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Identification of a small molecule SIRT2 inhibitor with selective tumor cytotoxicity

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ABSTRACT

As a member of the class III histone deacetylases, Sirtuin-2 (SIRT2) is critical in cell cycle regulation which makes it a potential target for cancer therapeutics. In this study, we identified a novel SIRT2 inhibitor, AC-93253, with IC_{50} of 6 μM *in vitro*. The compound is selective, inhibiting SIRT2 7.5- and 4-fold more potently than the closely related SIRT1 and SIRT3, respectively. AC-93253 significantly enhanced acetylation of tubulin, p53, and histone H4, confirming SIRT2 and SIRT1 as its cellular targets. AC-93253 as a single agent exhibited submicromolar selective cytotoxicity towards all four tumor cell lines tested with a therapeutic window up to 200-fold, comparing to any of the three normal cell types tested. Results from high content analysis suggested that AC-93253 significantly triggered apoptosis. Taken together, SIRT2 selective inhibitor AC-93253 may serve as a novel chemical scaffold for structure–activity relationship study and future lead development.

Introduction

Sirtuins are a seven-membered group (SIRT1-7) of proteins belonging to the silent information regulator 2 (Sir2) family [1–3]. They possess either histone deacetylase (SIRT1∼3, 5) or mono-ribosyltransferase activity (SIRT4 and SIRT6) which target histone and various nonhistone substrates in different subcellular locations [1]. Results from gene overexpression and knockout mice studies suggest that sirtuins, of which SIRT1 is the most studied, affect genome maintenance, aging, and metabolism via regulation of multiple disease-relevant pathways such as insulin signaling/secretion, cell cycle regulation, mitochondrial activity, and oxidative stress resistance [1,3]. Therefore, selectively targeting of individual sirtuin enzymes with small molecule modulators provides a new platform opportunity for drug discovery. Resveratrol, a small molecule SIRT1 activator, is currently in clinical trial for type 2 diabetes and cancer (www.clinicaltrials.gov).

SIRT2 is a NAD+-dependent protein deacetylase of several substrate proteins in the cytoplasm [1–4]. Particularly, SIRT2 deacetylates lysine 40 of α-tubulin both *in vitro* and *in vivo*, functioning together with another tubulin deacetylase, HDAC6 [4,5]. Thus, SIRT2 regulates the dynamics of microtubules and tubulin associated cellular events by controlling the levels of acetylated α-tubulin. Increased levels of SIRT2 protein were found in the nucleus in the G2/M transition of the cell cycle where the protein becomes phosphorylated and stabilized by an unknown kinase [6,7]. Overexpression of SIRT2, not a mutant SIRT2 without deacetylase activity, significantly prolonged the mitotic phase, thus delaying mitotic exit [6]. In addition, SIRT2 was reported to block the entry to chromosome condensation in glioma cell lines through nucleo-cytoplasmic shuttling in response to mitotic stress [8]. Thus, it has been proposed that SIRT2 might function as a mitotic checkpoint protein in G2/early metaphase to prevent chromosomal instability, particularly in response to microtubule inhibitor (MTI) induced mitotic stress, such as mitotic slippage [1,4]. Tumors with high levels of SIRT2 respond poorly to chemotherapy drugs, particularly microtubule poisons [4]. Therefore, SIRT2 inhibitors may have chemotherapeutic properties for selective cytotoxicity [4].

Progress has been made to identify SIRT2 inhibitors for cancer therapeutics [2]. Sirtinol and splitomicin, first reported as SIRT2 inhibitors through a yeast phenotypic screen [9,10], have been further optimized to more potent compounds [11,12]. Suramin and its analogs were later identified as inhibitors for both SIRT1 and SIRT2, with the binding of suramin to SIRT2 mainly through direct competition with either NAD+ or acetylated protein [13,14]. Using the crystal structure of SIRT5 [14], several *in silico* virtual screenings were reported with discovery of novel chemical scaffolds as SIRT2 inhibitors [15–18]. In addition, cambinol was identified to inhibit deacetylase activity of both SIRT1 and SIRT2. This compound was well tolerated in mice and significantly inhibited growth of Burkitt lymphoma xenografts [19], providing a proof-of-concept example that SIRT2 inhibition may be an effective treatment in clinical cancer therapeutics.

