Tenovin-D3, a Novel Small-Molecule Inhibitor of Sirtuin SirT2, Increases p21 (CDKN1A) Expression in a p53-Independent Manner

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Introduction

Histone deacetylases, or more precisely lysine deacetylases, can be divided into 4 major groups (1). Class I, II, and IV deacetylases are Zn2+-dependent, whereas class III HDACs require NAD+ as a co-factor. Class III includes the sirtuins, a family of NAD+-dependent lysine deacetylases and ADP-ribosyl transferases that link metabolic state to a variety of cellular processes including regulation of gene expression. Small-molecule inhibitors of class I and II HDACs are being used in the clinic for several conditions and for basic research. For example, SAHA (suberoylanilide hydroxamic acid/vorinostat) has been recently approved for the treatment of cutaneous T-cell lymphoma (2). Furthermore, trichostatin A (TSA), also a hydroxamic acid and a classic inhibitor of class I/II HDACs, improves the efficiency of protocols used to reprogram somatic cells into induced pluripotent stem (iPS) cells (3, 4).

While small-molecule inhibitors of class I/II histone deacetylases (HDAC) have been approved for cancer treatment, inhibitors of the sirtuins (a family of class III HDACs) still require further validation and optimization to enter clinical trials. Recent studies show that tenovin-6, a small-molecule inhibitor of sirtuins SirT1 and SirT2, reduces tumor growth in vivo and eliminates leukemic stem cells in a murine model for chronic myelogenous leukemia. Here, we describe a tenovin analogue, tenovin-D3, that preferentially inhibits sirtuin SirT2 and induces predicted phenotypes for SirT2 inhibition. Unlike tenovin-6 and in agreement with its weak effect on SirT1 (a p53 deacetylase), tenovin-D3 fails to increase p53 levels or transcription factor activity. However, tenovin-D3 promotes expression of the cell-cycle regulator and p53 target p21WAF1/CIP1 (CDKN1A) in a p53-independent manner. Structure–activity relationship studies strongly support that the ability of tenovin-D3 to inhibit SirT2 contributes to this p53-independent induction of p21. The ability of tenovin-D3 to increase p21 mRNA and protein levels is shared with class I/II HDAC inhibitors currently used in the clinic and therefore suggests that SirT2 inhibition and class I/II HDAC inhibitors have similar effects on cell-cycle progression. Mol Cancer Ther; 1–9. ©2013 AACR.

Abstract

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Interestingly, this feature is characteristic of inhibitors of class I and II HDACs (also called here non-sirtuin HDACs) such as TSA and the clinically approved SAHA (15, 16).

Materials and Methods

**Biological materials**

H1299, MCF7, MDAMB231, MDAMB468, U2OS, A431, HeLa, and HEK293 cells were obtained from the American Type Culture Collection. Normal human fibroblast cells (NHDF) were purchased from Promocell. ARN8, H1299-pCMV, and H1299-SirT2 overexpressing cells are described in ref. 6, and SAO2S cells were a kind gift from Klas Wiman (Karolinska Institutet, Stockholm, Sweden). HCT116 and HCT116-p53 null cells were a kind gift from Bert Vogelstein (Johns Hopkins University, Baltimore, MD). MDAMB468 cells expressing inactive SirT2 were generated by transfecting a vector expressing catalytically inactive SirT2 (pcDNA3-Neo-SirT2H187Y) and selecting with G418. Control cell lines were generated by transfecting a control vector (pcDNA3-Neo). All cells were grown in high-glucose Dulbecco’s Modified Eagles’ Media (DMEM) supplemented with 10% FBS and penicillin/streptomycin except H1299 and HCT116 cells (grown in RPMI-1640 medium and McCoy’s 5A modified medium respectively). Cells were grown at 37°C and 5% CO2 in a humidified atmosphere. The authors did not authenticate the cell lines for the present study. Cell lines were also routinely tested for mycoplasma (MycoAlert Detection Kit, Lonza).

Antibodies to the following targets were used: K40-acetylated α-tubulin (Sigma #T7451), α-tubulin (Sigma #T6199), SirT2 (Abcam #51023), GAPDH (Fitzgerald #RDI-TRK 564-6C5), and K38-acetylated p53 (Biologend #614202). Antibodies against p21 (118), p53 (DO1), and mdm2 (4B2) are described previously (6).

