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Discovery, In Vivo Activity, and Mechanism of Action of a Small-Molecule p53 Activator

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SUMMARY

We have carried out a cell-based screen aimed at discovering small molecules that activate p53 and have the potential to decrease tumor growth. Here, we describe one of our hit compounds, tenovin-1, along with a more water-soluble analog, tenovin-6. Via a yeast genetic screen, biochemical assays, and target validation studies in mammalian cells, we show that tenovins act through inhibition of the protein-deacetylating activities of SirT1 and SirT2, two important members of the sirtuin family. Tenovins are active on mammalian cells at one-digit micromolar concentrations and decrease tumor growth in vivo as single agents. This underscores the utility of these compounds as biological tools for the study of sirtuin function as well as their potential therapeutic interest.

INTRODUCTION

The forward chemical genetic (FCG) approach to drug discovery (Peterson and Mitchison, 2002; Schreiber, 2003) has a series of advantages over more classical methods based on biochemical screens but also involves important challenges. In the case of small-molecule screens carried out using a mammalian cell-based assay, the main advantage is that hit compounds show activity in cultured cells at concentrations that are acceptable for further experiments in organisms. The use of cell-based assays that require the expression of a reporter protein has the added advantage that the hit compounds are not general cytotoxics, as they are selected for their ability to increase a synthetic event. p53's tumor suppressor function depends on its ability to function as a transcription factor, and p53 is exquisitely sensitive to various stresses (Vousden and Lane, 2007). With all of this in mind, we set out to discover compounds that activate p53 in mammalian cells through the detection of an increase in expression of a reporter construct under the control of a p53-dependent promoter.

The major challenge in FCG is the elucidation of the precise mechanism of action of a hit compound (Peterson and Mitchison, 2002; Schreiber, 2003; Zheng et al., 2004). Searching for small-molecules that activate the transcriptional activity of p53 would be expected to lead to the discovery of both DNA-damaging agents and compounds that are specific for the p53 pathway, including agents that interact directly with p53 (Issaeva et al., 2004) or that inhibit mdm2 (Vassilev et al., 2004). The physiological role of mdm2 is to inhibit and destabilize p53 (Vousden and Lane, 2007). In addition, this approach was expected to identify compounds that interact with factors upstream of p53 and, therefore, also have effects on a variety of cellular networks.

SIGNIFICANCE

A major advantage of small-molecule cell-based screens is the identification of compounds that are bioactive at low concentrations. Here, we demonstrate that using p53 activation in cells as a primary screening assay leads to the discovery of small molecules with identifiable targets. To date, we have shown that two hit compounds from this screen are active in animal models and could lead to additional chemotherapeutics. This work expands current views in the drug discovery field regarding the utility of cell-based primary screens. The likelihood of success in the elucidation of the mechanism of action of hit compounds is rapidly growing with the development of sophisticated genetic screens and the plethora of findings and excellent reagents derived from basic research on cellular networks.

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Since the p53 tumor suppressor is activated in response to alterations in a wide variety of cellular events, identifying the protein target of a given p53-inducing compound can be viewed as a serious challenge. At the same time, given that many aspects of p53 regulation have been studied, we envisaged that identifying testable hypotheses relating to the mechanism of action of hit compounds in cells was achievable. Here, we describe the discovery and characterization of a bioactive small-molecule activator of p53 that we have named tenovin-1 and an analog with improved physical properties (tenovin-6). The antitumor activity these two compounds demonstrates that they are active in organisms and encouraged us to carry out experiments aimed at elucidating their precise mechanism of action. We show that tenovins inhibit the activities of human SirT1 and SirT2, two members of the NAD^+-dependent class III histone deacetylases that also belong to the sirtuin family.

Sir2p, one of the sirtuin homologs in yeast, helps connect metabolism to gene expression, and elevated Sir2p (or Sir2p-like) expression correlates with lifespan extension in several organisms. Mammals have seven sirtuin homologs (sirtuins, SirT1-7) with diverse NAD^+-dependent enzymatic activities (protein deacetylase and/or ADP-ribosyl transferase), cellular locations, and substrates (Haigis and Guarente, 2006; Michan and Sinclair, 2007). SirT1, SirtT2, and SirtT3 are highly homologous in sequence (Frye, 2000), show NAD^+-dependent protein deacetylase activity, and differ in their subcellular localization. SirT1 is nuclear and targets a variety of acetylated substrates (including p53) involved in gene expression, cell survival, differentiation, and metabolism. SirT2 is primarily cytoplasmic, targeting α-tubulin, but can also deacetylate histone H4. Finally, SirT3 is predominantly mitochondrial where it is proposed to regulate the function of acetyl-CoA synthetase 2. This study shows that using p53 as a sensor for compound activity in cells and exploiting the vast amount of available information on the regulation of p53 function can rapidly lead to the discovery of small-molecule tools with potential as therapeutics.

