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Inhibition of SIRT1 by a small molecule induces apoptosis in breast cancer cells

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ABSTRACT

Overexpression of SIRT1, a NAD^+-dependent class III histone deacetylases (HDACs), is implicated in many cancers and therefore could become a promising antitumor target. Here we demonstrate a small molecule SIRT1 inhibitor, ILS-JGB-1741(JGB1741) with potent inhibitory effects on the proliferation of human metastatic breast cancer cells, MDA-MB 231. The molecule has been designed using medicinal chemistry approach based on known SIRT1 inhibitor, sirtinol. The molecule showed a significant inhibition of SIRT1 activity compared to sirtinol. Studies on the antitumor effects of JGB on three different cancer cell lines, K562, HepG2 and MDA-MB 231 showed an IC_{50} of 1, 10 and 0.5 μM, respectively. Further studies on MDA-MB 231 cells showed a dose-dependent increase in K9 and K382 acetylation of H3 and p53, respectively. Results also demonstrated that JGB1741-induced apoptosis is associated with increase in cytochrome *c* release, modulation in Bax/Bcl2 ratio and cleavage of PARP. Flowcytometric analysis showed increased percentage of apoptotic cells, decrease in mitochondrial membrane potential and increase in multicaspase activation. In conclusion, the present study indicates the potent apoptotic effects of JGB1741 in MDA-MB 231 cells.

1. Introduction

Sirtuins are NAD^+-dependent class III histone deacetylases and are conserved from bacteria to eukaryotes [1,2]. The mammalian sirtuin family consists of seven members, SIRT1-7, characterized by a conserved 275 amino acid catalytic core and unique additional N-terminal and/or C-terminal sequences of variable length [2]. Among the seven human sirtuins, SIRT1 is the most well studied and is known to have more than dozen substrates including Ku70, p53, NF-κB, forkhead (FOXO) transcription factors, etc. to affect stress and DNA damage [3–8].

SIRT1 is indicated in the control of aging and longevity. It has also been involved in regulating lipid and glucose homeostasis by activating genes involved in mitochondrial biogenesis, fatty acid oxidation and respiration [9–11]. Role of SIRT1 in cancer biology was first implicated when p53 was identified as a direct substrate [3] and acetylation of Lys382 within p53 has been identified as a direct target of SIRT1. It has been reported that members of forkhead transcription factor family that are regulated by SIRT1 lead to increased resistance to stress and apoptosis causing cancer cell survival [12,13]. Findings from various studies suggest that SIRT1 modulators (inhibitors or activators) might become good therapeutic indications for cancer, inflammation and metabolic disorders like diabetes, obesity, etc. [14,15]. Many small molecule inhibitors of sirtuins, such as sirtinol, suramin, splitomicin and splitomicin analogs, have been identified through phenotypic screening in yeast [16,17].

In the present study, a novel small molecule (JGB1741) has been designed based on a known inhibitor of SIRT1, sirtinol, using bioinformatic tools. The molecule has been synthesized by medicinal chemistry approach and has been evaluated using biochemical enzymatic assay and cell-based assays. The results clearly demonstrated that JGB1741 inhibited SIRT1 in biochemical enzymatic assay, inhibited proliferation of cancer cells and induced apoptosis of human breast cancer cells, MDA-MB 231.

2. Materials and methods

2.1. Chemicals

Cyclohexanone, sulfur and NaOH were purchased from Lobachemie, India. Ethylcyanoacetate, morpholine, benzylamine were from Spectrochem, India. Phosphate-buffered saline (PBS), RPMI medium and fetal bovine serum (FBS) were purchased from Gibco

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BRL (CA, USA). Ethanol, 2-hydroxy1-naphthaldehyde, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) and propidium iodide, were from Sigma–Aldrich (Bangalore, India). *p*-Toluenesulfonic acid was from Merck. Silica gel (100–200 mesh) used for purification from SRL, India. Nitrocellulose membrane was from HyBond (Bangalore, India). Mouse monoclonal antibody against cytochrome *c* was from Chemicon (CA, USA). Monoclonal antibodies of PARP, Bax, Bcl2, H3, H3K9, p53 and Ac-p53 were from Upstate (Charlottesville, VA, USA). All the other chemicals and reagents (molecular biology grade) were procured locally.

