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Concurrent acetylation of FoxO1/3a and p53 due to sirtuins inhibition elicit Bim/PUMA mediated mitochondrial dysfunction and apoptosis in berberine-treated HepG2 cells

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ABSTRACT

Post-translational modifications i.e. phosphorylation and acetylation are pivotal requirements for proper functioning of eukaryotic proteins. The current study aimed to decode the impact of acetylation/deacetylation of non-histone targets i.e. FoxO1/3a and p53 of sirtuins (NAD^+ dependent enzymes with lysine deacetylase activity) in berberine treated human hepatoma cells. Berberine (100 μM) inhibited sirtuins significantly ($P < 0.05$) at transcriptional level as well as at translational level. Combination of nicotinamide (sirtuin inhibitor) with berberine potentiated sirtuins inhibition and increased the expression of FoxO1/3a and phosphorylation of p53 tumor suppressor protein. As sirtuins deacetylate non-histone targets including FoxO1/3a and p53, berberine increased the acetylation load of FoxO1/3a and p53 proteins. Acetylated FoxO and p53 proteins transcriptionally activate BH3-only proteins Bim and PUMA (3.89 and 3.87 fold respectively, $P<0.001$), which are known as direct activator of pro-apoptotic Bcl-2 family protein Bax that culminated into mitochondria mediated activation of apoptotic cascade. Bim/PUMA knock-down showed no changes in sirtuins' expression while cytotoxicity induced by berberine and nicotinamide was curtailed up to 28.3% ($P < 0.001$) and it restored pro/anti apoptotic protein ratio in HepG2 cells. Sirtuins inhibition was accompanied by decline in NAD^+/NADH ratio, ATP generation, enhanced ROS production and decreased mitochondrial membrane potential. TEM analysis confirmed mitochondrial deterioration and cell damage. SRT-1720 (1–10 μM), a SIRT-1 activator, when pre-treated with berberine (25 μM), reversed sirtuins expression comparable to control and significantly restored the cell viability ($P < 0.05$). Thus, our findings suggest that berberine mediated sirtuins inhibition resulting into FoxO1/3a and p53 acetylation followed by BH3-only protein Bim/PUMA activation may in part be responsible for mitochondria-mediated apoptosis.
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1. Introduction

The sirtuins (Silent Information regulator 2, SIR2) are members of evolutionarily well conserved proteins family, present in organisms extending from prokaryotes e.g. bacteria to eukaryotes e.g. plants & animals (Raynes et al., 2013). High degree of conservation among sirtuins from unicellular prokaryotic cells to complex, multicellular eukaryotic cells outline its fundamental importance (Milner, 2009). Earliest studies in budding yeast (*Saccharomyces cerevisiae*) suggested that overexpression of SIR2 gene enhanced longevity by 50% while its downregulation reduced lifespan of organism. Further studies in higher organisms e.g. worms and flies also demonstrated that SIR2 orthologues are actively involved in regulation of life span (Donmez and Outeiro, 2013). In mammals, SIR2 family members belong to NAD^+-dependent class III histone deacetylases which catalyze the removal of acetyl moiety from histones and various non-histone substrates resulting into more compact packaging of genetic material and subsequent transcriptional blockade (Raynes et al., 2013). Mammals have 7 sirtuin family members, SIRT-1

Abbreviations: BBR, berberine; NIC, nicotinamide; HepG2, human hepatocellular carcinoma cells; HCC, hepatocellular carcinoma; SIR2, Silent Information regulator 2; FoxO, forkhead-box that belong to the other (O) subfamily; FoxO1, forkhead-box O 1 transcription factor; FoxO3a, forkhead-box O 3a transcription factor; Bim, BH3-only member of Bcl-2 family; FBS, fetal bovine serum; ROS, reactive oxygen species; NAD^+, nicotinamide adenine dinucleotide; PI3K, phosphatidylinositol-3-kinase; PUMA, p53 upregulated modulator of apoptosis; $_P27^{kip1}$, cyclin dependent kinase inhibitor 1b or CDKN1B; GADD45a, growth arrest and DNA-damage inducible protein alpha; ERK, extracellular signal regulated kinase; PARP, Poly (ADP-ribose) polymerase; EDTA, ethylene-diamine tetraacetic acid; BSA, bovine serum albumin; PGC1α, peroxisome proliferator-activated receptor-γ coactivator 1α; TCA cycle, Tri-carboxylic acid cycle (Krebs cycle); MnSOD, Manganese Superoxide dismutase; NDUF9, NADH dehydrogenase (Ubiquinone) 1 Alpha Subcomplex, 9; ATP, adenosine tri-phosphate; ART, ADP-ribosyltransferase; p300, E1A binding protein p300; NAMPT, Nicotinamide phosphoribosyltransferase/visfatin; PI, propidium iodide.