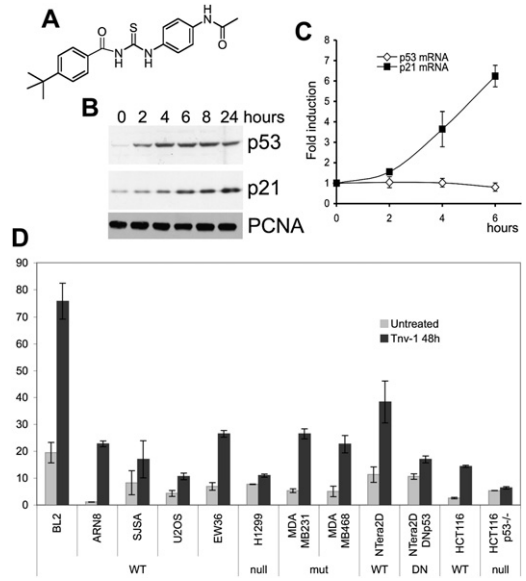

Figure 1. Effect of Tenovin-1 on Cultured Tumor Cell Lines

(A) Tenovin-1 structure.

(B) MCF-7 cells (expressing wild-type p53) were treated with 10 μM tenovin-1 for the indicated times. p53 levels and p21 levels were detected using DO1 (Bartkova et al., 1993) and 118 (Fredersdorf et al., 1996) mouse monoclonal antibodies, respectively. An antibody against α-tubulin (Cat. No. T9026, Sigma) was used to monitor loading efficiency.

(C) MCF-7 cells were treated with 10 μM tenovin-1 for the indicated times, and p53 and p21 mRNA levels were analyzed by Taqman-PCR as described (Saville et al., 2004). Error bars correspond to standard deviation values (n = 3).

(D) Toxicity of tenovin-1 on cultured tumor cells. Tumor cell lines were treated with DMSO (control) or with 10 μM tenovin-1 for 48 hr. Cell death (necrosis and apoptosis) was measured by annexin-V/propidium iodide labeling and FACS. Values correspond to the average of two independent experiments (±SD). p53 status in each cell line is indicated (DN, coexpressing the dominant-negative form of p53).

RESULTS

Discovery and Characterization of Tenovin-1

Following a pilot study with 4,000 compounds (Berkson et al., 2005), we screened 30,000 drug-like small molecules from the Chembridge DIVERSet for their ability to activate p53 in a robust, simple, and cheap primary cell-based screening assay. For details on the primary assay, secondary assays, and criteria used for prioritizing compounds, see the Supplemental Data available online. Here we describe the characterization of one hit compound from this screen, tenovin-1 (see Figure 1A for structure). As shown in Figure 1B, tenovin-1 elevates the amount of p53 protein within 2 hr of treatment. This compound also increases the levels of the p53-downstream target p21CIP/WAF1 protein (Figure 1B) and mRNA (Figure 1C), confirming that tenovin-1 can induce expression from an endogenous p53-dependent promoter. Tenovin-1 treatment does not alter p53 mRNA levels (Figure 1C), but increases p53 levels when p53 is coexpressed with mdm2 (see below and Figures 6A and 6F). This suggests that tenovin-1 protects p53 from mdm2-mediated degradation with little effect on p53 synthesis.

We observed that long-term treatment (4 days) with tenovin-1 decreases growth in all tumor cell lines tested. In order to identify those that are particularly sensitive to tenovin-1 for further in vivo studies, we compared the effects of a 48 hr treatment with tenovin-1 on the viability of a variety of tumor cell lines (Figure 1D). Treatment of BL2 Burkitt's lymphoma cells expressing wild-type p53 with 10 μM tenovin-1 for 48 hr leads to more than 75% cell death (Figure 1D). p53 levels in BL2 cells are increased by tenovin-1 (Figure 2A), and a 2 hr single treatment with tenovin-1 followed by 4 days of incubation in the absence of compound is sufficient to decrease growth and kill the majority of these cells in culture (Figure 2B). Initial in vivo experiments indicated that tenovin-1 impairs the growth of BL2-derived tumor xenografts (Figure S2). However, BL2-derived tumors grew slowly and at very different rates; hence, it was decided that this cell line was not ideal for further in vivo experiments. Among the cell lines studied in Figure 1D, ARN8 melanoma cells (p53 wild-type) showed the highest ratio between the percentage of dead cells in tenovin-1-treated and untreated cultures. ARN8 cells derive from the highly aggressive melanoma cell line A375, contain a p53-reporter gene that is induced by incubation with tenovin-1 (data not shown), and their p53 levels are responsive to tenovin-1 (Figure 2C). Furthermore, ARN8 cells give rise to fast growing tumors in SCID mice. Hence, these cells were chosen for in vivo studies (see below).

