Sirtuin inhibition induces apoptosis-like changes in platelets and thrombocytopenia*

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*Running title- Sirtuin inhibition induces platelet apoptosis

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Key words: Apoptosis, Histone deacetylase, Platelets, Sirtuin, Thrombocytopenia

Background: The role of sirtuins in regulating platelet aging is largely unexplored.

Results: Sirtuin inhibitors induced apoptosis-like changes in blood platelets associated with rise in active Bax and significant drop in platelet count.

Conclusion: Sirtuins act as central player in determination of platelet ageing.

Significance: This study rekindles attention to potential side effect of sirtuin inhibition in delimiting platelet life span and management of thrombosis.

ABSTRACT

Sirtuins are evolutionarily conserved NAD⁺-dependent acetyl-lysine deacetylases that belong to class III type of 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 have demonstrated that Sirt1, Sirt2 and Sirt3 are expressed in enucleate platelets. Both sirtinol or EX-527 induced apoptosis-like changes in platelets as revealed from enhanced annexin V binding, ROS production and drop in mitochondrial transmembrane potential. Above 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- as well as EX-527-treated platelets, thus implicating p53-Bax axis in apoptosis induced by sirtuin inhibitors. Administration of either sirtinol or EX-527 in mice led to reduction in both platelet count and number of reticulated platelets. Our results, for the first time, implicate sirtuins as a central player in determination of platelet aging. Since sirtuin inhibitors are being evaluated for their anti-tumor activity, this study rekindles attention to potential side effect of sirtuin inhibition in delimiting platelet life span and management of thrombosis.

INTRODUCTION

Histone deacetylases (HDAC) are classified in four classes depending on sequence identity and domain organization (1). Sirtuins are evolutionarily conserved NAD⁺-dependent acetyl-lysine deacetylases that belongs to class III type of HDAC. Sirtuins are involved in the regulation of metabolism and lifespan (2). It is also implicated in determining the balance between apoptosis, cell survival, and cell proliferation. In humans, seven sirtuins isoforms (Sirt1 to Sirt7) have been identified that localize either in the nucleus, cytoplasm or in mitochondria (3).

A number of studies have demonstrated that Sirt1 plays important role in the regulation of
cell fate and stress response in mammalian cells. Sirt1 promoted cell survival by inhibiting apoptosis or cellular senescence induced by stresses including DNA damage and oxidative stress. Like Sirt1, Sirt2 is a ubiquitous, nuclear and cytoplasmic protein deacetylase (4). The role of Sirt2 is implicated in tumorigenesis and cell cycle regulation. An increasing number of proteins have been identified as substrates of Sirt1 and Sirt2, which include p53, FoxO and peroxisome proliferator-activated receptor-\(\gamma\) (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 \(\mu\)m. 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 earlier been reported to affect platelet function (12-13). Inhibition of sirtuins with sirtinol attenuated the activation phenotype of platelets, which included agonist-induced platelet aggregation, rise in intracellular Ca\(^{2+}\) and 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 \textit{in vitro} and \textit{in vivo} conditions. Here we have demonstrated that Sirt1, Sirt2 and Sirt3 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 from enhanced annexin V binding to platelet surface, generation of reactive oxygen species (ROS) and disruption in mitochondrial transmembrane potential (\(\Delta\Psi_m\)). Apoptosis-like changes in platelets was associated with enhanced phagocytic clearance of cells by macrophages. Apoptosis-like phenotype in platelets induced by sirtuin inhibitors was attributable to p53-mediated transcription-independent induction of pro-apoptotic Bax and was calpain-dependent. Administration of either sirtinol or EX-527 in mouse resulted in decrease in both platelet counts as well as in number of reticulated platelets.

