SIRT Inhibitors Induce Cell Death and p53 Acetylation through Targeting Both SIRT1 and SIRT2

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Abstract

SIRT proteins play an important role in the survival and drug resistance of tumor cells, especially during chemotherapy. In this study, we investigated the potency, specificity, and cellular targets of three SIRT inhibitors, Sirtinol, Salermide, and EX527. Cell proliferative and cell cycle analyses showed that Sirtinol and Salermide, but not EX527, were effective in inducing cell death at concentrations of 50 μmol/L or over in MCF-7 cells. Instead, EX527 caused cell cycle arrest at G1 at comparable concentrations. In vitro SIRT assays using a p53 peptide substrate showed that all three compounds are potent SIRT1/2 inhibitors, with EX527 having the highest inhibitory activity for SIRT1. Computational docking analysis showed that Sirtinol and Salermide have high degrees of selectivity for SIRT1/2, whereas EX527 has high specificity for SIRT1 but not SIRT2. Consistently, Sirtinol and Salermide, but not EX527, treatment resulted in the in vivo acetylation of the SIRT1/2 target p53 and SIRT2 target tubulin in MCF-7 cells, suggesting that EX527 is ineffective in inhibiting SIRT2 and that p53 mediates the cytotoxic function of Sirtinol and Salermide. Studies using breast carcinoma cell lines and p53-deficient mouse fibroblasts confirmed that p53 is essential for the Sirtinol and Salermide-induced apoptosis. Further, we showed using small interfering RNA that silencing both SIRTs, but not SIRT1 and SIRT2 individually, can induce cell death in MCF-7 cells. Together, our results identify the specificity and cellular targets of these novel inhibitors and suggest that SIRT inhibitors require combined targeting of both SIRT1 and SIRT2 to induce p53 acetylation and cell death. Mol Cancer Ther; 9(4); 844–55. ©2010 AACR.

Introduction

The development and progression of cancer involves both epigenetic and genetic changes leading to the alteration of gene expression and thus cell phenotype. It is well established that alteration in the epigenome of the cell through promoter hypermethylation and histone deacetylation is a common facet of tumorigenesis. Indeed, previous studies have reported that the promoters of tumor suppressor genes, such as p16INK4A and p15INK4B, frequently show increases in DNA methylation and/or histone deacetylation, thereby leading to gene silencing (1). However, emerging evidence also suggests that the role of acetylation as a protein posttranslational modification, independent of histone modification, may also play a critical role in cell fate and thus tumorigenesis. For example, the activities of the tumor suppressors p53 and FOXO3a are both in part regulated by their acetylation status. The degree of acetylation is largely mediated by a balance between histone acetyl transferase and histone deacetylase (HDAC) activity, in a substrate-specific manner.

Sirtuins (also called SIRT) are NAD-positive–dependent class III HDACs that share extensive homologies with the yeast HDAC Silent Information Regulator 2 (Sir2; ref. 2). In yeast (3), nematode worms (4), and fruit flies (5), SIRT/Sir2 activity is crucial for life span extension in response to metabolic and other environmental stresses (6–9). In mammals, seven Sir2 homologues (Sirtuin 1-7 or SIRT1-7) have been identified, which possess primarily HDAC (SIRT1, SIRT2, SIRT3, SIRT5) or monoribosyltransferase activity (SIRT4 and SIRT6; refs. 10, 11), which target histone and various nonhistone proteins in distinct subcellular locations.

The mammalian SIRT1 is the direct homologue of the yeast Sir2 and has a wide range of substrates and cellular functions. SIRT1 can induce chromatin silencing through deacetylation of histones H1, H3, and H4 (12), and can modulate cell survival by regulating the transcriptional activities of p53 (13), NF-κB (14), FOXO proteins (15, 16), and p300 (17). In contrast to SIRT1,
SIRT2 is predominantly a cytoplasmic protein and is able to deacetylate several cytoplasmic substrates, including α-tubulin (18, 19).

