Note

Identification of a Selective SIRT2 Inhibitor and Its Anti-breast Cancer Activity

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SIRT2 is a member of the human sirtuin family of proteins and possesses nicotinamide adenine dinucleotide (NAD)-dependent lysine deacetylase activity. SIRT2 has been involved in various cellular processes including gene transcription, genome constancy, and the cell cycle. In addition, SIRT2 is deeply implicated in diverse diseases including cancer. In this study, we identified a small molecule inhibitor of SIRT2 with a structure different from known SIRT2 inhibitors by screening from a chemical library. The hit compound showed a high selectivity toward SIRT2 as it only inhibited SIRT2, and not other sirtuins including SIRT1 and SIRT3 or zinc-dependent histone deacetylases (HDACs) including HDAC1 and HDAC6, *in vitro*. The compound increased the acetylation level of eukaryotic translation initiation factor 5A (eIF5A), a physiological substrate of SIRT2, and reduced cell viability of human breast cancer cells accompanied with a decrease in c-Myc expression. These results suggest that the compound is cellular effective and has potential for development as a therapeutic agent against breast cancers by specific inhibition of SIRT2.

Key words SIRT2; c-Myc; breast cancer; high-throughput screening; eukaryotic translation initiation factor 5A

Sirtuins are a family of nicotinamide adenine dinucleotide (NAD)-dependent lysine deacetylases shown to play biological and physiological roles in diverse cellular processes such as metabolism, transcription, and DNA repair. Mammals have seven sirtuins that display different subcellular localizations and functions. SIRT2 is a predominantly cytosolic protein and was originally reported as a microtubule deacetylase,[1] but further studies have revealed that SIRT2 acts as a deacetylase for histones and a number of non-histone proteins. This wide variety of substrates might be correlated with physiological roles of SIRT2 in diverse biological processes such as the cell cycle, autophagy, and energy metabolism.[2]

Many studies suggest an important involvement of SIRT2 in neurodegeneration, inflammation, bacterial infection, and cancer, and that modulation of SIRT2 activity could be novel strategy for therapies against these disorders.[3–5] However, pharmacological evidence for SIRT2 as a valid therapeutic target has not yet been shown. Therefore, potent and selective SIRT2 inhibitors are required for initial proof of concept studies. Although there are several reports describing SIRT2 inhibitors to date,[6] specificity and cellular potency of most present SIRT2 inhibitors appear to be insufficient for elucidating *in vivo* SIRT2 functions. In this regard, development of potent and highly specific SIRT2 inhibitors has been recently achieved.[7,8] Here, we identified a potent and specific SIRT2 inhibitor by high-throughput screening. Because the fundamental structure of the compound is different from that of existing SIRT2 inhibitors, it may serve as a novel class of chemical tool for exploring SIRT2 functions in cells.

MATERIALS AND METHODS

Materials and Cell Culture RK-9123016 was purchased from Vitas-M Laboratory (Hong Kong, China) through a local distributor. SirReal2 and nicotinamide (NA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Trichostatin A (TSA) was prepared as described previously.[9] Mouse monoclonal eukaryotic translation initiation factor 5A (eIF5A) antibody was obtained from BD Bioscience (San Jose, CA, U.S.A.). Rabbit polyclonal antibody against acetylated eIF5A (Ac-eIF5A) was raised as described previously.[10] Mouse monoclonal α-tubulin antibody (B-5-1-2) was obtained from Sigma-Aldrich. Mouse monoclonal c-Myc (9E10) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). MCF-7 cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% heat inactivated fetal bovine serum (FBS) and antibiotics at 37°C, 5% CO_2 in a humidified incubator.