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to SIRT-7; which are characterized by highly conserved NAD^+-binding domain and common catalytic region. Sirtuin members differ in their N- and C-terminal region, sub-cellular localization and in utilizing different substrates and protein binding partners. SIRT-1, 6 and 7 are predominantly nuclear, SIRT-2 is cytoplasmic but occasionally found in nucleus and SIRT-3, 4 & 5 are mainly mitochondrial proteins. In contrast to their weak deacetylase activity, SIRT-4 & SIRT-6 possess unique ADP-ribosyltransferase activity while SIRT-5 appears to be effective demalonylase and desuccinylase (Stünkel and Campbell, 2011; Donmez and Outeiro, 2013; Yuan et al., 2013).

SIRT-1, being the closest homolog among all mammalian sirtuins to yeast SIR2, is most studied and well characterized member of the sirtuin family. SIRT-1 deacetylates histones H1, H3 and H4 proteins. Various non-histone proteins including Forkhead box O (FoxO) family proteins, peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α), p53 and p300 have been reported as SIRT-1 down targets. Deacetylating potential of SIRT-1 to these substrates seems to be crucial for cell survival, metabolic demands, genetic stability and autophagy, all of which is somehow related with longevity, a function allotted initially to SIR2 in yeast (Yi and Luo, 2010; Bruzzone et al., 2013; Langley and Sauve, 2013). Among all SIRTs; SIRT-3, SIRT-4 and SIRT-5 are mitochondrial proteins having mitochondrial-targeting sequences. Of these, only SIRT-3 shows potent deacetylase activity. In addition, SIRT-3-deficient animals show considerable increase in acetylation of mitochondrial proteins, while no significant changes are observed in protein acetylation of SIRT-4 −/− and SIRT-5 −/− mitochondria (Chen et al., 2013). SIRT-3 has been shown to deacetylate different components of TCA cycle, oxidative phosphorylation (NDUF9 and ATP synthase), fatty acid oxidation (long-chain acyl CoA dehydrogenase, LCAD), and reactive oxygen species (ROS) homeostasis [Manganese Superoxide dismutase (MnSOD) and isocitrate dehydrogenase 2]. Through these functions, SIRT-3 is suggested to maintain energy demands and suppresses ROS generation and their consequences; thus preparing cell to combat oxidative stress and promote cell survival (Cha and Kim, 2013). SIRT-3 may be considered as mitochondria localized tumor suppressor due to its ability to inhibit mitochondrial ROS generation as ROS are involved in both the initiation and promotion of tumorigenesis (Kakkar and Singh, 2007).

There are reports that SIRT-1 is upregulated in hepatocellular carcinoma, acute myeloid leukemia (AML), prostate cancer and non-melanoma skin cancer, while increased expression of SIRT-3 was found in breast cancer (Deng, 2009). Exogenously expressed SIRT-1 provides chemoresistance to doxorubicin (anticancer drug) in various cancer cell lines, whereas SIRT-1 knockdown partially reverses the drug resistant phenotype (Chen et al., 2011; Barneda-Zahonero and Parra, 2012). SIRT-1 inhibits pro-apoptotic effects of many tumor suppressor genes including FoxOs (Pramanik et al., 2014) and p53 (Kloster et al., 2013) by its deacetylase action, thus promoting cell survival. Recent study provided a clue that SIRT-1 coprecipitates with p53 and deacetylate C-terminal lysines residue, affecting pro-apotoic function of p53 (Lee and Gu, 2013). In addition, FoxOs have also been reported to be deacetylated resulting into loss of their apoptotic function (Frazzi et al., 2013). These molecular mechanisms have been verified in many cultures and in-vivo systems. For instance, SIRT-1-deficient thymocytes showed aggravated apoptosis after irradiation, suggesting that p53 is activated through acetylation (Lee and Gu, 2013). Acetylated form of FoxOs and p53 transcriptionally activate BH3-only proteins Bim and PUMA respectively, which are considered potent executioners of mitochondria mediated cell death (Hoffmann et al., 2014; Pramanik et al., 2014; Shi et al., 2015; Wang et al., 2015).