Both SIRT1 and SIRT2 may have a role in the development of cancer. For example, SIRT1 can activate stress defense and DNA repair mechanisms, thus allowing the preservation of the genomic integrity (20). Conversely, it has also been shown that SIRT1 overexpression can enhance tumor growth and promote cell survival in response to stress and drug resistance. Nevertheless, SIRT1 is upregulated in a spectrum of cancers, including lymphomas, leukemia and soft tissue sarcomas, prostate cancer, and lung and colon carcinomas (21–23). Overexpression of SIRT2 can significantly prolong the mitotic (M) phase and delay mitotic exit. In consequence, it has been proposed that SIRT2 might function as a mitotic checkpoint protein in G2-M to prevent the induction of chromosomal instability, particularly in response to microtubule inhibitor–mediated mitotic stress (24). Consistently, tumors with high levels of SIRT2 are refractory to chemotherapy, especially microtubule poisons (25).

The dual role of SIRTs in modulating the acetylation of tumor suppressor proteins and chromatin renders them attractive therapeutic targets for anticancer drug development (20). However, despite the development of several effective SIRT inhibitors, little is known about the specific mechanisms of action and cellular targets of these proteins. In the present study, we have used breast cancer cells as a model system, and profiled the in vitro and in vivo activity of several recently developed potent SIRT inhibitors. Moreover, we have identified the tumor suppressor p53 as the critical target of the SIRT inhibitor–induced cell death in breast cancer cells.

Materials and Methods

Cells and cell culture
The human breast carcinoma cell lines MCF-7 originated from the American Type Culture Collection and were acquired from Cancer Research UK, in which they were tested and authenticated. These procedures include cross-species checks, DNA authentication, and quarantine. Cell lines used in the present study were in culture for <6 mo and were maintained in DMEM/F12 media (Sigma) containing 10% FCS and 2 mmol/L glutamine (10% CO₂; 37°C). All final concentrations of solvent were identical between and within experiments; SIRT inhibitors were dissolved in stock solutions of 20 mmol/L in DMSO, and for treatments, the compounds were diluted in DMSO to the appropriate concentrations and added to cells [0.5% (v/v) final DMSO concentration]. All cells were <70% confluent and growing exponentially before transfection and treatment. The number of cells seeded was adjusted to attain this level of confluency depending on the culture volume and cell type, but was consistent within experiments. The population doubling time was 16 to 19 h with cell confluency maintained at <80%.

Compound synthesis
SIRT inhibitors EX527 (6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide; ref. 26), Sirtinol (2-[2-Hydroxynaphthalen-1-ylmethylene]amino]-N-(1-phenethyl) benzamide; ref. 27), and Salermide (N-[3-[2-hydroxynaphthalen-1-ylmethylene]-amino]-phenyl)-2-phenylpropionamide; ref. 28) were prepared according to reported procedures. Nicotinamide was acquired from Sigma UK.

Small interfering RNA transfection
MCF-7 cells were transfected with 100 nmol/L SIRT1 or SIRT2 small interfering RNA (siRNA; Dharmacon) using oligofectamine (Invitrogen). Twenty hours post-transfection, transfected cells were examined by cell cycle analysis.

Sulforhodamine B assay
Approximately 3,000 cells were seeded in each well of the 96-well plate. All treatment compounds were added 24 h following seeding; this is designated 0 h in the sulforhodamine B (SRB) assays. After culture, 100 μL of trichloroacetic acid were added to each well and incubated for 1 h at 4°C. The plates were then washed with deionized water thrice before incubation at room temperature for 1 h with 0.4% SRB in 1% acetic acid. The plates were then washed with deionized water and air dried. Tris base (10 mmol/L) was then added to the wells to solubilize the bound SRB dye, and the plates were then read at 492 nm using the Anthos 2001 plate read (Jencons Scientific Ltd.).

Cell cycle analysis
Cell cycle analysis was done by propidium iodide staining, as previously described (29). Both adherent and floating cells were harvested and stained with propidium iodide (0.2 mg/mL) in the presence of DNase-free RNase for flow cytometry analysis. The cell cycle profile was analyzed using the Cell Diva software (Becton Dickinson UK Ltd.).