2.2. Design and synthesis of JGB1741

The three dimensional catalytic domain of the SIRT1 (Uniprot code: Q96EB6; 244-498 amino acid residues) was developed by comparative modeling using three template structures: SIRT2 (PDB 1J8F, chain A), Hst2 (PDB 1Q1A, chain A) and Sir2-Af1 (PDB 1M2G, chain A) as described earlier [18]. The protein structure was build using PRIME homology modeling program (Schrodinger L.L.C., USA). All energy minimizations were also performed by the same program. Docking studies were done using AutoDock 4.0 [19,20] and also GLIDE (Schrodinger L.L.C., USA) programs. JGB1741 was synthesized by medicinal chemistry approach in four steps (Supplementary data).

2.3. Recombinant SIRT1 purification and activity assay

Full length SIRT1 clone was a generous gift from Dr. Ronen Marmorstein, Wistar Institute, Philadelphia, USA. SIRT1 expression was accomplished in BL21 *(DE3) PLysS cells with induction at an A_{600} of 0.6 with a final concentration of IPTG at 1 mM for 16–20 h at 15 °C. SIRT1 was purified using a Ni^{+2}–NTA chelating column followed by anion exchange chromatography as previously described [21]. Typically 3–5 mg of ~70% purified SIRT1 was obtained per liter culture. The SIRT1 activity assay was done using recombinant purified SIRT1 and p53K382 fluorophore-labeled substrate using BioMol fluorimetric activity assay kit from Biomol Inc., USA according to the manufacturer's protocol. SIRT3 activity assay was done using SIRT3 activity assay kit from Biomol Inc., USA according to manufacturer's protocol. SIRT2 recombinant enzyme was a generous gift from Aptuit Laurus Pvt. Ltd., India and p53K320 fluorophore-labeled substrate was from Biomol Inc., USA.

2.4. Cell lines, culture conditions and treatment

Human metastatic breast cancer cells, MDA-MB 231, human chronic myeloid leukemia cells, K562, human hepatocellular carcinoma cells, HepG2, and human embryonic kidney cells, HEK293 cells were procured from National Center for Cell Sciences, Pune, India. All cells were grown in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were subcultured twice each week, seeding at a density of about 2×10^3 cells/ml. Cell viability was determined by the trypan blue dye exclusion method. IC_{50} was calculated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [22]. Morphological changes of cells treated with different concentrations of JGB1741 were observed by phase-contrast inverted microscope. Flowcytometric analysis was done using Guava Personal Cell Analysis-96 (PCA-96) System apparatus (Guava Technologies, San Francisco, USA). To calculate the percentage of apoptotic cells, the Multiple caspase activity (Guava® MultiCaspase Assay), changes in mitochondrial membrane potential (Guava® Mitochondrial Depolarization Assay) and cell cycle analysis (Guava Cell Cycle Assay) were carried out according to manufacturer's protocol.

2.5. Immunoblot analysis

For immunoblot analysis, 5×10^6 cells were treated with JGB1741 at different concentrations (0, 10, 100, 500 and 1000 nM). After 24 h of treatment, cells were harvested and lysed in RIPA lysis buffer (Sigma–Aldrich) containing 1× protease inhibitor cocktail (GE Amersham,). After 30 min of shaking at 4 °C, the mixtures were centrifuged (10,000*g*) for 10 min, and the supernatants were used as the whole-cell extracts. The protein content was determined according to the Bradford method [23]. Protein samples were separated by 10–15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) along with protein molecular weight standards and transferred to nitrocellulose membrane. Membranes were stained with 0.5% Ponceau *S* in 1% acetic acid to check the transfer. The membranes were blocked with 5% (w/v) non-fat dry milk and then probed with a relevant antibody (Bax 1:2000 dilution, Bcl2 1:2000 dilution, PARP 1:1000 dilution, Ac-p53K382 1:1000 dilution, p53 1:3000 dilution and PCNA 1:1000 dilution) followed by chemiluminescence detection using peroxidase-conjugated secondary antibodies. Equal protein loading was detected by probing the membrane with β-actin or H3 antibodies.

Release of cytochrome *c* from mitochondria to cytosol was measured by Western blot as previously described [24] using cytochrome *c* antibody (1:1000 dilution).