Berberine, a quaternary isoquinoline alkaloid, is derived from the root and stem bark of numerous clinically important medicinal plants of *Berberidaceae* family (Guamán Ortiz et al., 2014; Kaboli et al., 2014). Berberine has been reported to demonstrate significant antimicrobial, antifungal and antiviral activity (Basha et al., 2002; Wu et al., 2011; Kong et al., 2012). It has also been reported to have a multitude of biological effects including anti-diarrheal, (Bandyopadhyay et al., 2013) anti-malarial, (Le Tran et al., 2003) anti-hypertensive, (Lau et al., 2001) anti-arrhythmic, (Chen et al., 2014) anti-hyperglycemic, (Singh and Kakkar, 2009; Kapoor et al., 2014) antitumor, (Fu et al., 2013; Zhu et al., 2014) anti-inflammatory, (Mo et al., 2014), anti-oxidative, (Li et al., 2014) and cerebro-protective activities (Pires et al., 2014). In this study, we report that berberine inhibits sirtuins mRNA as well as protein expression. Owing to its inhibitory effect over SIRT-1, non-histone targets viz. p53 and FoxOs proteins get hyperacetylated resulting into activation of pro-apoptotic genes and genes responsible for cell cycle arrest, while SIRT-3 inhibition leads to excessive ROS generation, altered ATP production, energy homeostasis, and mitochondrial abnormality culminating into death of human hepatoma cells. Sirtuins inhibition followed by hyperacetylated FoxO's and p53 mediated BH3-only protein Bim/PUMA dependent cell death provide a glimpse of signaling in inhibition of uncontrolled cell proliferation and sensitization of cancer cells.

2. Materials and methods

2.1. Chemicals

3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT), Hoechst 33258, 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), dihydroethidium (DHE), propidium iodide (PI) and RNase A were purchased from Sigma-Aldrich (St. Louis, MO, USA). SRT-1720 was procured from BioVision, USA. Antibodies specific for Bcl-2, Bax, β-actin, p53, phosphorylated p53, SOD2 and horseradish peroxidase conjugated anti-goat and anti-mouse antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody for SIRT-1, SIRT-2, SIRT-3, FoxO1, FoxO3a, Bim, PUMA, Apaf-1, cleaved poly [ADP-ribose] polymerase (cleaved PARP), acetylated lysine and peroxidase labeled anti-rabbit immunoglobulin, Alexafluor-555 conjugated anti-rabbit immunoglobulin were purchased from Cell signaling Technology (CST, Beverly, MA, USA). All other chemicals and reagents were of analytical grade and were obtained from Sigma-Aldrich until specifically indicated.

2.2. Cell line and culture conditions

Human hepatoma cells, HepG2 was procured from American type culture collection (ATCC; Manassas, VA, USA) and maintained in Minimum Essential Medium Eagle (Sigma) supplemented with 10% heat inactivated fetal bovine serum, (Gibco Life Technologies Ltd., Grand Island, NY, USA) 1% *v/v* antibiotic — antimycotic, (Gibco) 1 mM sodium pyruvate and 2 mM L-glutamine under standard conditions of culture at 37 °C in humidified atmosphere of 5% CO_2 and 95% air (ThermoForma, model no. 371). All experiments were carried out on cells having viability more than 95%. After seeding, cells were incubated overnight to adhere and then treated with berberine, nicotinamide, SRT-1720 and combinations at different concentrations and time intervals. Berberine chloride and nicotinamide were obtained from Sigma-Aldrich and stock solutions were made in Milli Q ultrapure water.

2.3. Tetrazolium dye reduction assay

Cell viability of vehicle and treated hepatoma cells was assessed by tetrazolium dye MTT reduction assay as described by Mosmann (1983). Briefly, cells were plated in 96-well plates (1×10^4 cells/well) and treated with a range of nicotinamide concentrations (0 to 50 mM), SRT-1720 concentrations (1–10 μM) or in combination with berberine for different time periods (24 & 48 h). After indicated incubation time, media was replenished; MTT (0.5 mg/mL) was added and incubated for 4 h. Thereafter, media was aspirated and 200 μL of DMSO was added to dissolve the formazan crystals and plate was read at 570 nm using a Spectramax PLUS 384 microplate reader (SoftMaxPro

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