Western blotting
Both adherent and floating cells were harvested for Western blot analysis and 20 μg of protein were loaded of each sample. Cells were lysed and SDS-PAGE gel electrophoresis was done as previously described (30). The antibodies against β-tubulin (H-235), p21 (SC-6248), and SIRT2 (H-95) were purchased from Santa Cruz Biotechnology (Autogen Bioclear). Antibodies against acetyl-Lysine (#9441), acetyl-p53 (lys382; #2525), and p53 (IC12) were purchased from Cell Signaling Technology. The antibody against SIRT1 (E104) was purchased from Abcam. Primary antibodies were detected using horseradish peroxidase linked to anti-mouse and antirabbit conjugates as appropriate (DAKO), and visualized...
using the enhanced chemiluminescence detection system (Amersham Biosciences).

**In vitro SIRT activity assay**

The Biomol hSIRT1/hSIRT2 activity assay (AK-555/AK-556) was used to deduce the activity potential of SIRT inhibitors. The assay was done essentially as described (31) and according to the manufacturer’s instructions (Biomol International). All compounds were preincubated with the hSIRT1/hSIRT2 before commencing the reaction through the addition of the “Fluor de Lys” deacetylase substrate. Deacetylation of K382-p53/K320-p53 was used as a marker of HDAC activity. All compounds were prepared fresh in DMSO to 10 mmol/L, 24 h before the assay was performed. Fluorescence was read (excitation 360; emission, 460) using the Fluoroskan Ascent FL fluorometer (Thermo Fisher) using the Ascent software. The dose concentrations were done in triplicate. The amounts of acetylation were derived from the levels of fluorescence (displayed in arbitrary fluorescence units). In addition, the percentages of inhibition were calculated from the arbitrary fluorescence unit of the treated assays and nontreated controls.

**Three-dimension modeling**

To prepare the docking-related files including protein and inhibitors, the three dimensional structure of Human SIRT1 was modeled by the Protein Homology/analogy Recognition Engine (Phyre; ref. 32). The modeled structure of SIRT1 is based on the structure of yeast Sir2 (PDB entry is 2HJH) already containing nicotinamide and Zn, with the original human SIRT1 amino acid sequence from residues 212 to 529. The three-dimensional structure of SIRT2 used for docking was taken from the human Sirtuin C-pocket (PDB entry is 1J8F). The three inhibitor structures, Sirtinol, Salermide, and EX527, were generated by the Dundee PRODRG2 Server (33). The docking studies were done using the site feature docking algorithm from the LibDock (34) software of the Discovery Studio package (version 2.1).

The force field was applied to the SIRT1 and SIRT2 using the in-built CHARMM force field calculation. The binding region for the docking of SIRT2 is the same a previously described (28), which was a 10-Å sphere around the Gln167. The docking region used for SIRT1 was designed in a 10-Å sphere around the residue of Gln345, which is genetically conserved to the Gln167 of SIRT2. All the docking studies were carried out by default setting of the LibDock software and then the generated results of these docking studies were subjected to the “Score Ligand Poses” built-in function of the Discovery Studio to evaluate and rank the docking results. The best pose of each docking study was chosen based on the LigScore2 (35). Furthermore, the best pose of each docking study was energy minimized with the protein structure by the “Ligand Minimization” function of the Discovery Studio. All the structural pre-
efficacy of some of these SIRT inhibitors on SIRT1 and SIRT2 (26, 27, 36). However, these in vitro data could not completely explain for the failure of EX527 to induce cell death, as EX527 has higher SIRT2 inhibitory activity than Sirtinol and higher SIRT1 inhibitory activity compared with Sirtinol and Salermide yet does not induce cell death.