**EXPERIMENTAL PROCEDURES**

ABT-737 was purchased from Selleck Chemicals. Annexin V-FITC was from BD Pharmingen. Rabbit polyclonal anti-p53, Acetyl-p53, Sirt1 and Sirt3 antibodies were procured from Cell Signaling Technology. N-hydroxysuccinimidoobiotin (NHS-biotin), PESTrepavidin, JC-1, thiazole orange, carbonyl cyanide-3-chlorophenylhydrazone (CCCP), 6-carboxy-2',7'-dichlorodihydrofluorescein (H\(_2\)DCF-DA), apyrase, ethylene glycol tetraacetic acid (EGTA), ethylenediamine-tetraacetic acid (EDTA) sodium orthovanadate, acetysalicylic acid, bovine serum albumin (fraction V), rabbit polyclonal antiactin, Triton X-100, Thrombin, protease inhibitors, DMSO, Sirtinol, EX527, AGK2, Tetramethylrhodamine, methyl ester (TMRM), Acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (AC-DEVD-AMC), rabbit polyclonal Sirt2 and mouse monoclonal anti-Bax (6A7) antibody, were purchased from Sigma. Calcein-AM, TRIzol and tbutoxy-carbonyl-Leu Metchloromethyl-coumarin were from Invitrogen. RPMI-1640 and hysep were purchased from HiMedia. Diethylpyrocarbonate (DEPC) was bought from Amresco. High-capacity cDNA reverse transcription kit was from Applied Biosystem. Sets of forward and reverse primers were purchased from Eurofins, Operon. 2X 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. Horseradish peroxidase (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 (16). The final cell count was adjusted to 0.5-0.8 × 10^9/ml with a Cell Counter (Beckman Coulter model Multisizer 4). All steps were carried out under sterile conditions and precautions were taken to maintain the cells in resting condition. Approval for the animal experiment was approved by the university ethics review board and all human participants have given written informed consent. The study was conducted according to Declaration of Helsinki.

Cytofluorimetric analysis of mitochondrial transmembrane potential

Mitochondrial transmembrane potential (ΔΨ_m) was evaluated using the potential-sensitive fluorochrome JC-1, which selectively moves across polarized mitochondrial membrane and forms aggregates (red). As membrane potential collapses, color changes from red to green due to release of monomeric dye (17). In order to study ΔΨ_m, platelets were pre-treated with either sirtinol (50 or 100 μM), CCCP (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 dark. Cells were washed in phosphate-buffered saline (PBS) and JC-1 fluorescence was analyzed in FL1 and FL2 channels of flow cytometer (model FACSCalibur, Becton Dickinson) for detection of 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 multi-platelet particles. The ratio of red to green (FL2/FL1) fluorescence reflected 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 loss of mitochondrial transmembrane potential, platelets were incubated with 1 μg/ml TMRM for 15 min prior to treatment with reagents (CCCP, 100 μM; sirtinol, 100 μM). TMRM-stained platelets were analyzed in FL2 channel of the flow cytometer.

Flow cytometric measurement of ROS

Platelets, pre-treated with either sirtinol (50 and 100 μM), AGK2 (100 μM) or EX-527 (10 or 50 μM) or vehicle (DMSO) were washed with PBS and incubated with H2DCF-DA (1 μM) for 30 min at 37 °C in dark. For positive control, platelets were treated with H2O2 (1 %) for 10 min. Cells were again washed and fluorescence was analyzed in FL1 channel of the flow cytometer. Sirtinol being itself is fluorescent; values for sirtinol were deducted from the observed values in experiments.

Measurement of annexin V binding by flow cytometry

Platelets (1 x 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) or EX-527 (10 or 50 μM) or vehicle (DMSO). For positive control, platelets were treated with thrombin (1 U/ml) for 10 min without stirring and resuspended in annexin V-binding buffer. Samples were incubated with 5 μl FITC-labeled annexin V at RT for 30 min in 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 (18-19). Briefly, blood from healthy donors was collected in citrate and peripheral blood mononuclear cells (PBMCs) were isolated using Hysep according to manufacturer’s instructions. Monocytes were further isolated by plating the PBMCs on polystyrene-coated tissue culture flasks for 4 h at 37 °C, followed by 3 washes with PBS to remove non-adherent lymphocytes. Monocytes (2, 50,000 in 500 μl volume) were then plated on 6-well plates in RPMI 1640 supplemented with 10 % fetal bovine serum and cultured for 7 days to obtain the monocyte-derived macrophages (MDMs). 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 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. MDMs were recovered by trypsin/EDTA treatment for 15 min at 37 °C and subjected to flow cytometric and epifluorescence microscopic analysis.

**Western Blotting**

Proteins were separated by 10 % SDS-PAGE and electrophoretically transferred onto PVDF membrane for 2 h at 0.8 mA/cm² in a semidyry blotter (TE 77 PWR, GE Healthcare). In order 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 primary antibodies, blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, diluted 1:10000 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 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) or EX-527 (10 and 50 µM) or vehicle (DMSO) and lysed with equal amount of 2X radioimmune precipitation assay (RIPA) buffer. After a 10-min incubation in ice, an equal volume of 2X substrate buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1 % CHAPS, 5 mM DTT, and 10 µM caspase substrate AC-DEVD-AMC) 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-Metchloromethylcoumarin (10 µM). After 30 min incubation, cellular fluorescence was quantified with a fluorescence microplate reader (BioTek model FLx800) at 37 °C (excitation, 351 nm; emission, 430 nm).