**Compounds**

Tenovins D3 and D1 were synthesized as described in the Supplementary Information. Tenovins-6, 30a, 30b, 30d, 30n 30j, 30k, 33, and 39 are described previously (17), whereas AM158 is described (6). TSA (#T8552), nutshell-3 (#N6287, racemic), and 4-AA (#100528) were purchased from Sigma-Aldrich, whereas 5406085 was sourced from ChemBridge (#5406085) and tubacin (#BML-GR362) from Enzo Life Sciences. Compound stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 40 mmol/L and stored at −20°C.

**SirT2 expression and purification**

SirT2 cDNA (residues 34–356) was cloned into the PGEX-6P-1 vector (GE Life Sciences) and overexpressed in BL21(DE3)T1R bacterial cells (Sigma) as a glutathione S-transferase (GST) fusion protein. Competent bacteria were transformed with the SirT2 plasmid and starter cultures (10 mL) grown overnight at 37°C in LB medium with ampicillin (100 mg/L). The overnight starter culture was added to 1 L of LB media containing ampicillin, which was grown at 37°C until the OD600 reached 0.9. IPTG (0.1 mmol/L) and Zn(OAc)2 (40 μmol/L) were added and the culture induced at 24°C overnight. The cells were harvested by centrifugation at 11,899 × g for 25 minutes before storing the resulting pellets at −20°C. SirT2-containing pellets were resuspended in lysis buffer (50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 5 mmol/L β-mercaptoethanol, 1% Triton-X-100, Roche EDTA-free mini protease inhibitor cocktail) and incubated on ice for 30 minutes with lysozyme (1 mg/mL). The suspension was sonicated for 30 seconds (8×). The soluble fraction was collected after centrifugation of the lysed pellet suspension at 20,442 × g for 20 minutes. The resulting supernatant was filtered, added to prewashed glutathione sepharose (GE LifeSciences), and rotated gently at 4°C for 2 hours. The supernatant was removed after centrifugation at 2,010 × g for 5 minutes and the sepharose washed with buffer (50 mmol/L Tris, pH 8, and 150 mmol/L NaCl) to remove nonspecifically bound proteins. The GST fusion protein was then eluted with elution buffer (50 mmol/L Tris, pH 8, 10 mmol/L reduced L-glutathione). Fractions containing GST-SirT2 were pooled and dialysed into buffer (50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 1 mmol/L β-mercaptoethanol). Cleavage of the fusion protein was achieved using PreScission protease (GE LifeSciences) in fresh buffer overnight. SirT2 was separated from GST via glutathione sepharose chromatography (GST-Trap, GE LifeSciences), and the SirT2-containing fractions were pooled and further purified via gel filtration chromatography.

**Sirtuin and HDAC8 activity assays**

**Fluorescence-based assays.** In vitro deacetylation assays were conducted using components from the Fluor de Lys Fluorescent (FdL) Assay Systems (Biomol kits #AK555, #AK556, #AK557, and #AK518) as described (6). SirT1, SirT2, and SirT3 FdL substrates were used at 15 mmol/L, whereas HDAC8 FdL substrate was used at 25 mmol/L. IC50 values were determined as described (17).

**Assay using cellular extract containing acetylated α-tubulin.** Cytoplasmic extracts were prepared following the Dignam method and combined with dd H2O, 10× complete EDTA-free protease inhibitor (Roche), 527 mmol/L Tris, pH 7.5, 5 mmol/L NAD+, 5 mmol/L dithiothreitol (DTT), 750 mmol/L NaCl, 0.5 mmol/L TSA, and 2 mmol/L phenylmethylsulfonylfluoride (PMSF) and incubated on ice before the addition of premixed recombinant SirT2 (0.027 mg/mL) and tenovin-D3. Levels of K40-acetylated α-tubulin and total α-tubulin were determined by Western blot analysis.

**Western blotting.** Western blot analysis was conducted as described previously (18). DTT (Sigma D60632) was added to the samples to a final concentration of 100 mmol/L. Proteins were separated on NuPAGE 4%–12% bis-tris gels (Invitrogen) in 1× NuPAGE MOPS buffer (Invitrogen #NP000102) supplemented with NuPAGE antioxidant (Invitrogen #NP0005). The mobility of the proteins of interest was determined using molecular weight markers (SeeBlue Plus2, Invitrogen #LC9295). Protein transfer to polyvinylidene difluoride (PVDF) membranes (Millipore #PVH00010) was done with 1×
NuPAGE transfer buffer (Invitrogen #NP0006). Following incubation with primary and horseradish peroxidase-conjugated secondary antibodies, chemiluminescent signals were detected with ECL solution (Amersham Biosciences). Adobe Photoshop was used to adjust the brightness and contrast of scanned blot images. All lanes were treated in the same manner.