Modeling of SIRT1/2 docking predicts sirtinol and salermide binding efficiently to SIRT1/2, and of EX527 to SIRT1 alone

Next, we analyzed the inhibitory functions of these SIRT inhibitors using computational docking studies. Preliminary, computational docking studies were carried out on Sirtinol, Salermide, and EX527 using both Gold...
and LibDock in an effort to rationalize the observed SIRT1/SIRT2 selectivity differences. For these studies, the crystal structure of hSIRT2 (PDB code: 1J8F) was used along with a hSIRT1 model, and the inhibitors docked into the C-pocket (Fig. 3). The hSIRT1 model was obtained from the Phyre Web site (www.sbg.bio.ic.ac.uk/phyre/; structure 2HJH) and based on the homologous yeast protein Sir2 (Fig. 3A). In terms of SIRT1 binding, both Sirtinol and Salermide show significant hydrogen bonding interactions with Gln 345 and His 363 (Fig. 3B). EX527 also displays hydrogen bonding to Gln 345, with additional π-π interactions with His 363 and Phe 273. In terms of SIRT2 binding, Sirtinol and Salermide show comparable hydrogen bonding interactions with Gln 345 and His 363 of SIRT1 and SIRT2. EX527 also displays hydrogen bonding to Gln 345 of SIRT1, with additional π-π interactions with His 363 and Phe 273 of SIRT1. However, EX527 does not form strong hydrogen bonding interactions with His 363 of SIRT2. Taken together, these findings suggest that Sirtinol and Salermide have high affinity for SIRT1 and SIRT2, whereas EX527 is selective for SIRT1 but not SIRT2. These findings also suggested that both SIRT1 and SIRT2 have to be inhibited to induce cell death in vivo as they target SIRT1 and SIRT2, whereas EX527 induced cell cycle arrest at G1 because it targets only SIRT1.

Sirtinol and salermide but not EX527 can induce p53 acetylation in vivo

To investigate further the distinct effects of the SIRT inhibitors on cell fate, we performed Western blot analysis on MCF-7 cells treated with 50 μmol/L for 0, 24, and 48 hours, to study their effects on the acetylation status of the SIRT1/2 target p53 (Fig. 4A). Interestingly, Sirtinol and Salermide but not EX527 could induce p53 acetylation at lysine 382, which has been shown to correlate with p53 activation. Consistently, the p53 acetylation was also
accompanied by an induction of p53 stability. The control nicotinamide was ineffective in inducing p53 acetylation at the concentrations studied. To examine the effects of the compounds on SIRT2 activity, we investigated the expression levels of acetylated tubulin, which is a specific SIRT2 target (18). The results showed that only Sirtinol and Salermide but not EX527 and nicotinamide could induce tubulin acetylation. We next treated MCF-7 cells for 24 hours with a range of concentrations of SIRT inhibitors and harvested the cells for Western blot analysis (Fig. 4B). Similar to earlier data, the results showed that at concentrations of ≥50 μmol/L, Sirtinol and Salermide, but not EX527 and nicotinamide, are able to induce p53 acetylation and that only Sirtinol and Salermide but not EX527 and nicotinamide can induce tubulin acetylation. Notably, there was also a general decrease in protein levels in MCF-7 cells after treatment with 100 μmol/L Salermide and was likely to be a result of protein degradation due to cell death after drug treatment. The failure of EX527 to induce p53 and

Figure 3. The three-dimensional structures of SIRT1 and SIRT2. A, the crystal structure of hSIRT2 (PDB code: 1J8F) was used along with a hSIRT1 model, and the inhibitors docked into the C-pocket. The hSIRT1 model was obtained from the Phyre Web site (structure 2HJH) and based on the homologous yeast protein Sir2. B, structures of the complexes of inhibitors with SIRT1 and SIRT2 modeled by docking the inhibitors onto the C-pocket of SIRT1 and SIRT2 structure after minimization.
tubulin acetylation was not because of its lack of SIRT-inhibitory activity as it could effectively cause global protein acetylation as revealed by the acetyl-Lysine antibody.

Together, these results suggest that although both Sirtinol and Salermide target SIRT1 and SIRT2, EX527 only inhibits SIRT1 and not SIRT2 effectively and that p53 acetylation requires inhibition of both SIRT1 and SIRT2.