Lysine Deacetylase Assay Measurements of *in vitro* enzymatic activities of zinc-dependent histone deacetylases (HDACs) including HDAC1 and HDAC6 were performed by a fluorogenic assay as described previously.[11] Measurements of *in vitro* deacetylase activities of human sirtuins including SIRT1, 2 and 3 were carried out by an electrophoretic mobility shift assay. Recombinant sirtuin proteins were incubated with a carboxyfluorescein (FAM)-labeled fluorescent peptide (1.5 μM FAM-RHKK(Ac)LM for SIRT1 and SIRT2; 1.5 μM FAM-QPKK(Ac)KPL for SIRT3) and 1 mM NAD in 50 μL of assay buffer (25 mM Tris–HCl [pH 9.0], 137 mM sodium chloride (NaCl), 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mM bovine serum albumin (BSA) in 384 well plates. After 60 min at 37°C, the reaction was stopped by adding nicotinamide (final concentration 10 mM) in 50 μL of the stop buffer (100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) [pH 7.5], 10 mM ethylenediaminetetraacetic acid (EDTA), 0.25% CR-3). The samples were analyzed using a LabChip EZ Reader II (PerkinElmer, Inc., Waltham, MA, U.S.A.). Percent conversion is defined as 100×P/(P+S), where P and S are peak

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heights of product and peptide substrate, respectively.

Immunoblotting Whole cell lysates were prepared by collecting cells directly in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and heating at 95°C for 10 min. Immunoblotting was performed as described previously.[12)]

Cell Viability Assay MCF-7 cells (1×10^3) were grown in a 96-well plate and treated with dimethyl sulfoxide (DMSO) or compounds for 96 h. Cell viability was measured by the ATPlite luminescence assay system (PerkinElmer, Inc., Waltham, MA, U.S.A.) using a Synergy H4 hybrid reader (BioTek, Tokyo, Japan).

RESULTS AND DISCUSSION

In search for novel SIRT2 inhibitors, we screened a chemical library consisting of approximately 140000 compounds from The University of Tokyo using an *in vitro* assay based on fluorogenic acetyl-lysine peptides[11)] and identified RK-9123016 (Fig. 1A) as a potent and selective SIRT2 inhibitor. *In vitro* electrophoretic mobility shift assay revealed that RK-9123016 inhibited the enzymatic activity of SIRT2 with an IC_{50} value of 0.18 μM but not other human sirtuin members including SIRT1 and SIRT3 at 100 μM (Fig. 1B). In addition, RK-9123016 at 100 μM did not affect activities of zinc-dependent HDACs including HDAC1 and HDAC6, while TSA,[9)] a pan-inhibitor for zinc-dependent HDACs, inhibited enzymatic activities of both enzymes (Fig. 1C).

In order to evaluate *in vivo* SIRT2 inhibition by RK-9123016 we examined the acetylation level of eIF5A, a physiological substrate of SIRT2. We previously reported that both HDAC6 and SIRT2 regulate deacetylation of eIF5A and that inhibition of both enzymes, but not either one alone, is necessary for a noticeable increase in eIF5A acetylation in cells.[10,13)]