2.6. Acid extraction of histones

Histones were isolated from cells as previously described [25]. Of histones (20 μg) were separated on 15% SDS gel, transferred onto nitrocellulose membrane, blocked using 5% non-fat milk solution and probed with Acetylated H3K9 (1:1000 dilution) or H3 (1:3000 dilution) primary antibodies. The acetylation levels were detected by chemiluminescence.

2.7. Statistical analysis

Data were reported as the mean ± S.D. of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered as significant.

3. Results and discussion

3.1. Design and synthesis of JGB1741

Many small molecule inhibitors of SIRT1 have been identified and one of the molecules with strongest antitumor activity known is sirtinol. In this study we have designed and developed a small molecule inhibitor of SIRT1, JGB1741, based on sirtinol structure. The classical bioisosteric equivalence between benzene and thiophene [26] prompted us to replace the benzene of 3-amino benzamide of sirtinol with thiophene which might give a molecule with better or similar activity to sirtinol. Since the crystal structure of SIRT1 is not available, the best model of the catalytic core of SIRT1 was developed and the fitness of the model was checked by PROCHECK program [27] (Fig. 1A). This model has 82.7% residues in most favored regions and ProSA-Web Z score of −6.37 similar to the template structure scores. A series of thiophene derivatives of sirtinol were docked with modeled SIRT1 catalytic domain. Docking studies using Auto Dock program, showed that E-N-benzyl-2-[(E)-[(2-hydroxynaphthalen-1-yl) methylidene] amino]-4,5,6,7-tetrahydro-1-benzothiophene-3-carboxamide (JGB1741) has given a better binding score (−7.53 kCal/mol) compared to sirtinol (−6.9 kCal/mol). Moreover, the interacting amino acid residues were also almost similar in both the molecules