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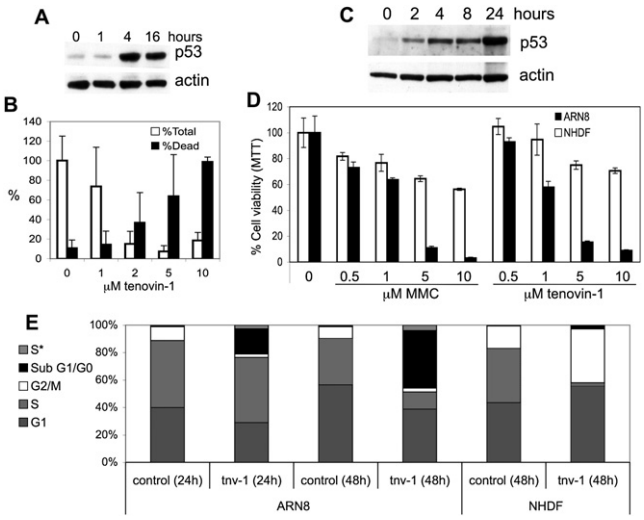

Figure 2. Effect of Tenovin-1 on BL2 and ARN8 Tumor Cell Lines and on Normal Human Dermal Fibroblasts

(A) BL2 Burkitt lymphoma cells were treated with 10 μM tenovin-1 for the indicated times, and p53 and actin were detected with DO1 or antibody CP01 (Calbiochem), respectively.

(B) BL2 cells were treated with increasing concentrations of tenovin-1 for 2 hr, after which tenovin-1 was removed and cells were cultured for 4 days in fresh medium. Cells were stained with trypan blue and counted. White bars represent the total number of cells in each sample (viable and nonviable) expressed as a percentage of the average number of cells in the untreated control samples. Black bars represent the percentage of dead cells in each sample (trypan blue-stained cells). Values correspond to the average of four independent experiments ± SD.

(C) ARN8 cells were treated with 10 μM tenovin-1 for the indicated times. p53 and actin were detected.

(D) Subconfluent ARN8 tumor cells or normal human dermal fibroblasts (NHDF) were treated with the indicated amounts of mitomycin C or tenovin-1 for 48 hr. Cell growth was determined by MTT assay (Smart et al., 1999). Values correspond to the average of three independent experiments ± SD.

(E) ARN8 tumor cells or normal human dermal fibroblasts (NHDF) were left untreated or treated with 10 μM tenovin-1 for the indicated times. Cell-cycle distribution was analyzed by BrdU labeling and FACS. S* indicates cells that do not incorporate BrdU and have a DNA content between 2N and 4N. Note that the proportion of cells with a sub-G1/G0 DNA content dramatically increases in the tenovin-1-treated ARN8 tumor cells. Instead, the effect of tenovin-1 on NHDFs is primarily cytostatic with little increase in the proportion of dead cells.

It is worth noting here that tenovin-1 is as potent at decreasing ARN8 cell growth as the DNA-damaging agent mitomycin C (Figure 2D) but shows no indication of activation of the DNA damage response (see below and Figure S4). Also, normal human dermal fibroblasts are significantly more resistant to high concentrations of tenovin-1 than ARN8 cells (Figure 2D). In this normal cell type, the effect of tenovin-1 treatment is primarily cytostatic (Figure 2E) and reversible after removal of the compound from the medium (S. Chowdry, M.A., and S.L., unpublished data).

The experiments summarized in Figure 1D can also be used to evaluate the role played by p53 on the sensitivity of tumor cells to tenovin-1. Confirming the results obtained in our secondary assays (see Supplemental Experimental Procedures and Figure S1), a 48 hr treatment with tenovin-1 kills NTera2D cells (wild-type p53) more effectively than NTera2D-DNp53 cells (containing p53 together with a dominant negative p53 fragment). Furthermore, HCT116 cells expressing wild-type p53 are more susceptible to tenovin-1-induced cell death than the HCT116 $p53^{-/-}$ isogenic cells after a 48 hr treatment (Figure 1D and Figure S1). These experiments clearly show that wild-type p53 contributes to the cytotoxic effect of tenovins. We then asked whether wild-type p53 is essential for tenovin-induced cell death. On the one hand, we observed that two breast cancer cell lines with mutant p53 (MDA-MB231 and MDA-MB468) were among the most sensitive to this compound in Figure 1D. On the other, long-term treatment (4 days) with tenovin-1 inhibits growth of p53 null cells (Figure S1B). Hence, it is likely that functional p53 contributes to increase the rate of cell killing but is not essential for the long-term killing effect of tenovin-1. This suggests that tenovin-1 targets a factor(s) upstream of p53 that not only modulates p53 function but also other cellular pathways.