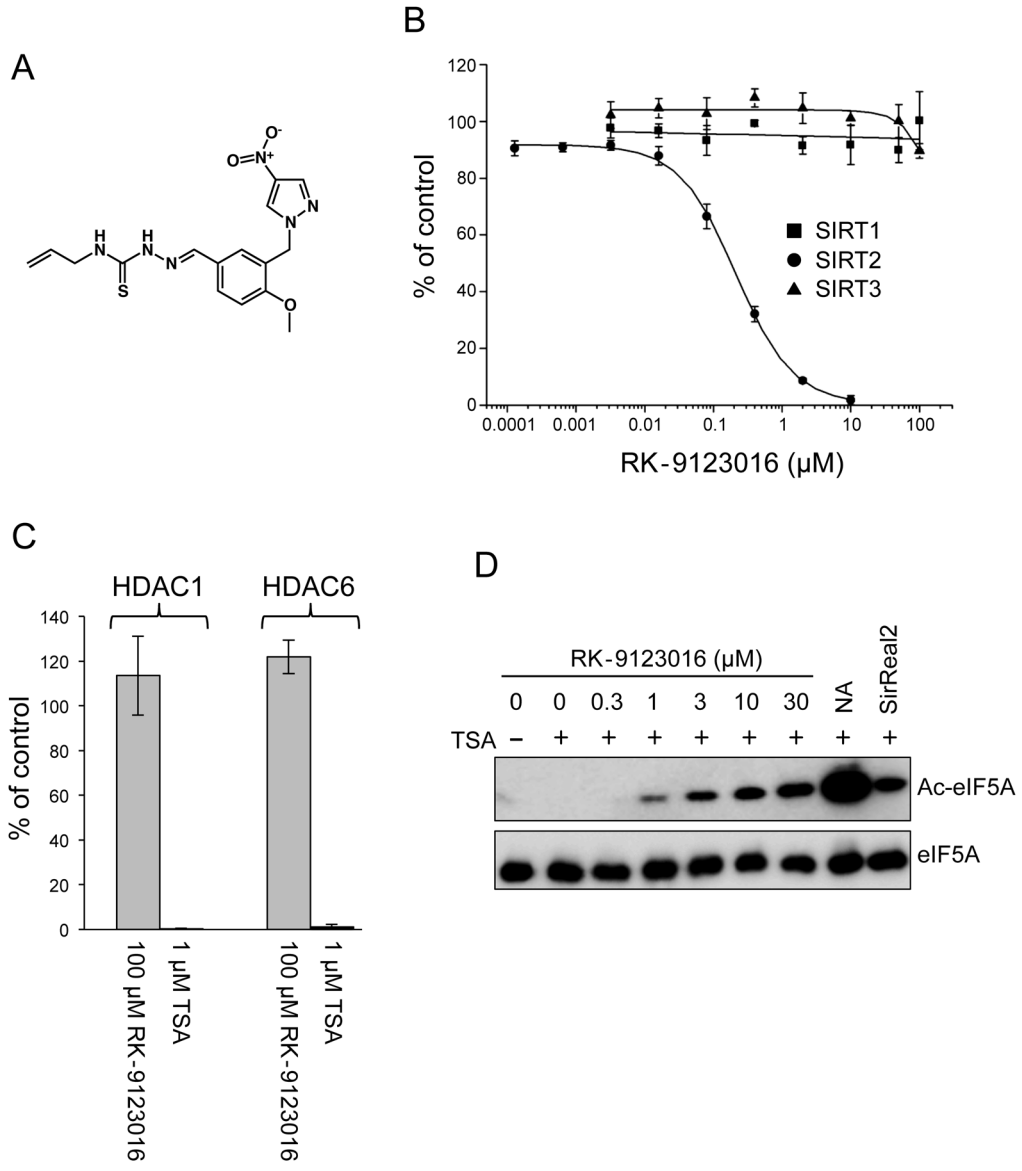


Fig. 1. Inhibition of SIRT2 Activity by RK-9123016

(A) Structure of RK-9123016. (B and C) Effect of RK-9123016 on lysine deacetylases *in vitro*. The activity of RK-9123016 to inhibit SIRT1, 2, and 3 (B) or HDAC1 and 6 was estimated using an electrophoretic mobility shift assay or a fluorogenic assay, respectively. Error bars represent the standard deviation of three independent assays. (D) Effect of RK-9123016 on the acetylation of eIF5A in MCF-7 cells. MCF-7 cells were treated with different concentrations of RK-9123016, 5 mM nicotinamide (NA), or 30 μM SirReal2 as positive controls in the presence of 1 μM TSA for 24 h. The lysates were immunoblotted with the indicated antibodies.