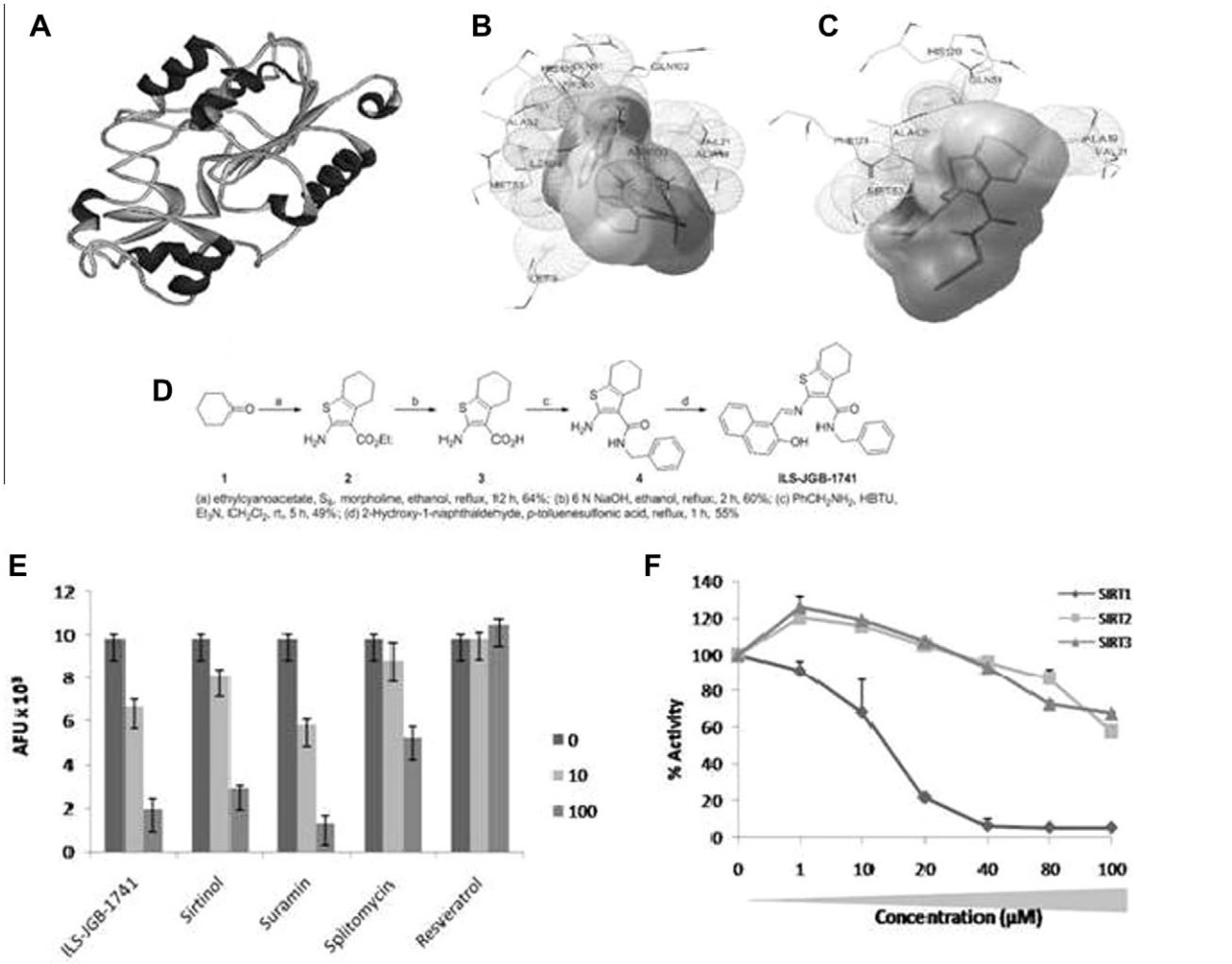

Fig. 1. Design and synthesis of JGB1741. (A) Model of the SIRT1 protein catalytic core obtained using PRIME. (B) Interacting amino acid residues of sirtinol and SIRT1 catalytic core. (C) Interacting amino acid residues of JGB1741 and SIRT1 catalytic core. (d) Schematic representation of synthesis of JGB1741. (E) A representative graph of three independent experiments with each point taken in triplicates showing the effect of JGB1741on SIRT1 activity with known inhibitors of SIRT1 (Suramin, splitomycin and sirtinol) using Biomol kit. (F) The graph shows the percent activity of the enzymes SIRT1, SIRT2 and SIRT3 in the presence of different concentrations of JGB1741as determined using Biomol fluorimetric activity assay kits. The error-bar represents the SD calculated with the triplicates.

(Fig. 1B and C). Therefore we synthesized the molecule, JGB1741, using medicinal chemistry approach.

Synthesis of the inhibitor JGB1741 was carried out from cyclohexanone in four steps (Fig. 1D). In the first step, multi-component Gewald reaction [28] of cyclohexanone (1) was carried out in presence of ethylcyanoacetate and sulfur that resulted in thiophene ester (2). The compound (2) on base-catalyzed hydrolysis produced thiophene acid (3) that was subsequently converted to amide (4) by reacting with benzylamine in presence of HBTU [29]. Finally, the compound (4) on treatment with 2-hydroxy-1-naphthaldehyde in presence of the catalyst, *p*-toluenesulfonic acid, furnished the inhibitor molecule JGB1741 [30]. The structure of JGB1741 was confirmed by spectroscopic data and purity of the compound (99.47%) was confirmed by HPLC (Column: Symmetry Sheild C-18, 250 × 4.6 mm, 5 μm, Mobile Phase: H2O/CH3CN (20:80), flow rate: 1.0 ml/min) prior to biological assay.

3.2. JGB1741 inhibited SIRT1 activity

The activity of the synthesized small molecule, JGB1741, was determined in a cell-free biochemical assay. The small molecule was incubated with the purified SIRT1 in presence of a fluorophore-labeled acetylated p53 peptide substrate and the deacetylase activity was determined by measuring the fluorescence at 350–460 nm. The results clearly demonstrated that JGB1741 inhibited SIRT1 activity maximally at 100 μM concentration (95%). The calculated IC_{50} of JGB1741 was ~15 μM. JGB1741 showed better inhibition of SIRT1 compared to sirtinol and splitomycin at 10 μM concentrations (Fig. 1E). Furthermore, the molecule was a weak inhibitor of SIRT2 and SIRT3 with an IC_{50} > 100 μM concentration (Fig. 1F). However the molecule showed a lower potency when compared to suramin, a known SIRT1 inhibitor [31]. Suramin was used as a positive control in further experiments.