**Quantitative reverse transcriptase real-time PCR**

(i) RNA extraction

Platelets were isolated from human blood as described above. Precaution was taken to prevent leukocyte contamination. Cells were counted in Beckman Coulter multizer 4. Total RNA was extracted from platelets (2.5-2.8 X 10^8 cells per ml) using TRIzol reagent according to manufacturer’s protocol and suspended in DEPC-treated water.

(ii) Reverse transcription

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

(iii) Quantitative real–time PCR

Primers were designed using latest version of Primer3 Input software. The primers for target gene (p53) were AGAGGAAGAG-AATCTCCGCA (F) and GTTTCTTCTT-TGGCTGGGGA (R). GAPDH was used as the reference gene and the primer sequences for GAPDH were GAAGGTG-AAGGTCGGAGTC (F) and GAAGAT-GGTGATGGGATTT (R). We performed real-time PCR employing SYBR Green SuperMix in CFX-96 real-time PCR System (Bio-Rad). Thermal cycling conditions were 95 °C for 3 min followed by 40 cycles
consisting of 10 sec of denaturation at 95 °C and 10 sec of annealing (at 56 °C in case of GAPDH & 59 °C in case of p53 genes) and extension at 72 °C. Melt peak analysis of amplicons was carried to rule out non-specific amplifications.

**Platelet clearance analysis**

NHS-biotin (600 mg) was injected in 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 retro-orbital blood was drawn from each mouse under control as well as treated groups, mixed with 200 µl buffered saline glucose-citrate buffer (116 mM NaCl, 13.6 mM trisodium citrate, 8.6 mM Na$_2$HPO$_4$, 1.6 mM KH$_2$PO$_4$, 0.9 mM EDTA, 11.1 mM glucose, pH 7.3), followed by 1 ml 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$_2$PO$_4$, 0.8 mM K$_2$HPO$_4$, 3 % bovine calf serum). Cells were pelleted at 1400 x g for 10 min and resuspended in 300 µl sheath fluid. They were stained with FITC-conjugated rat anti-CD41, which label only platelets, followed by PE-streptavidin for 1 h on ice, washed in balanced salt solution and analysed by flow cytometry to determine the fraction of platelet population labeled with PE (14).

**Labeling of reticulated platelets**

Mice were injected intravenously with either DMSO or sirtinol (15 mg/kg) or AGK2 (15 mg/kg) or EX-527 (20 mg/kg). Blood was collected from 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 blood with 50 µl thiazole orange (0.1 mg/ml in PBS) and 1 µl PE-conjugated CD41 antibody for 15 min at RT in dark, followed by fixation with 1 ml 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. Platelet count was carried out using a Cell Counter (Beckman Coulter model Multisizer 4) at different time points (0, 12, 36, 60, and 84 h). After 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 (t test) were used for evaluation and tests were considered significant at $P < 0.05$ (2-tailed tests). All statistical tests were carried out using Sigma Plot, version 11 statistics software. Data are presented as means ± SD of at least three individual experiments from different blood donors.

**RESULTS**

**Human platelets express Sirt1, Sirt2 and Sirt3**

As sirtinol is known to inhibit multiple sirtuin isoforms, we checked for the presence of Sirt1, Sirt2 and Sirt3 in human platelets by Western analysis. An earlier study has already reported expression of Sirt1 in human platelets (22). As presented in Fig.1, all the 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 anti-tumor activity and to induce apoptosis in several cancer cell lines (7-9). In order to examine the effect of sirtuin inhibitors on platelets, we studied mitochondrial transmembrane potential ($\Delta \Psi_m$), 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.2A). CCCP-treated platelets were employed as positive control. Similar results were observed when we substituted JC-1 with TMRM, a pharmacologically distinct mitochondrial membrane potential probe (data not shown).
Phosphatidylserine (PS) redistribution from the inner to the outer leaflet of surface membrane is an early and widespread event during apoptosis (23). Annexin V-FITC has a strong, Ca\(^{2+}\)-dependent affinity for PS and therefore is used as a probe for detection of apoptosis. Pretreatment of platelets with increasing concentrations of either sirtinol (50 and 100 µM) or EX-527 (10 and 50 µM) respectively resulted in 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) as compared to the untreated (control) cells, indicative of induction of apoptosis-like events upon inhibition of sirtuin (Fig. 2B). Thrombin–treated platelets were employed as positive control.