Therefore, MCF-7 cells were treated with RK-9123016 at various concentrations in the presence of TSA to inhibit HDAC6. We also used two known small molecules that inhibit SIRT2, nicotinamide, a pan-sirtuin inhibitor, and SirReal2, a specific SIRT2 inhibitor,[7] as positive controls. The level of acetylated eIF5A was then determined by immunoblotting using an antibody that specifically recognizes the acetylated form of eIF5A.[10] In agreement with previous reports, the acetylation of eIF5A was barely observed under treatment with TSA alone (Fig. 1D). On the other hand, treatment with RK-9123016 together with TSA increased the level of eIF5A acetylation in a dose-dependent manner (Fig. 1D). These results suggest that RK-9123016 inhibits the SIRT2 activity in cells.

Recent studies indicate that selective SIRT2 inhibitors decrease cell viability in various types of cancer cells including human breast cancer MCF-7 cells by promoting degradation of the oncoprotein c-Myc.[8] Therefore, we examined the effect of RK-9123016 on cell viability of MCF-7 cells by measuring cellular amounts of ATP. Consistent with previous results, RK-9123016 as well as SirReal2 reduced the viability of MCF-7 cells in a concentration-dependent manner (Fig. 2A). Importantly, as shown in Fig. 2B, the expression of c-Myc protein was reduced by RK-9123016 in a dose range similar to that required for an increase in eIF5A acetylation (Fig. 2B).

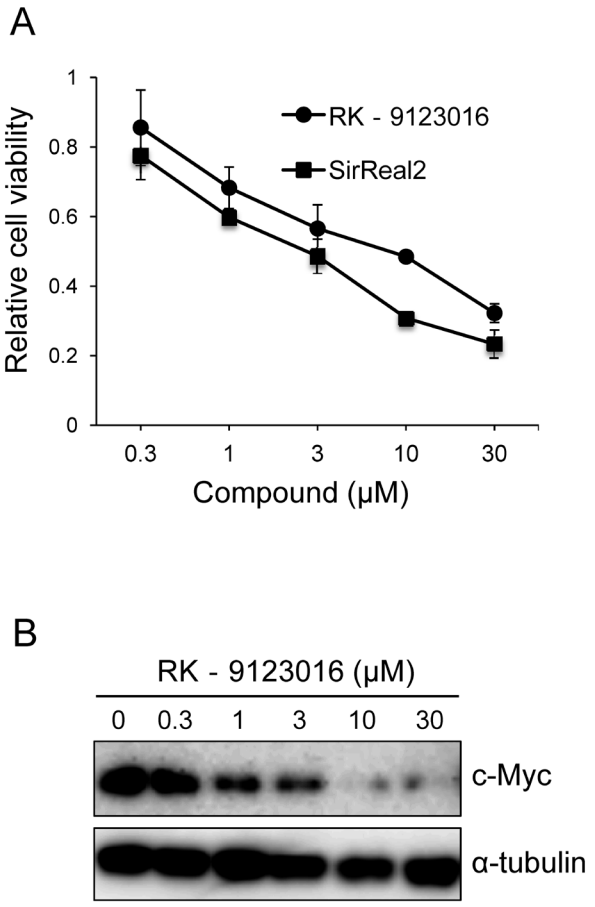


Fig. 2. Inhibition of Cell Viability by RK-9123016 in MCF-7 Cells

(A) MCF-7 cells were treated with various concentrations of RK-9123016 or SirReal2 for 96 h. Cell viability was measured by the ATPlite luminescence assay. Error bars represent the standard deviation of three independent assays. (B) Effect of RK-9123016 on c-Myc expression. MCF-7 cells were treated with different concentrations of RK-9123016 for 24 h. The lysates were immunoblotted with the indicated antibodies.

These findings suggest that RK-9123016 exhibits anti-cancer activity against breast cancer MCF-7 cells by promoting SIRT2 inhibition-mediated degradation of c-Myc oncoprotein.