The cytotoxic function of sirtinol and salermide is dependent on the presence of functional p53

To examine the role of p53 in mediating the cytotoxic and cytostatic effects of the SIRT inhibitors, we treated breast carcinoma cell lines (37), MCF-7 (wild-type p53), MDA-MB-231 (mutant p53), BT474 (mutant p53), and ZR-75-1 (very low p53 expression) with 50 μmol/L of SIRT inhibitors for 0, 24, and 48 hours. Flow analysis results showed that both Sirtinol and Salermide effectively induced cell death in MCF-7 cells, but the ability of Sirtinol and Salermide to induce cell death is compromised in other breast cancer cell lines without functional p53 (Fig. 5A). Notably, EX527 treatment caused a substantial increase in G1 cell population with little cell death in all breast carcinoma cell lines, with the exception of BT-474. To further confirm these results, we also studied the effects of the SIRT inhibitors on the cell cycle status of mouse embryo fibroblasts (MEF) derived from wild-type and p53-deficient mice (Fig. 5B). Consistent with the breast carcinoma cell line data, the flow cytometric results indicated that although EX527 treatment caused a substantial increase in G1 population with little cell death in both the wild-type and p53−/− MEFs, Sirtinol and Salermide only induced cell death efficiently in wild-type but not p53−/− MEFs. We next examined the expression of acetylated lysine, acetylated p53, and acetylated tubulin in the wild-type and p53−/− MEFs following SIRT inhibitor treatment. The Western blot results showed that Sirtinol and Salermide, but not EX527, are able to induce p53 acetylation in the wild-type MEFs, and that Sirtinol and Salermide but not EX527 can induce tubulin acetylation in both the wild-type and p53−/− MEFs. Western blotting using the acetyl-Lysine antibody again revealed that EX527 is more effective than Sirtinol and Salermide in inducing the acetylation of some proteins and some of these EX527 targets might account for its ability to induce G1 arrest. Cleaved caspase-3 was used as a marker of apoptosis, and the Western blot results showed that the loss of p53 decreases the cleavage and activation of caspase-3, reiterating our findings from the cell cycle analysis. Together, these results suggest that functional p53 is required for the cytotoxic function of Sirtinol and Salermide but is dispensable for the cytostatic effects of EX527.
Figure 5. Effects of SIRT inhibitors on cell cycle status and p53, tubulin, and global lysine acetylation. A, MCF-7, MDA-MB-231, BT474, and ZR-75-1 breast carcinoma cells as well as the wild-type (WT) and p53−/− MEFs were treated with 50 μmol/L of SIRT inhibitors for 0, 24, and 48 h of Sirtinol, EX527, and Salermide. Flow cytometric analysis of DNA content was done after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2-M) is indicated. Representative data from three independent experiments are shown. B, the expression of cleaved (active) caspase-3, acetylated lysine, acetylated p53, p53, acetylated tubulin, and tubulin was analyzed by Western blotting in the SIRT inhibitor–treated wild-type and p53−/− MEFs.
Silencing of both SIRT1 and SIRT2 is required for inducing MCF-7 cell death

Our earlier results suggested that induction of cell death by SIRT inhibitors in vivo requires the inactivation of both SIRT1 and SIRT2. To examine this conjecture, MCF-7 cells were transfected with either control siRNA or siRNA against SIRT1, SIRT2 or SIRT1 and SIRT2. The transfected cells were then incubation with or without 10 nmol/L of the chemotherapeutic drug paclitaxel for 24 hour before the cells were subjected to cell cycle and Western blot analyses. The cell cycle analysis showed that the siRNA-mediated knockdown of endogenous SIRT1 or SIRT2 individually had no discernable effect on the cell cycle status of MCF-7 cells, but the silencing of endogenous SIRT1 and SIRT2 simultaneously induced a noticeable G2-M phase cell cycle arrest as well as cell death (Fig. 6A). Interestingly, treatment of MCF-7 with 10 nmol/L paclitaxel also elicited a cell death and cell cycle arrest profile similar to that induced by SIRT1/2 knockdown. In addition, we discovered that silencing of SIRT2 alone actually abolished the paclitaxel-induced G2-M cell cycle arrest and cell death. The Western blot analysis confirmed successful SIRT1 and/or SIRT2 knockdown and showed that the SIRT1/2 knockdown-induced G2-M arrest and cell death was associated with an increase in p53 acetylation (Fig. 6B). Intriguingly, paclitaxel treatment also resulted in a similar increase in p53 acetylation, and this p53 hyper-acetylation was again accompanied by cells undergoing G2-M arrest and cell death. In addition, we discovered that silencing of SIRT2 alone by specific siRNA suppressed paclitaxel-induced cell cycle arrest and cell death as well as p53 acetylation. Together these results suggest that paclitaxel mediates the G2-M arrest-associated cell death through inducing the acetylation and activation of p53.