3.3. JGB1741 specifically inhibited MDA-MB 231 cell proliferation

Several SIRT1 inhibitors were shown to induce apoptosis in various cancer cells [32–35]. The cell-free biochemical assay demonstrated JGB1741 to be a potent inhibitor of SIRT1 and so its anticancer properties were evaluated on three different cancer cell lines, K562, HepG2 and MDA-MB 231 and on non-tumorigenic cell line, HEK293, as a control. The preliminary MTT assay showed that

JGB1741 inhibited MDA-MB 231 cell proliferation more potently with an IC_{50} of 0.5 μM than K562 and HepG2 cell proliferation (IC_{50} > 1 μM) (Fig. 2A).

Further experiments were therefore carried out on MDA-MB 231 cells to explore the potency of JGB1741 in inhibiting cancer cell growth. The IC_{50} of the molecule in MDA-MB 231 cells was determined to be 512 nM after 24 h (Fig. 2B). Although, JGB1741 showed lower potency than suramin in biochemical enzymatic assay, in cell-based assay JGB1741 showed much higher potency with lower IC_{50} of 512 nM compared to suramin with IC_{50} of 7 μM [36]. Moreover, the molecule did not show any cytotoxicity on HEK293 cells even after 48 h incubation, demonstrating its specificity towards cancer cells (Fig. 2C).

Phase-contrast inverted microscopic observation of the cells treated with JGB1741 showed a variety of morphological changes that were proportional to the concentration. With an increase in the concentration of JGB1741, the MDA-MB 231 cells lost their differentiated properties of epithelial cells, became round, decreased in size and finally entered into apoptosis (Fig. 2D). Our results are in consistent with earlier results [33].

3.4. JGB1741 inhibited SIRT1 in cells

Previous studies have shown that SIRT1 deacetylates histone proteins with a preference for histone H4 at lysine 16 (H4K16) and histone H3 at lysine 9 (H3K9) [37]. Therefore, in presence of SIRT1 inhibitor, JGB1741, the acetylation levels of H3K9 were studied by Western blot. An increase in the global acetylation of H3K9 was observed in JGB1741-treated MDA-MB 231 cells dose dependently (Fig. 3A). Moreover, since all the experiments were carried out in presence of sodium butyrate, any deacetylase activity of class I and II HDACs on acetylated H3K9 is inhibited. These results clearly indicate the cell-based inhibition of SIRT1 deacetylase activity by JGB1741. Also, signal compensation in Ac-H3K9 levels was observed in cells when co-treated with JGB1741 and a SIRT1 activator hit (Activator X) clearly indicating the target-inhibition of JGB1741 in cells (Fig. 3B).

One of the primary targets of SIRT1 is p53 and deacetylated p53 abrogates the p53-mediated cell death pathway during DNA damage and stress [3,4]. Therefore, the inhibitory activity of JGB1741 on SIRT1 was evaluated by Western blot analysis using p53 and acetylated p53K382 antibodies. The results showed an increase in both p53 expression and acetylated p53K382 levels in JGB1741-treated MDA-MB 231 cells in a concentration dependent manner (Fig. 3c). The results are in agreement with earlier studies [35,38,39]. This data clearly demonstrate the inhibition of SIRT1 by JGB1741 in cells.

3.5. JGB1741-induced apoptosis of MDA-MB 231 cells

Further to understand the mechanism of inhibition of cell proliferation of MDA-MB 231 cells by JGB1741, cell death was quantified in terms of percentage of apoptotic cells by flowcytometry. Analysis of JGB1741-treated cells showed an increase in the percent apoptotic cells in a dose-dependent fashion with ~70% apoptosis at 1 μM concentration of JGB1741. The MDA-MB 231 cells treated with JGB1741 also showed a cell cycle arrest at G1 phase with more and more cells entering into sub G0/G1 phase, the apoptotic phase, in a dose-dependent fashion (Fig. 4A). Similar results were obtained when MCF-7 cells were treated with sirtinol [35].