**p53-dependent transcription measurements**

ARN8 human melanoma cell lines expressing β-gal under the control of a p53-dependent promoter (RGCΔFos-LacZ) were incubated with compounds for 16 hours. Cells were lysed and β-galactosidase activity detected using CPRG as a substrate as described (19).

**RNA preparation and TaqMan-PCR analysis**

Total RNA was extracted, cDNA prepared, and real-time PCR carried out as previously described (18).

**Transwell assay**

Following treatment for 2 hours, 10^5 cells in 100 μL serum-free medium were seeded into the upper wells and 600 μL growth medium containing 10% v/v FBS added as a chemo-attractant to the lower wells of 6.5 mm Transwells with 8.0-μm pores (Corning). After a 4-hour incubation at 37°C to allow for migration, cells in the upper wells were removed with cotton swabs. Subsequently, membranes were fixed with 1% v/v formaldehyde in PBS and nuclei stained with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector). Pictures of each 7 representative fields per membrane were taken with a fluorescent microscope (Leica) at ×40 magnification and nuclei were counted.

**Immunocytochemistry**

Cells seeded in 2-well glass chamber slides (Nunc) were treated and stained as previously described (7).

**Results**

**Discovery and characterization of a SirT2 selective analogue of tenovin-6**

Figure 1A describes the synthesis of tenovin-D3. Further experimental details and purity analysis are specified in Supplementary Methods, Supplementary Fig. S1, and Supplementary Table S1. Inhibition of purified SirT1 and SirT2 deacetylase activity is shown in Figure 1B. Tenovin-D3 inhibited SirT2 deacetylase activity with an IC50 of 21.8 μmol/L, while SirT1 was less affected (IC50 > 90 μmol/L). Error bars correspond to SDs, n = 3. C, tenovin-6 and D3 inhibit SirT2-mediated deacetylation of full-length α-tubulin at lysine 40 from a cytoplasmic extract.

Figure 1. Synthesis and characterization of tenovin-D3 in biochemical assays. A, synthesis of tenovin-D3 and structure of tenovin-6. Reagents and conditions: (i) NaSCN, acetone, rt, 16 hours; (ii) acetone, rt, 16 hours then (iii) 2 mol/L HCl in diethyl ether, 64%. B, inhibition of purified SirT1 and SirT2 by tenovin-D3 using fluorescent peptide substrates at a concentration of 15 μmol/L (SirT1 IC50 > 90 μmol/L, SirT2 IC50 = 21.8 ± 2 μmol/L). Error bars correspond to SDs, n = 3. C, tenovin-6 and D3 inhibit SirT2-mediated deacetylation of full-length α-tubulin at lysine 40 from a cytoplasmic extract.
SirT2 by tenovin-D3 was tested using fluorescently labeled peptide substrates. As shown in Fig. 1B and Supplementary Fig. S2, tenovin-D3 inhibits purified SirT2 deacetylase activity, whereas it is significantly less potent against SirT1. The efficiency of inhibition is dependent on the substrate concentration as observed with tenovin-6 (6).

To avoid potential artifacts associated with using fluorescently labeled peptides as previously reported (20, 21), we developed a biochemical assay that uses full-length α-tubulin as a substrate for SirT2. As shown in Fig. 1C, both tenovin-6 and tenovin-D3 inhibit recombinant SirT2 in this assay.

In cells, tenovin-D3 increases the levels of acetylated α-tubulin, a well-established substrate for SirT2 (ref. 10; Fig. 2A), and this increase is reversed when SirT2 is overexpressed (Fig. 2B). This supports that tenovin-D3 inhibits SirT2 in cells.

**Tenovin-D3 does not increase p53 levels or induce p53-dependent transcription**

Tenovin-6 was identified by its ability to increase p53 levels as well as transcription from p53 target genes such as the CDKN1A gene encoding p21 (6, 18). In addition, it was shown that tenovin-6 inhibits purified SirT1 deacetylase activity and increases the levels of acetylated p53 in cells (6, 18). As SirT1 deacetylates and inactivates p53 (8, 9), we proposed a model in which tenovin-6 causes the induction of p53 through its ability to inhibit SirT1 (6, 18). This has been recently supported by a series of structure–activity relationship (SAR) studies (17). Here, we strengthen this model further by showing that, unlike tenovin-6 and in agreement with its lack of SirT1 inhibition, tenovin-D3 does not increase p53 levels or transcription factor function (Fig. 2C and D; ref. 17).