Discussion

In this study, we have for the first time compared the potencies, mechanisms of action, and cellular targets of the three recently developed SIRT inhibitors, Sirtinol, Salermide, and EX527. From cell proliferation and cell cycle assays, it was found that although Sirtinol and Salermide can effectively induce cell death, EX527 does not trigger cell death but a cell cycle arrest. The in vitro SIRT inhibitory assay shows that all three compounds potently inhibited SIRT1, with EX527 at least two orders of magnitude weaker than Sirtinol and Salermide.
of magnitude more potent than Sirtinol and Salermide. This finding is supported by computational docking studies showing that all three inhibitors bind SIRT1 with high affinity. However, despite the in vitro SIRT inhibitory assay showing high degrees of inhibitory activity against SIRT2 for all three compounds, only Sirtinol and Salermide exhibit high affinity for SIRT2 in the structural studies. The structural-docking study results are supported by the in vivo finding that EX527 failed to cause the reacetylation of tubulin, a critical cellular target of SIRT2 (38). This finding is also consistent with our in vitro results indicating that inactivation of both SIRT1 and SIRT2 is required for the induction of cell death and p53 acetylation and that EX527 neither triggers cell death nor p53 acetylation in MCF-7 cells. The structural in vitro activity discrepancy could be due to the fact that in the in vitro SIRT2 inhibitory assay, the recombinant human SIRT2 is the single predominant Sirtuin species existing at high concentrations and the specificity might be lost as a result. Alternatively, EX527 could have an altered selectivity toward the recombinant SIRT2 protein under in vitro experimental conditions. Moreover, the in vitro assays used a synthetic p53 peptide instead of a wild-type protein. These observations showed that the in vitro SIRT assays are not always consistent with findings in vivo, which may be based on cell cycle analysis, analysis of global acetylation profiles, SIRT isoform-specific target acetylation, and docking studies. Nevertheless, this also highlights the fact that the in vitro SIRT assay data have to be interpreted with caution and should not be used alone in studying SIRT or inhibitor activity. It is important to note, however, that although the structural and computational docking studies provide a qualitative appreciation of possible sources of inhibitor selectivity, further detailed biological study is required (for example, site-selective mutations) to verify these results in vivo. For example, although our results provide qualitative comparisons with a recent report by Lara et al. (28), the precise binding modes of the inhibitors differ. Moreover, computational studies by Huhtiniemi et al. (39) suggest that the conformational freedom of the “flexible loop” region of the C-pocket may play a crucial role in substrate binding, something that is not taken into account using static docking techniques.