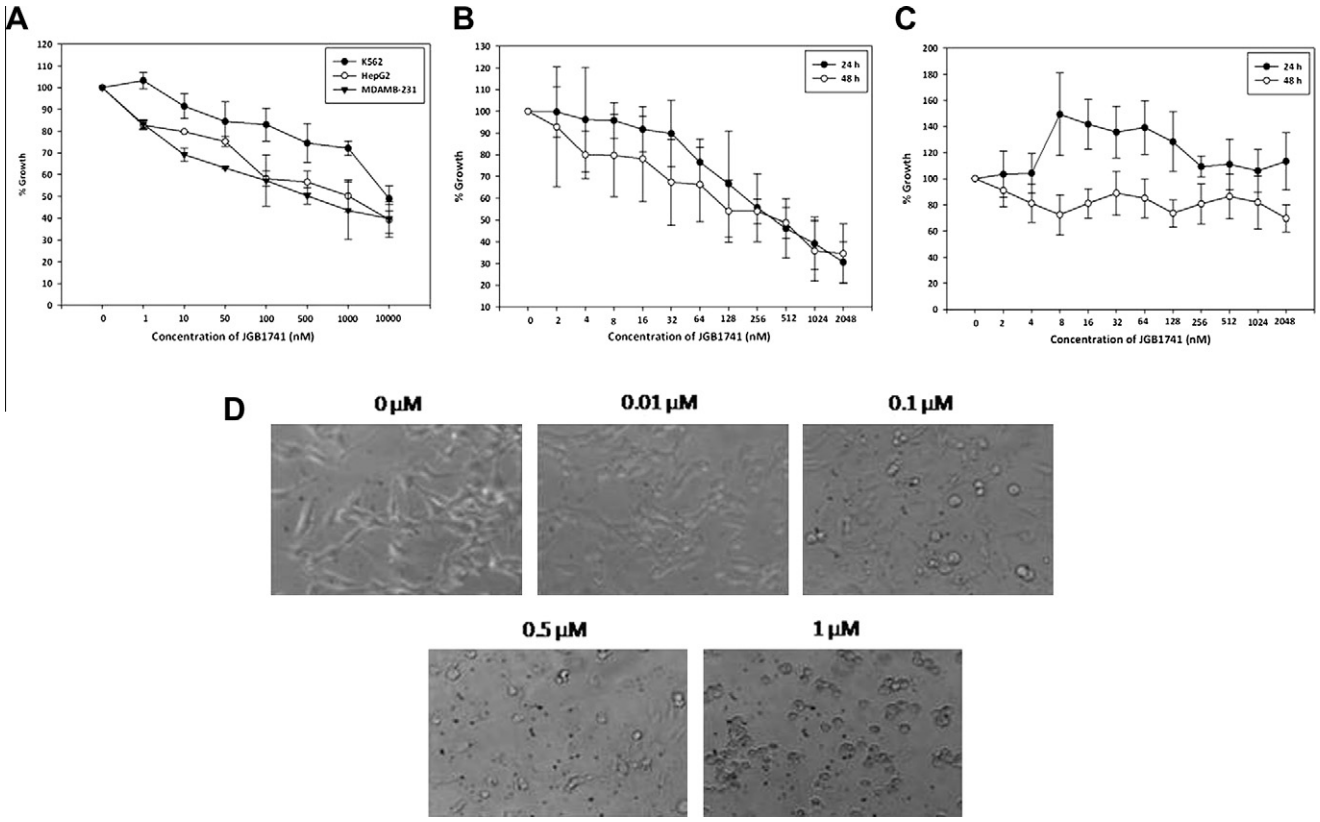

Fig. 2. Anti-proliferative effects of JGB1741. (a) The effect of JGB1741 at different concentrations on K562, HepG2 and MDA-MB 231 cell proliferation was determined by MTT assay and the results showed more potent effect of JGB1741 on MDA-MB 231 cells compared to K562 and HepG2 cells. (B) MTT assay of JGB1741 in MDA-MB 231 cells for 24 and 48 h. The IC50 of JGB1741 in MDA-MB 231 cell proliferation was determined to be 512 nM at 24 h. (C) A graph illustrating no cytotoxicity of JGB1741 on HEK293 cell proliferation. (D) Morphological changes due to induction of apoptosis by JGB1741 were observed by phase-contrast microscopy. The MDA-MB 231 cells lost their characteristics of epithelial cells with increasing concentrations of JGB1741.