**Tenovin-D3 upregulates p21 expression in a p53-independent fashion**

Despite its inability to activate p53, we found that a short treatment with tenovin-D3 led to an increase in p21 protein in some tumor cell lines while not in others (Fig. 3A and Supplementary Fig. S3). Interestingly, this effect of tenovin-D3 is independent of p53 status and particularly noticeable in MDAMB468 cells, which express the transcriptionally inactive p53R273H mutant (Fig. 3A).

In MDAMB468 cells, p21 mRNA levels were also markedly increased upon short-term treatment with tenovin-D3, whereas the mRNAs of other p53-dependent

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**Figure 2.** Tenovin-D3 (tnvD3) induces phenotypes consistent with SirT2 inhibition in cells. A, tenovin-D3 increases the levels of acetylated α-tubulin in cells. H1299 cells were treated with the indicated amounts of tenovin-D3 for 16 hours. TSA (40 nmol/L) was added to reduce background due to non-sirtuin HDAC activity. K40-acetylated α-tubulin and total α-tubulin were analyzed by Western blotting. B, H1299 cells stably overexpressing SirT2 (H1299-SirT2) or empty vector transfected (H1299-pCMV; ref. 6) were treated with the indicated amounts of tenovin-D3 for 16 hours and analyzed as in A. C, MCF7 cells expressing wild-type p53 were treated with the indicated compounds for 8 hours. p21 and p53 were detected. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as a control. D, MCF7 cells stably overexpressing SirT2 (H1299-SirT2) were treated with the indicated amounts of tenovin-D3 for 16 hours and analyzed as in A. E, MCF7 cells expressing wild-type p53 were treated with the indicated compounds for 24 hours. β-Gal p53 reporter activity was measured. MCF7 cells expressing wild-type p53 were treated with the indicated compounds for 6 hours. Nutlin-3 is a small-molecule activator of p53 (30). mRNA levels of p53-downstream targets were detected. β-Actin mRNA was analyzed as a control. Error bars correspond to SDs, n = 3.
genes remained unchanged (Fig. 4A). In cell lines where the effect of tenovin-D3 on p21 protein levels was lower, weaker effects of this compound on p21 mRNA levels were observed.

To provide evidence that SirT2 inhibition can actually lead to an increase in p21 levels, we conducted the following SAR studies. Tenovin analogues that are deficient for SirT2 inhibition (17, 6) also fail to increase p21 levels in MDAMB468 cells (Fig. 3B). In contrast, treatment with tenovin-6, tenovin-39, and other tenovin-based inhibitors of SirT2 resulted in an increase in p21 protein levels. As shown in Fig. 4B, tenovin-6 also leads to increased p21 mRNA levels in p53-mutant cell lines. These SAR studies strongly support that the effect of tenovin-D3 on p21 expression is related to its ability to inhibit SirT2. Two analogues, tenovin-30d and tenovin-30n failed to increase p21 levels in MDAMB468 despite the fact they inhibit purified SirT2 (17). However, these 2 compounds are poor at increasing tubulin acetylation in cells. This strongly suggests that their lack of activity on p21 levels is due to cell permeability or stability issues. In addition, we observed that expression of a catalytically inactive and dominant-negative SirT2 mutant (10) increases p21 levels in MDAMB468 p53-mutant cells (Fig. 3D).

As shown above, the effect of tenovin-D3 on acetylated tubulin was prevented in SirT2-overexpressing cells (Fig. 2B). However, the increase in p21 levels by tenovin-D3 was only partially reversed by SirT2 overexpression in the same cell system (Fig. 3C). This could imply that tenovin-D3 effects other than SirT2 inhibition are involved in the increase in p21, including effects on other sirtuins or on other factors. The amino acid sequences of catalytic sites in sirtuins and non-sirtuin HDACs are not related. Therefore, it is unlikely that tenovins inhibit non-sirtuin HDACs efficiently. As a control experiment, we tested tenovin-D3