Here we report the identification of a SIRT2 inhibitor, AC-93253. This compound showed selective inhibition of SIRT2 with IC_{50}

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value of 6.0 μM, with lower potency for two closely related SIRT family members, SIRT1 and SIRT3. AC-93253 increased acetylated α-tubulin protein levels and resulted in selective cancer cell cytotoxicity apparently by triggering apoptosis.

Materials and methods

Reagents and cell lines. AC-93253, trichostatin A (TSA), α-tubulin Lys40 antibody, total α-tubulin antibody, DMSO were purchased from Sigma (St. Louis, MO). Histone H4 Lys16 antibody and total histone H4 antibody were obtained from Millipore (Billerica, MA). p53 Lys382 antibody and actin antibody were from Cell Signaling Technology (Danvers, MA). Total p53 antibody (DO-1) was from BD Pharmingen (San Diego, CA). Secondary antibodies labeled with IRdye800 infrared fluorescent dye were obtained from LI-COR Biosciences (Lincoln, NE). Tetrazolium salt powder (MTS) was purchased from Promega (Madison, WI). Hoechst 33342, Propidium iodide (PI) and Yopro were purchased from Invitrogen (Carlsbad, CA). A fluorogenic, acetylated peptide substrate Arg-His-Lys-Lys (Ac) (derived from a regulatory acetylation site of p53 amino acid 379–382) was ordered from BPS Bioscience (San Diego, CA). Purified Sirtuin-2 (amino acid 50–356), and Sirtuin-3 (amino acid 102–399) were ordered from BPS Bioscience (San Diego, CA). Nicotinamide adenine dinucleotide (NAD+, oxidized form) and purified human Sirtuin-1 (amino acid 1–747) were purchased from Biomol (Plymouth Meeting, PA).

HeLa, DU-145, A549, MiaPaCa2, and NCI-H460 were purchased from ATCC (Manassas, VA). All above cell lines were supplemented with 10% fetal bovine serum (FBS; v/v, Invitrogen) and 10 mM L-glutamine (Invitrogen). Human umbilical vein endothelial cells (HuVEC), human primary mammary epithelial cells (HMEC) and human primary prostate epithelial cells (PrEC) were purchased from Lonza (Walkersville, MD).

Biochemical assays for sirtuin activity and small molecule screening. All sirtuin activity assays were performed in a low binding NUNC plate (Catalog No. 62408-936, VWR) in a reaction buffer containing 50 mM Tris–HCl (pH8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$. 1 mg/ml BSA and 1% DMSO were added to the reaction buffer before use. 2× Sirtuin enzyme was added to wells followed by addition of compounds with concentration ranging from 0.01 μM to 10 mM with suramin as a positive control. The total reaction volume is 50 μL. 2× fluorogenic substrate (50 μL) was added to initiate the reaction which was allowed to incubate at 30 °C for 2 h. Nicotinamide was added to stop the reaction followed by kinetic measurements of fluorescent signal (Ex 360 nm/Em 460 nm) for 1.5 h with 15 min intervals in Envision Excite multichannel reader (Perkin Elmer, Wellesley, MA). IC_{50} values and curve fitting were performed using Prism 5.0 (GraphPad Software, San Diego, CA) with nonlinear regression analysis.

Western blot analysis. HeLa cells or NCI-H460 cells were seeded at a density of 2.5×10^5 in 6-well plates with MEM plus 10% FBS for overnight. Cells were treated with various concentrations of AC-93253 for 16 h in the absence or presence of 40 nM TSA After 16 h, cells were lysed in RIPA buffer (Sigma) containing protease inhibitor cocktail (Roche, Palo Alto, CA) and phosphatase inhibitors (Sigma). Total cell lysates were denatured and resolved on SDS–polyacrylamide gels, and transferred onto nitrocellulose membranes. Western blot was performed with 1:1000 dilution of primary antibodies and 1:10000 dilution of secondary antibodies. Image scanning and quantification was performed with Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) according to manufacturers' instructions.