Reactive oxygen species (ROS) play an important role in the initiation and execution of apoptosis (24). We determined the effect of sirtuin inhibition on level of cytosolic ROS. The dye H\(_2\)DCF-DA was used for measurement of ROS, which was oxidized to DCF by reactive oxygen species. The results revealed a dose-dependent increase in ROS in platelets treated with either sirtinol or EX-527 (Fig. 2C). Hydrogen peroxide-treated platelets were employed as 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 reticulo-endothelial system through process of phagocytosis that eventually leads to deletion of senescent platelets (25). We evaluated macrophage-assisted clearance of platelets following Sirt1/Sirt2 inhibition. Calcein-stained platelets, either 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 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-pretreated platelets than those with untreated cells (Fig. 3A). 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. 3B).

Apoptosis-like changes induced by sirtuin inhibitors are mediated through activation of pro-apoptotic 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 expression of active Bax in human platelets using an antibody specific for conformationally active form of the protein (clone 6A7). As expected, 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. 4A), suggestive of critical role of Bax in sirtuin-mediated changes in human platelets.

Acetyl-p53 is known to regulate transcription-independent pathway of apoptosis (27). The best characterized pro-apoptotic function of p53 involves its translocation to mitochondria 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 thus promotes its stability by protecting from proteasomal degradation (27). In order to understand molecular underpinnings of sirtinol- or EX-527-induced changes, we studied 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. 4B), thus implicating p53 and Bax in sirtuin inhibition-induced apoptosis-like
signaling in platelets. Since total p53 level was unchanged in presence of inhibitors, we checked for the presence of p53 mRNA in platelets by real-time qPCR. The critical quantity (Cq) 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 significant presence of p53 mRNA in platelets that could replenish protein loss due to proteasomal cleavage. Melt peak analysis was performed to rule out non-specific amplification. Amplicons of GAPDH and p53 exhibited single sharp peaks at 83.5 °C and 85 °C, respectively, suggestive of lack of non-specific amplification. To the best of our knowledge this is the first report of expression of p53 mRNA in human platelets.

Bax and other pro-apoptotic proteins induce release of mitochondrial cytochrome c into cytosol, which eventually leads to caspase-3 activation (28). However, a growing weight of evidences has suggested that not all forms of programmed cell death are caspase-mediated. Platelets specifically are known undergo caspase-independent cell death associated with activation of calpains (15, 20, 29-31). In order 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 as compared to control cells (Fig. 4E), consistent with 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 significantly higher proteolytic activity of calpain (by 21.5 ± 5 % and 49 ± 5 %, respectively) than their vehicle-treated counterparts (Fig. 4F), thus implicating calpain in sirtinol-mediated platelet cell death. Similar results were observed in EX-527-treated platelets.

Sirtuin inhibition leads to reduction in 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. Since sirtuin inhibition transforms platelets to apoptotic phenotype, we investigated the impact of sirtinol on platelet count and life span under in vivo condition. Reticulated platelets are RNA-containing younger cell population, which are stained with thiazole orange (12). Administration of either sirtinol (15 mg/kg) or EX-527 (20 mg/kg) into mice resulted in decrease in the number of reticulated platelets (by 36.5 % and 40.5 %, respectively, after 36 h) (Fig. 5A) as well as significant reduction in platelet count (by 59.94 % in 12 h in sirtinol-administered mice and by 44 % in 36 h in EX-527-administered mice) (Fig. 5B). After 36 h of sirtinol or EX-527 administration, number of reticulated platelets eventually normalized (Fig. 5A). However, our experiments do not rule out platelet damage as a cause of sirtinol-induced thrombocytopenia in mice. 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, when compared with the in vitro situation using human platelets, whereas same serum concentration was sufficient to suppress platelet production and cause thrombocytopenia. Secondly, possibility of impaired clearance of mice platelets in presence of sirtinol cannot be ruled out. Similar result was observed in AGK-2 (15 mg/kg) administered mice (data not shown).