In this study, we identified a synthetic compound named RK-9123016 as a potent and selective SIRT2 inhibitor with a structure different from all of known SIRT2 inhibitors. Recent crystallographic analysis of the SIRT2 complex with SirReal2 suggests that potency and high selectivity of SirReal2 are based on its unique inhibitory mechanism by which SirReal2 binds the pocket created by a ligand-induced structural rearrangement of the active site.[7] Because potency (IC_{50}: 0.18 μM) and selectivity against other sirtuin members (>500 fold) of RK-9123016 are comparable to those of SirReal2, it is interesting to test whether RK-9123016 inhibits SIRT2 in a similar fashion to SirReal2. Elucidation of precise mechanism of SIRT2 inhibition by RK-9123016 as the basis for its potency and high selectivity is a subject for a further study. Together with results showing an increase in acetylation of eIF5A by RK-9123016 in breast cancer cells (Fig. 1D), RK-9123016 could be a valuable small-molecule tool for exploring SIRT2 functions in cells.

Involvement of SIRT2 in tumorigenesis seems to be controversial. Genetic studies using SIRT2 knockout (KO) mice indicate that SIRT2 may function as a tumor suppressor.[14] In contrast, growing evidence suggests that SIRT2 promotes tumorigenesis.[15–19] The reason for this discrepancy is unknown, but it may depend on cancer progression stage or cell type. Recent reports using a selective SIRT2 inhibitor demonstrated that SIRT2 inhibition exhibits anti-tumor activity against breast cancer cells by degrading c-Myc oncoprotein.[8] Our results support this observation, as two selective SIRT2 inhibitors, RK-9123016 and SirReal2,[7] inhibited cell growth of human breast cancer MCF-7 cells accompanied by c-Myc degradation (Fig. 2), although their chemical structures are completely different. Taken together, pharmacological evidence using several selective SIRT2 inhibitors with different structures strongly suggests that SIRT2 is a promising target for breast cancer therapy. Thus, RK-9123016 may serve as a valuable tool for validation of SIRT2 as a therapeutic target.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) North BJ, Marshall BL, Borra MT, Denu JM, Verdin E. The human Sir2 ortholog, SIRT2, is an NAD^+-dependent tubulin deacetylase. *Mol. Cell*, **11**, 437–444 (2003).