It is notable that in this study, we examined the cellular potency and targets of the SIRT inhibitors using the MCF-7 breast carcinoma cell line because they have substantial levels of SIRT1 and SIRT2 as well as a functional p53. In mammalian cells, the transcription factor p53 functions as a tumor suppressor and is a target of acetylation as well as SIRT1 and SIRT2. The acetylation of functional p53 leads to its activation and is usually triggered in response to oncogenic as well as environmental stress signals, including oxidative stress and chemotherapeutic drug treatments (40). Once activated, p53 triggers the apoptotic program to induce cell death. In cell-based in vivo studies, both Sirtinol and Salermide but not EX527 can efficiently induce p53 acetylation and this was associated with the induction of cell death in MCF-7 cells. Moreover, further studies using breast carcinoma cell lines with different p53 status and wild-type and p53-deficient MEFs confirmed that Sirtinol and Salermide are ineffective in inducing apoptosis in the absence of functional p53 expression. These findings suggest that the tumor suppressor p53 is integral to the cytotoxic activity of the SIRT inhibitors Sirtinol and Salermide, and the failure of EX527 to trigger cell death is because of its inability to acetylate and activate the endogenous p53. A cooperative role for SIRT1 and SIRT2 to regulate stress-induced cell death pathways in a p53-independent manner has also been described (25). It is also likely that SIRT inhibitors, such as Sirtinol and Salermide, also target SIRTs other than SIRT1 and SIRT2. Nevertheless, SIRT1 and SIRT2 will probably have a more prominent role in controlling cell growth and survival as they exist in the same intracellular compartments (i.e., nucleus and cytoplasm) as most of the cell cycle and death regulators (41). The reason why Sirtinol and Salermide are ineffective in causing global lysine acetylation is unclear. However, because these global lysine acetylation patterns are associated with treatment with the very potent SIRT1 inhibitor EX527, it could be that these acetylated patterns might represent proteins preferentially targeted by SIRT1 and/or other HDACs.

Data from SIRT inhibitor experiments also suggest that both SIRT1 and SIRT2 have to be inactivated to induce cell death in vivo, at least in SIRT1- and SIRT2-positive cells. This is confirmed by the siRNA-silencing experiments showing that not one but both SIRT1 and SIRT2 have to be deleted to cause cell death. This observation is consistent with a recent study showing that despite the interaction of SIRT1 with p53, deletion of SIRT1 in MEFs fails to induce p53 acetylation and activate the apoptotic function of p53 (42). Notably, cell cycle profiles of MCF-7 cells are different in response to treatment with EX-527 compared with SIRT-1 siRNA, and this might suggest that additional mechanisms of induction of cell cycle arrest by EX-527, which could be mediated by inhibiting other HDACs. Intriguingly, treatment with the chemotherapeutic drug paclitaxel also induces a G2-M arrest-associated cell death indistinguishable from that triggered by silencing of SIRT1 and SIRT2. The fact that this was also accompanied by p53 acetylation suggests that SIRT inhibitors, such as Sirtinol and Salermide, and paclitaxel function through common pathways and mediate their cytotoxic effects through targeting p53 and acetylation. Unexpectedly, silencing of SIRT2 is sufficient to repress the cytotoxic effects of paclitaxel and abolish p53 hyperacetylation. Similarly, SIRT1 knockdown could also prevent paclitaxel from causing the reacetylation of tubulin. The molecular mechanisms responsible are unknown and might involve compensatory mechanisms between different SIRT proteins. It is also likely that SIRT2 silencing will cause cell cycle arrest and chromatin condensation as revealed by sirt2-deficient mouse studies.
(24). As a consequence, SIRT2 silencing alone may lessen the potency of chemotherapeutic drugs, such as paclitaxel, which preferentially target proliferating cells. Nevertheless, this observation further highlights the importance of p53 acetylation in the induction of cell death and that SIRT inhibitors and paclitaxel might have synergistic or additive effects at suboptimal concentrations. In summary, we have used a combination of in vitro SIRT inhibition assay, structural studies, and in vivo cell-based systems to delineate the substrate specificity and efficacy of three novel SIRT inhibitors, Sirtinol, Salermide, and EX527. Together, the results also provide evidence to show that the cytotoxic effect of SIRT inhibitors is mediated predominantly through the inactivation of both SIRT1 and SIRT2 and the subsequent acetylation of the tumor suppressor p53. These data also suggest that a redundancy of functions may exist between SIRT1 and SIRT2, and that SIRT1 and SIRT2 cooperate to deacetylate the tumor suppressor protein p53 to attenuate cell death.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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