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

Acetylation of proteins is a post-translational 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 which transfer 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 NAD$^+$-dependent acetyl-lysine deacetylase that belongs to the HDAC-III family and is expressed in prokaryotes as well as eukaryotes. Mammalian sirtuins are linked to healthy ageing, and thus 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, relevance of sirtuin-mediated signalling remains obscure in these cells. Here we, for the first time, report 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 drop in mitochondrial transmembrane potential ($\Delta$$\Psi$$_m$), enhanced surface exposure of PS and rise in cytosolic ROS. Calpain but not caspase was 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). As platelet apoptosis is known to be mediated through pro-apoptotic members of Bcl-2 (14-15), we evaluated the effect of sirtuin inhibition on activation of Bax. Either sirtinol- or EX-527-pretreated platelets were found to express significantly higher level of conformationally active Bax than the control (untreated) counterparts. As 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 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 from flow cytometry and epifluorescence microscopy studies. Administration of mice with either sirtinol or EX-527 led to thrombocytopenia as well as decrease in number of reticulated platelets, though sirtinol had no significant effect on platelet life span in mice. Drop in number of reticulated platelets could be attributed to decreased production of platelets as was 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 anti-apoptotic members of Bcl-2 family (14-15). Proteasomal peptidase activity promotes platelet survival through constitutive elimination of the conformationally active Bax. Acetyl-p53 is known to play determining role in transcription-independent pathway of Bax-mediated apoptosis (27). Acetyl-p53 is a substrate of sirtuin and an upstream positive regulator of Bax (36-37). Based on evidences 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 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 severity of thrombosis or thrombocytosis. It has been recently reported that platelets play critical role in cancer cell proliferation and thus sirtuin inhibition can indirectly contribute to anticancer effect by restricting platelet count (38). Since sirtuin inhibitors are being actively evaluated for their anti-tumor activity, (7-9) our observations also call for careful consideration of their potential adverse effect on platelets while exploiting the benefits of sirtuin inhibition in cancer therapeutics.
ACKNOWLEDGEMENT

This research was supported by grants received by D. Dash from the Department of Biotechnology (DBT) and the Department of Science and Technology (DST), Government of India, DST-FIST Programme, the Indian Council of Medical Research (ICMR) and the Council of Scientific & Industrial Research (CSIR). D. Dash thankfully acknowledges Tata Innovation Fellowship grant received from the DBT. S. Kumari is a recipient of research fellowship from CSIR.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTION

S.K., S.N.C., M.K.N. and R.L.M. performed different experiments. D.D. and S.K designed the research, analyzed data and wrote manuscript. D.D supervised the entire study.

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FIGURE LEGENDS

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.

Figure 2. Study of apoptosis-like features in platelets treated either with sirtinol or EX-527. Mitochondrial transmembrane potential (FL2/FL1 ratio) (A), PS exposure (FITC-annexin V binding) (B), and ROS generation (C) were studied in control platelets (RP), as well as in cells pre-treated either with sirtinol or EX-527 as indicated. Bar diagram represents mean±SD of five different experiments. (*p < 0.05 as compared to DMSO-pretreated resting platelets).

Figure 3. Phagocytic uptake of platelets by autologous macrophages.

Flow cytometry (A) and epifluorescence microscopy (B) of macrophages co-incubated with calcein-labeled platelets pretreated either with sirtinol (100 µM) or DMSO (control). Panels 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 (upper panel) and β-actin (loading control) (lower panel) in platelets pretreated with either DMSO (RP), sirtinol, EX-527 or ABT-737. (B) Western blots representing expression of acetyl-p53 (upper panel) and p53 (loading control) (lower panel) in platelets pretreated with either DMSO (RP), 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 fluorogenic substrate, AC-DEVD-AMC, in platelets pretreated with DMSO (RP), sirtinol, EX-527 or ABT-737. (F) Calpain activity determined from the extent of cleavage of fluorogenic substrate, t-butoxycarbonyl-Leu-Metchloromethylcoumarin, in platelets pretreated with DMSO, sirtinol, EX-527 or A23187. Bar diagram represents mean±SD of five different experiments. (*p < 0.05 as compared to DMSO-pretreated resting platelets). (ns; non-significant)

Figure 5. Sirt1 inhibition leads to reduction in reticulated platelets and platelet count.

(A) Relative number of reticulated platelets in peripheral blood sample drawn from either DMSO (vehicle) or sirtinol (15 mg/kg) or EX-527 (20 mg/kg) -pretreated mice 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 peripheral blood sample drawn from either DMSO (vehicle) or sirtinol (15 mg/kg)- pretreated mice at intervals of 0, 12, 36, 60, and 84 h. (Data represents mean±SD of five different experiments. (*p < 0.05 as compared to DMSO-pretreated resting platelets).

Figure 6. Proposed role of sirtuin in determination of platelet lifespan.

Sirtuins inhibitor enhances acetylation of p53, which eventually leads to activation of pro-apoptotic Bax and apoptosis-like signaling in platelets. In absence of acetylation p53 is ubiquitinated and degraded by 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). Thus proteasome and sirtuin both regulate platelet apoptosis and survival.
Figures:

Figure 1

Individual 1  Individual 2

Sirt1

Sirt2

Sirt3
Figure 2
Figure 3
Figure 4
Figure 6