2) Gomes P, Outeiro TF, Cavadas C. Emerging role of sirtuin 2 in the regulation of mammalian metabolism. *Trends Pharmacol. Sci.*, **36**, 756–768 (2015).
3) de Oliveira RM, Sarkander J, Kazantsev AG, Outeiro TF. SIRT2 as a therapeutic target for age-related disorders. *Front. Pharmacol.*, **3**, 82 (2012).
4) Pais TF, Szego EM, Marques O, Miller-Fleming L, Antas P, Guerreiro P, de Oliveira RM, Kasapoglu B, Outeiro TF. The NAD-dependent deacetylase sirtuin 2 is a suppressor of microglial activation and brain inflammation. *EMBO J.*, **32**, 2603–2616 (2013).
5) Eskandarian HA, Impens F, Nahori MA, Soubigou G, Coppée JY, Cossart P, Hamon MA. A role for SIRT2-dependent histone H3K18 deacetylation in bacterial infection. *Science*, **341**, 1238858 (2013).
6) Hu J, Jing H, Lin H. Sirtuin inhibitors as anticancer agents. *Future Med. Chem.*, **6**, 945–966 (2014).
7) Rumpf T, Schiedel M, Karaman B, Roessler C, North BJ, Lehotzky A, Oláh J, Ladwein KI, Schmidtkunz K, Gajer M, Pannek M, Steegborn C, Sinclair DA, Gerhardt S, Ovádi J, Schutkowski M, Sippl W, Einsle O, Jung M. Selective Sirt2 inhibition by ligand-induced rearrangement of the active site. *Nat. Commun.*, **6**, 6263 (2015).
8) Jing H, Hu J, He B, Negrón Abril YL, Stupinski J, Weiser K, Carbonaro M, Chiang YL, Southard T, Giannakakou P, Weiss RS, Lin HA. SIRT2-selective inhibitor promotes c-Myc oncoprotein degradation and exhibits broad anticancer activity. *Cancer Cell*, **29**, 297–310 (2016).
9) Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J. Biol. Chem.*, **265**, 17174–17179 (1990).
10) Ishfaq M, Maeta K, Maeda S, Natsume T, Ito A, Yoshida M. Acetylation regulates subcellular localization of eukaryotic translation initiation factor 5A (eIF5A). *FEBS Lett.*, **586**, 3236–3241 (2012).
11) Narita K, Matsuhara K, Itoh J, Akiyama Y, Dan S, Yamori T, Ito A, Yoshida M, Katoh T. Synthesis and biological evaluation of novel FK228 analogues as potential isoform selective HDAC inhibitors. *Eur. J. Med. Chem.*, **121**, 592–609 (2016).
12) Ito A, Shimazu T, Maeda S, Shah AA, Tsunoda T, Iemura S, Natsume T, Suzuki T, Motohashi H, Yamamoto M, Yoshida M. The subcellular localization and activity of cortactin is regulated by acetylation and interaction with Keap1. *Sci. Signal.*, **8**, ra120 (2015).
13) Ishfaq M, Maeta K, Maeda S, Natsume T, Ito A, Yoshida M. The role of acetylation in the subcellular localization of an oncogenic isoform of translation factor eIF5A. *Biosci. Biotechnol. Biochem.*, **76**, 2165–2167 (2012).
14) Kim HS, Vassilopoulos A, Wang RH, Lahusen T, Xiao Z, Xu X, Li C, Veenstra TD, Li B, Yu H, Ji J, Wang XW, Park SH, Cha YI, Gius D, Deng CX. SIRT2 maintains genome integrity and suppresses tumorigenesis through regulating APC/C activity. *Cancer Cell*, **20**, 487–499 (2011).
15) Chen J, Chan AW, To KF, Chen W, Zhang Z, Ren J, Song C, Cheung YS, Lai PB, Cheng SH, Ng MH, Huang A, Ko BC. SIRT2 overexpression in hepatocellular carcinoma mediates epithelial to mesenchymal transition by protein kinase B/glycogen synthase kinase-3beta/beta-catenin signaling. *Hepatology*, **57**, 2287–2298 (2013).
16) Liu PY, Xu N, Malyukova A, Scarlett CJ, Sun YT, Zhang XD, Ling D, Su SP, Nelson C, Chang DK, Koach J, Tee AE, Haber M, Norris MD, Toon C, Rooman I, Xue C, Cheung BB, Kumar S, Marshall GM, Biankin AV, Liu T. The histone deacetylase SIRT2 stabilizes Myc oncoproteins. *Cell Death Differ.*, **20**, 503–514 (2013).
17) Yang MH, Laurent G, Bause AS, Spang R, German N, Haigis MC, Haigis KM. HDAC6 and SIRT2 regulate the acetylation state and oncogenic activity of mutant K-RAS. *Mol. Cancer Res.*, **11**, 1072–1077 (2013).
18) Sunami Y, Araki M, Hironaka Y, Morishita S, Kobayashi M, Liew EL, Edahiro Y, Tsutsui M, Ohsaka A, Komatsu N. Inhibition of the NAD-dependent protein deacetylase SIRT2 induces granulocytic differentiation in human leukemia cells. *PLoS ONE*, **8**, e57633 (2013).
19) Zhao D, Zou SW, Liu Y, Zhou X, Mo Y, Wang P, Xu YH, Dong B, Xiong Y, Lei QY, Guan KL. Lysine-5 acetylation negatively regulates lactate dehydrogenase A and is decreased in pancreatic cancer. *Cancer Cell*, **23**, 464–476 (2013).