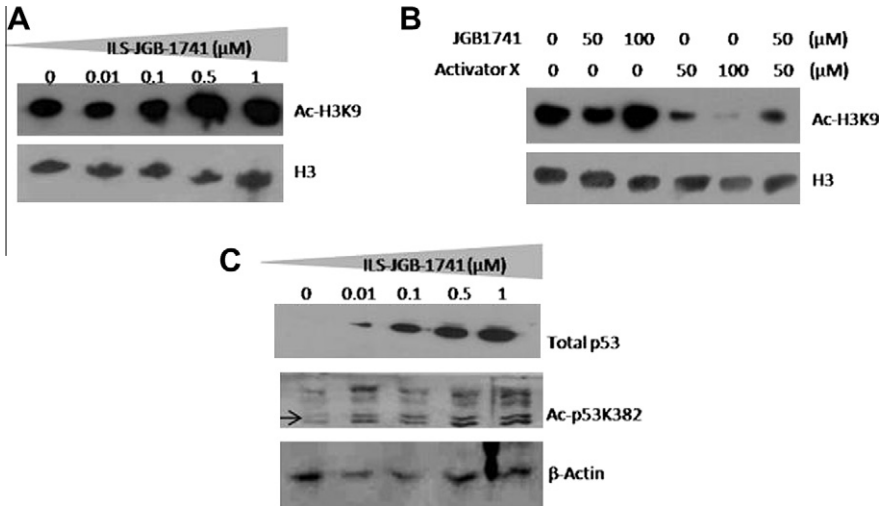

Fig. 3. JGB1741 inhibited SIRT1 in cells. (A) A representative Western blot showing the expression levels of global Ac-H3K9 and Histone H3 levels. (B) Western blot showing the signal compensation in the levels of Ac-H3K9 in presence of JGB1741 and an Activator X. (C) Inhibition of SIRT1 by JGB1741 resulted in increase of both total p53 and Ac-p53 levels dose dependently.