High content image based assay. For the live cell imaging assay, HeLa cells were treated with AC-93253 at different concentrations at 37 °C for 48 h. Fluorescent labeling was achieved with addition of 0.5 μg/ml Hoechst 33342, 1 μg/mL PI, and 0.5 μg/mL Yopro for 15 min. Image acquisition was performed with an INcell 1000 with a 10x objective (GE Healthcare, Piscataway, NJ). The program was set to take four images per well with exposure time set at 100 ms for Hoechst and 50 ms for PI or Yopro. Yopro intensity CV (coefficient of variation) was chosen as a parameter for quantification of apoptotic cells. The thresholds for segmentation of apoptotic cells were determined with gating values equivalent to the average of DMSO treated samples plus 2 standard deviations.

MTS cytotoxicity assay. MTS assay was performed as previously described [20]. Briefly, cells were seeded in 96-well plates (Catalog No. Costar 3598, Cornings, MA) and incubated overnight. Compounds were diluted in the corresponding media to designated concentrations (final DMSO concentration is 0.5% v/v). After 72 h incubation, MTS/PMS (Promega, Madison, WI) were added to the culture medium for 3 h. SDS was then added to a final concentration of 1.25% (w/v %) to stop the reaction. Plates were measured for absorbance at 492 nm using Envision Excite. The absorbance (A) at 492 nm is directly proportional to the viable cells in the culture. Inhibition% was calculated using the following formula:

$$\text{Inhibition}\% = (A_{\text{DMSO}} - A_{\text{compound}})/A_{\text{DMSO}} \times 100\%$$

IC_{50} values and curve fitting were performed using Prism 5.0.

Anchorage independent growth assay. Inhibition of anchorage independent growth of HeLa cells was determined by using CytoSelect™ 96-Well Cell Transformation Assay Kit (Cell Biolabs, San Diego, CA), following manufacturers' instruction. In brief, 10,000 HeLa cells were plated in 0.4% soft agar on top of 0.6% base agar layer in 96-well plates. The next day, cells were treated with compounds and colonies were allowed to grow for 7 days under 37 °C and 5% CO_2. The agar was then solubilized and cells were lysed with lysis buffer. Cell lysates were stained with CyQuant and fluorescent signal was measured at 485/520 nm using Envision Excite.

Colony formation assay of bone marrow cells and A549 lung cancer cells. Frozen bone marrow cells (Stemcell Technologies, Vancouver, BC, Canada) were recovered in RPMI1640 medium supplemented with 10% FBS. Cells were treated with AC-93253 at concentrations ranging from 1 μM to 1.4 nM for 18 h. The medium containing compound was then removed and cells were plated into 35 mm dishes in Methocult medium (H4434, Stemcell Technologies). Erythroid and myeloid colonies were manually counted after 14 days. A549 lung cancer cells were plated into 35 mm dishes and were treated with AC-93253 at the same concentration range as that of bone marrow cell for 18 h. Compounds were then removed and A549 colonies were counted after 7 days. Each concentration of the compound was tested in duplicated dishes for both bone marrow cells and A549 cells.

Results

AC-93253 selectively inhibits deacetylase activity of SIRT2 in vitro

Using a fluorogenic substrate, we performed a biochemical based assay with purified SIRT1, 2, and 3. Suramin was used as a positive control for the assay with IC_{50} values of 20.5 and 2.6 μM for SIRT2 (Fig. 1B) and SIRT1 (Fig. 1C), respectively, agreeing well with previous published results [Biomol International LP; 14]. Suramin did not inhibit deacetylase activity of SIRT3 at the concentrations tested (Fig. 1D). AC-93253 (Fig. 1A) showed potent inhibition of SIRT2 deacetylase activity with IC_{50} value of 6.0 μM (Fig. 1B). AC-93253 also inhibited two closely related members SIRT1 and SIRT3 with IC_{50} values at 45.3 μM (Fig. 1C) and 24.6 μM (Fig. 1D), which are 7.5- and 4-fold less potent, respectively, than SIRT2. Therefore, AC-93253 exhibited selective inhibitory activity to SIRT2 with a chemical scaffold distinct from small molecules identified previously [2].