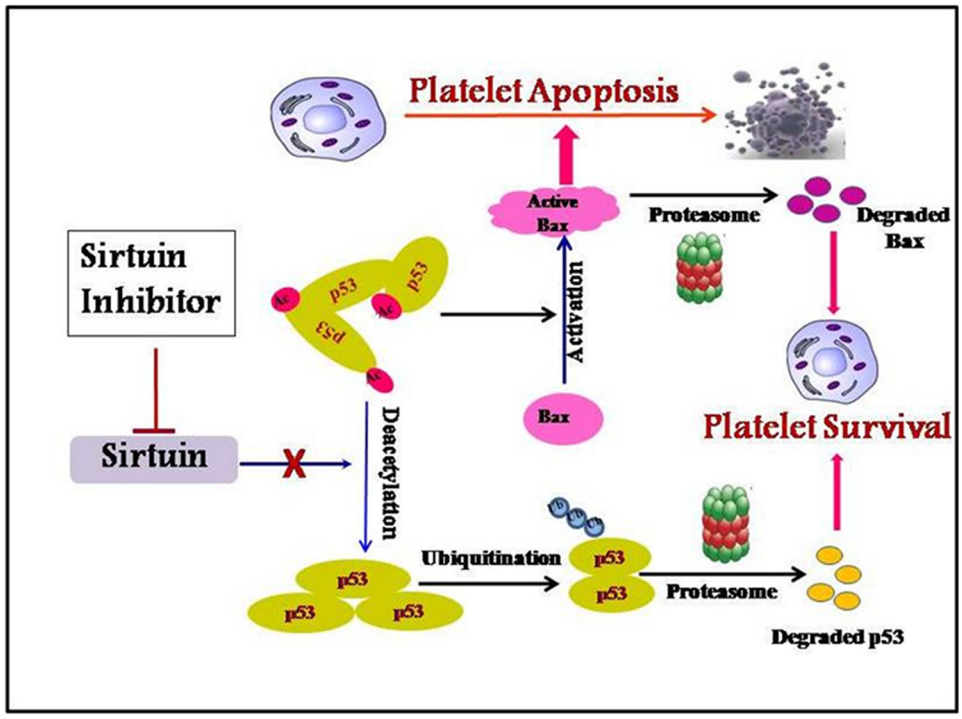

FIGURE 6. **The proposed role of sirtuin in the determination of platelet life span.** The sirtuin inhibitor enhances acetylation of p53, which eventually leads to the activation of proapoptotic Bax and apoptosis-like signaling in platelets. In the absence of acetylation, p53 is ubiquitinated and degraded by the proteasome, which facilitates cell survival (27). As reported in our earlier studies, proteasomal peptidase activity promotes platelet survival through constitutive elimination of the conformationally active Bax (15). Therefore, the proteasome and sirtuin both regulate platelet apoptosis and survival.

belongs to the HDAC III family and is expressed in prokaryotes as well as eukaryotes. Mammalian sirtuins are linked to healthy aging, and, therefore, have possible therapeutic implications in age-related pathologies, metabolic and cardiovascular disorders, and inflammation (4, 35). Platelets are major players in hemostasis and thrombosis. Although Sirt1, Sirt2, and Sirt3 are expressed in platelets, the relevance of sirtuin-mediated signaling remains obscure in these cells. Here we report, for the first time, the regulation of platelet cell death by sirtuins. In addition to Sirt1, Sirt2 and Sirt3 were also found to be expressed in enucleate platelets. We demonstrate that sirtinol, AGK2, and EX-527, pharmacologically distinct inhibitors of Sirt1/Sirt2, induced apoptosis-like changes in platelets *in vitro*, which included a drop in $\Delta\Psi_m$, enhanced surface exposure of phosphatidylserine, and a rise in cytosolic ROS. Calpain, but not caspase, has been found to be activated in sirtinol or EX-527-treated platelets, which is consistent with caspase-independent cell death described in different cells, including platelets (15, 20, 28–30). Because platelet apoptosis is known to be mediated through proapoptotic members of Bcl-2 (14–15), we evaluated the effect of sirtuin inhibition on the activation of Bax. Sirtinol- or EX-527-pretreated platelets were found to express a significantly higher level of conformationally active Bax than the control (untreated) counterparts. Because p53 is a known substrate of sirtuin and an upstream regulator of Bax (36–37), we asked next whether Sirt1 inhibition would affect the level of post-translationally modified p53 in platelets. In line with the above reasoning, enhanced acetylation of p53 was observed in sirtinol or EX-527-treated cells. Consistent with apoptosis-like phenotypes, sirtinol-treated human platelets were found to be phagocytosed more efficiently by macrophages, as demonstrated *in vitro* by flow cytometry and epifluorescence microscopy. Administration of mice with either sirtinol or EX-527 led to thrombocytopenia as well as a decrease in the number of reticulated platelets, although sirtinol had no significant effect on platelet life span in mice. The drop in number of reticulated platelets could be attributed to decreased production of platelets, as reported earlier in mice treated with HDAC1/2-specific inhibitors (12).

Earlier studies, including ours, have attributed delimitation of platelet life span to activities of pro- and antiapoptotic members of the Bcl-2 family (14, 15). Proteasomal peptidase activity promotes platelet survival through the constitutive elimination of the conformationally active Bax. Acetyl-p53 is known to play a determining role in the transcription-independent pathway of Bax-mediated apoptosis (27). Acetyl-p53 is a substrate of sirtuin and an upstream positive regulator of Bax (36–37). On the basis of the evidence provided in this study, it may be surmised that sirtuin deacetylase activity regulates platelet life span through inhibition of p53 acetylation, which, as a consequence, precludes Bax activation and platelet cell death (Fig. 6). Our observations support the possibility of enhancement of platelet life span in the presence of sirtuin activators, whereas platelets from $sirt^{-/-}$ mice are anticipated to undergo apoptosis-like signaling and early clearance. Sirtuin may, therefore, be a potential therapeutic target to induce apoptosis-like events in platelets and to reduce the severity of thrombosis or thrombocytosis. It has been reported recently that platelets play a critical role in cancer cell proliferation, and, therefore, sirtuin inhibition can indirectly contribute to the anticancer effect by

restricting the platelet count (38). Because sirtuin inhibitors are being actively evaluated for their antitumor activity (7–9), our observations also call for the careful consideration of their potentially adverse effect on platelets while exploiting the benefits of sirtuin inhibition in cancer therapeutics.

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Sirtuin Inhibition Induces Apoptosis-like Changes in Platelets and Thrombocytopenia

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J. Biol. Chem. 2015, 290:12290-12299.
doi: 10.1074/jbc.M114.615948 originally published online March 31, 2015