![Figure 3. Tenovin-D3 does not activate p53 but can increase p21 levels. A, tenovin-D3 increases p21 levels independently of p53. Cell lines with differing p53 status were treated with tenovin-D3 for 8 hours. Indicated proteins were detected. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as a loading control. B, tenovins that are poor inhibitors of SirT2 do not increase p21 levels. Other tenovins that inhibit SirT2 do increase p21 levels. MDAMB468 cells were treated with 15 mM of the indicated compounds for 6 hours. p21 and α-tubulin were detected. C, stable cell lines generated from H1299 cells (p53-null) following transfection with a control plasmid (H1299-pCMV) or a plasmid-overexpressing SirT2 (H1299-SirT2) were treated with tenovin-D3 in the presence of 40 nM TSA and the indicated proteins detected. The intensity of the p21 bands was measured. D, extracts from MDAMB468 cells stably transfected with a vector expressing catalytically inactive SirT2 (lanes 1 and 4) or control vector (lanes 2 and 3) were probed with antibodies against the indicated targets. D, DMSO vehicle.](https://www.aacrjournals.org/tc/article-pdf/2013/1/OF5/329215/329215.pdf)
in an HDAC8 assay. As shown in Supplementary Fig. S4, and in agreement with our previous data (6), both tenovin-6 and tenovin-D3 are poor inhibitors of HDAC8. Biochemical assays for all the sirtuins are not yet available. Therefore, aside from SirT1 and SirT2, we have only been able to test the effect of tenovin-D3 on one additional sirtuin. As shown in Supplementary Fig. S4, tenovin-D3 inhibits SirT3 by only 20% at 60 μmol/L.

**Similarities between the effects of tenovin-D3 and class I/II HDAC inhibitors**

Several inhibitors of class I/II HDACs protect α-tubulin from deacetylation, an event thought to be mediated by HDAC6 (22). Confirming this, in our hands, both TSA (a general inhibitor of class I/II HDACs) and tubacin (a more selective inhibitor of HDAC6; ref. 22) increase levels of acetylated α-tubulin in MDAMB468 cells (Fig. 6C). Modulation of the acetylation status of tubulin has been implicated in the negative effects of these inhibitors on cell migration (22, 23). Because tenovin-D3 increases the levels of acetylated α-tubulin, we tested whether it also affects cell migration. As shown in Fig. 5A, a short-term treatment with tenovin-D3 does indeed reduce cell migration in a Transwell assay.

It is still unclear whether inhibitors of class I and II HDACs activate p53 function as single agents (24). A survey of the existing literature suggests that p53 activation by these agents may depend on the compound tested, the cell lineage, and the incubation time. Long-term treatments with these inhibitors could lead to activation of p53 through indirect mechanisms. To clarify this issue and to compare the effects of tenovin-D3 with non-sirtuin inhibitors, we tested whether short-term treatment of MCF7 cells with TSA affects p53 levels and p53-dependent transcription (Sachweh and colleagues, in revision). Under these conditions, and as shown above for tenovin-D3, TSA does not activate p53 (Sachweh and colleagues, in revision and Fig. 5B), whereas under the same conditions, TSA clearly increases α-tubulin acetylation (Fig. 5C). Tubacin also failed to boost p53-dependent transcription in ARN8 cells (Fig. 5D). We conclude that in our experimental settings, tenovin-D3, TSA, and tubacin fail to activate p53.

It is also important to note that induction of p21 expression in a p53-independent manner is characteristic of non-sirtuin HDAC inhibitors including TSA (15, 16, and Supplementary Fig. S6). Confirming these reports, and as shown here with tenovin-D3, TSA treatment leads to the accumulation of p21 protein and p21 mRNA in the MDAMB468 cell line (Fig. 6A and B and Supplementary Fig. S7). Under the same conditions, the HDAC6 inhibitor tubacin has a weaker effect on p21 levels in MDAMB468.
cells (Fig. 6C), suggesting that inhibition of HDAC6 is not sufficient to explain the induction of p21 by TSA. Finally, we show that tenovin-D3 still increases p21 levels in MDA-MB-468 cells even in the presence of high doses of TSA (Fig. 6A). This indicates that tenovin-D3 and TSA complement each other’s effect on p21 expression.

In summary, these results show that there are similarities between the responses to SirT2 inhibitor and non-sirtuin HDAC inhibitor treatments.