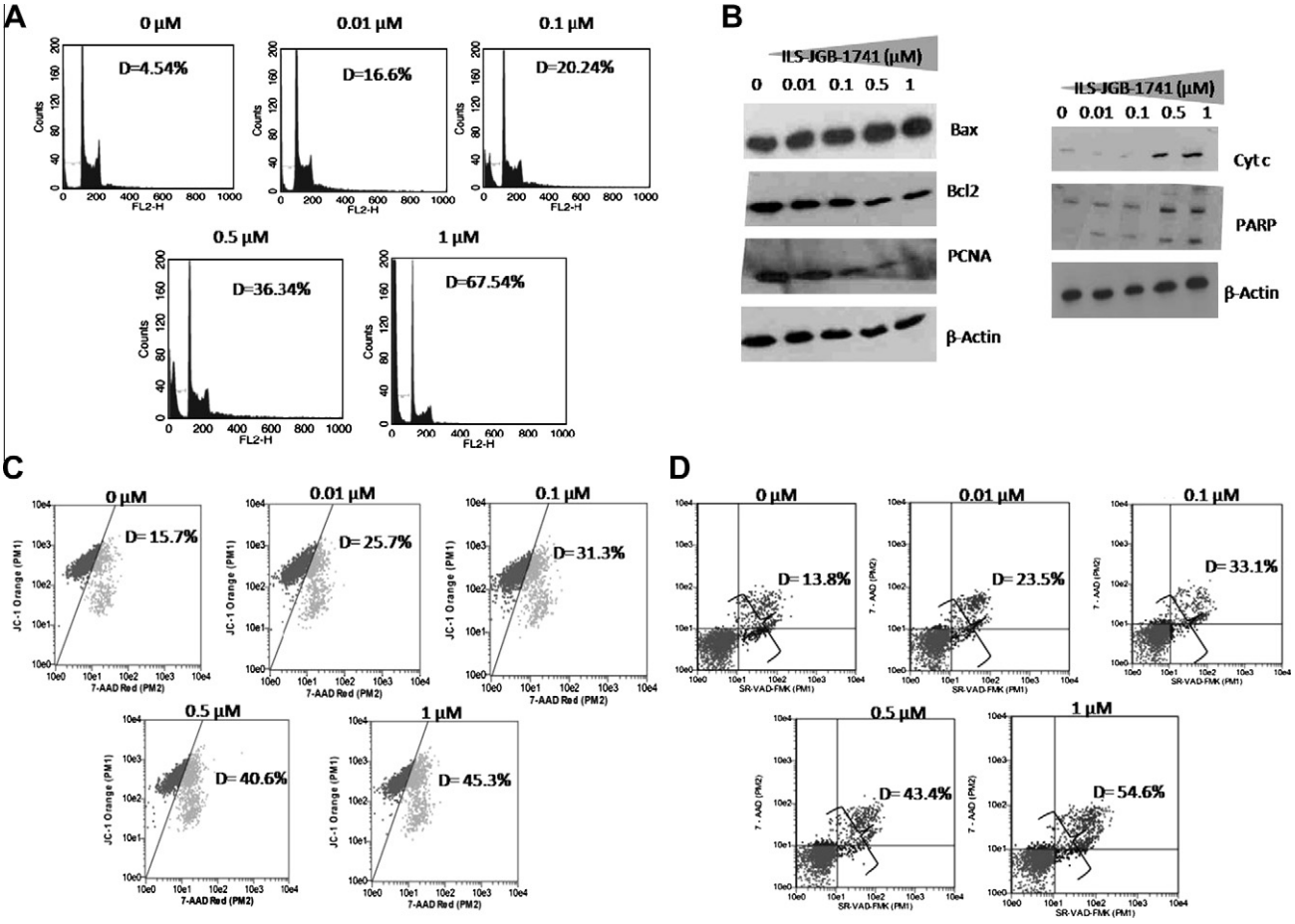

Fig. 4. JGB1741-induced apoptosis of MDA-MB 231 cells. (A) Apoptosis of MDA-MB 231 cells treated with JGB1741 was quantified using Flowcytometry and showed an increase in the percent apoptotic cells dose dependently. (B) Western blots showing the modulation of apoptotic marker ratio, Bax–Bcl2 ratio, with a dose-dependent decrease anti-apoptotic protein Bcl2 and marginal increase in the expression levels of pro-apoptotic protein Bax, a significant decrease in the proliferating cell nuclear antigen (PCNA), release of cytochrome *c* from mitochondria and cleavage of PARP. β-Actin was used as control. (C) Dot plot graphs showing the flowcytometric analysis of the mitochondrial membrane potential of JGB1741-treated MDA-MB 231 cells. (D) Dot plots from the flowcytometer analysis of the dose-dependent increase in the percent dead cells in JGB1741-treated MDA-MB 231 cells as a result of multicaspase activation. *D* = Percent dead cells.

Further to understand the underlying molecular mechanism of apoptosis induced by JGB1741, activation of the p53-mediated signaling network via inhibition of SIRT1 was evaluated. Bax homodimers are inducers of apoptosis whereas Bax–Bcl2 heterodimer

formation results in cell survival. Both Bax and Bcl2 are transcriptional targets of p53 [40,41]. The levels of the pro-apoptotic protein Bax and anti-apoptotic protein Bcl2 were measured by Western blot analysis. There is a significant decrease in the levels of Bcl2 protein with a marginal increase in the Bax protein levels indicating formation of Bax homodimers and thus inducing apoptosis. Also, Western blot analysis of the cytoplasmic extracts of the cells treated with JGB1741 also showed a dose-dependent increase in the cytochrome *c* levels and cleavage of PARP indicating induction of apoptosis (Fig. 4B). The marker of dividing cells, proliferating cell nuclear antigen (PCNA) was also studied by Western blot and the result showed a dose-dependent decrease in the expression of this protein suggesting induction of apoptosis (Fig. 4B). All these results are in consent with earlier studies on SIRT1 inhibitor [38].

During apoptosis, membrane potential of the mitochondria decrease due to which cytochrome *c* gets released into cytoplasm thereby activating caspases. So the mitopotential of the apoptotic cells and activation of caspases were determined by flowcytometry and the results showed a significant decrease in the membrane potential (Fig. 4C) and increase in the caspase activation (Fig. 4D).

4. Conclusion

In conclusion, here we demonstrate a potent inhibitor of SIRT1, JGB1741. Although the biochemical assay indicates JGB1741 as less potent inhibitor of SIRT1 compared to suramin, the in vitro cell-based data clearly indicates that it is more potent in inhibiting cancer cell proliferation, specifically metastatic breast cancer cells MDA-MB 231, at much lower IC_{50} (512 nM) compared to suramin (IC_{50} 7 μM). The study also demonstrates that JGB1741 is a specific inhibitor of SIRT1 and thereby increases the acetylated p53 levels leading to p53-mediated apoptosis with modulation of Bax/Bcl2 ratio, cytochrome *c* release and PARP cleavage. Thus JGB1741 could become a potential therapeutic hit for the treatment of breast cancer.

5. Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.118.

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