Discussion

Elucidating molecular mechanisms for compounds identified by phenotypic screens is a difficult endeavor and constitutes the major challenge in Forward Chemical Genetic approaches (25). However, recent technological advances have provided more examples where the identification of the target(s) for small molecules in cells has been successful. In drug discovery, the challenging target ID process is followed by the no less demanding task of confirming that the phenotypic changes induced in cells are associated with modulation of the putative target by the small molecule.

We have previously identified tenovin-6 as an inhibitor of both SirT1 and SirT2 deacetylase activities (6). Here, we describe the synthesis of tenovin-D3 and its characterization as a selective SirT2 inhibitor. We show that this compound induces cell phenotypes associated with SirT2 depletion but fails to activate p53 in all cell lines tested, which correlates with its inability to inhibit purified SirT1. In addition, we have used tenovin-D3 as a chemical tool to show that SirT2 regulates the acetylation status of p53 in the cytoplasmic compartment (where SirT2 is primarily localized) and not in the nucleus (van Leeuwen and colleagues, in revision).

Unexpectedly, tenovin-D3 induces expression of the p21 cell-cycle inhibitor, a downstream target of the transcription factor function of p53 in a p53-independent fashion. Suggesting that this is due to the ability of tenovin-D3 to inhibit SirT2, other tenovin analogues that do not inhibit SirT2 in vitro (17) also fail to increase p21 levels in cells expressing inactive p53. Furthermore, p21 levels are increased in MDA-MB-468 p53-mutant cells harboring a catalytically inactive SirT2 mutant that is thought to have a dominant-negative effect. However, unlike the effect of tenovin-D3 on tubulin acetylation, the increase in p21 levels was only partially reversed by overexpression of SirT2, which suggests that tenovin-D3 may have other targets in addition to SirT2.

Non-sirtuin HDAC inhibitors as well as SirT2 inhibitors may be of interest to reduce neurodegenerative processes (26–28). In the cancer field, non-sirtuin HDAC inhibitors have attracted significant attention during the last years and, in the case of inhibitors of HDAC I and II families, some have reached clinical trials. One of the most consistent findings with inhibitors of class I and II HDACs is

Figure 5. Similarities between tenovin-D3 and non-sirtuin HDAC inhibitors. A. ARN8 melanoma cells were treated as indicated and their migration analyzed in a Transwell assay. Error bars correspond to SDs, n = 4. B. MCF7 cells were incubated as indicated for 6 hours and mRNA levels measured. Error bars correspond to SD, n = 3. C. MCF7 cells were treated with vehicle (EtOH) or TSA as indicated. Acetylated α-tubulin was detected by immunocytochemistry. DNA was stained with Hoechst. D. ARN8 cells were treated with nutlin (2 µmol/L) or tubacin for 16 hours and p53-dependent transcription detected as in Fig. 2D. Error bars correspond to SDs, n = 6. Structure of nutlin-3.
their ability to increase p21 levels irrespective of p53 status (15, 16, and Supplementary Fig. S6). This includes treatments with TSA and SAHA, which is approved for cancer treatment (29). Which of the HDACs inhibited by these compounds contribute to their effect on p21 still remains to be elucidated and most likely involves inhibition of several HDACs.

These findings show that there are similarities between the effects of non-sirtuin HDAC inhibitors and the effects of tenovin-D3, suggesting that targeting SirT2 may enhance the therapeutic outcome of HDAC inhibitors in the clinic. For these combination studies to be clinically relevant, they should be conducted using the clinically approved SAHA and T-cell cutaneous lymphoma models.

Finally, it is necessary to determine all the events leading to p21 induction in response to tenovin-D3. This requires better characterization of sirtuin substrates and reagents to detect them in cells, as well as biochemical assays for all sirtuin family members. Regarding the characterization of non-sirtuin–related effects of tenovins, we have preliminary data showing that some tenovin analogues affect redox signaling. Further SAR studies are needed to determine whether this property of certain tenovins contributes to the induction of p21 expression.

Given the promising results obtained with tenovin-6 in imatinib-resistant CML stem cells (11, 14), it is of great importance to identify and understand all the effects that the tenovins are inducing in cells.

Disclosure of Potential Conflicts of Interest

N.J. Westwood has ownership interest (including patents) in a patent application in progress on compounds disclosed in the article. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: A.R. McCarthy, N.J. Westwood, S. Lain
Development of methodology: A.R. McCarthy, N.J. Westwood, S. Lain
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.R. McCarthy, M.C.C. Sachweh, J. Campbell, C. J. Drummond, L. Pirrie, M.J.G.W. Ladds, N.J. Westwood
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