Increasing Tenovin-1 Water Solubility and Tumor Growth Inhibitory Effect

In preliminary experiments, daily administration of tenovin-1 (92 mg/kg) showed indications of reducing growth of tumors derived from BL2 cells or ARN8 cells (Figure S2). However, tenovin-1's poor water solubility in the highly concentrated stock solutions needed for these experiments limited its use in vivo. Structure activity relationship (SAR) studies were used to guide the synthesis of an analog of tenovin-1 with increased water solubility (Table 1; Table S1). Tenovin-2 and -3, which differ from tenovin-1 in the R^2 substituent only, both retain the desired biological activity. As no other changes to the structure of tenovin-1 are apparently tolerated, it was decided to attach a water solubilizing group at the R^2 position resulting in the synthesis of tenovin-6 (Table 1).

Tenovin-6, which is seven times more water soluble than tenovin-1, is slightly more effective than tenovin-1 at increasing p53 levels in cells (Figure 3A). As observed with tenovin-1, functional p53 contributes to tenovin-6 cytotoxicity (Figure 3D and Figure S1A), but p53 is not essential for its long-term killing effect (Figure S1B). As expected, tenovin-6 is more toxic to ARN8 melanoma cells than tenovin-1 (Figure 3B), decreases their growth after a single short exposure (Figure 3C), and delays growth of ARN8-derived xenograft tumors at 50 mg/kg (Figure 3E). Tenovin-6's better water solubility also allowed improving the quality of its pharmacokinetic valuation (Table S2). The in vivo antitumor activity of tenovin-6 prompted us to elucidate its precise mechanism of action as required for further optimization studies.

Target Identification Studies

Compound-induced haploinsufficiency profiling utilizes the finding that yeast strains heterozygous for gene knockouts affecting the target of a compound frequently confer compound hypersensitivity by reducing the level of the target protein that is present in the cell (Giaever et al., 1999), and screening a genome-wide collection of such heterozygous strains is a powerful way to determine candidate targets for inhibitors that are active against yeast (Lum et al., 2004). To identify candidate targets for tenovins, we carried out a genetic screen using the Euroscarf collection of diploid *S. cerevisiae* strains that are each heterozygous for a specific gene deletion, covering over 94% of protein-coding genes between them. For a detailed description of the genetic screening procedure, see the Supplemental Data.

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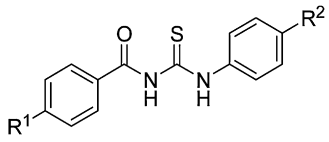
Table 1. Tenovin SAR Studies Aimed at Increasing Water Solubility



ID	R^1	R^2	p53 Increase[a]	K40Ac Tubulin Increase[b]	SirT2 Inhibition[c]
Tenovin-1	^{t}Bu	$NHCOCH_3$	+	+	+
Tenovin-2	^{t}Bu	$NHCOCH(CH_3)_2$	+	+	+
Tenovin-3	^{t}Bu	NH_2	+	+	+
Tenovin-4	N_3	$NHCOCH_3$	–	–	–
Tenovin-5	^{t}Bu	NHCOPh	+	+	+
Tenovin-6	^{t}Bu	$NHCO(CH_2)_4NMe_2.HCl$	+	+	+

[a] Ability to increase p53 levels was determined in MCF-7 cells treated for 6 hr at 10 μM as described in Figure 1B.
[b] Ability to increase K40 Ac-tubulin levels was determined in H1299 cells treated as described in Figure 7B.
[c] SirT2 inhibition assessed as described in Figure 5B using a compound concentrations of 10 or 30 μM depending on the compounds solubility in the assay buffer.

Tenovin-6 inhibits the growth of *S. cerevisiae* cultures with an IC_{50} of 30 μM and is more toxic to yeast than the less water-soluble tenovin-1. We therefore screened 6,261 yeast strains for hypersensitivity to tenovin-6 and identified a strain heterozygous for a partial deletion of *SIR2* among the most hypersensitive strains (Figure 4). This suggested that Sir2p homologs could be targets for tenovin-6 in mammalian cells. Two genes encoding proteins that directly or indirectly interact with Sir2p were also in the list of 16 hit candidate genes (see Discussion and Table S3).

Activity of Tenovins on Purified Human Sirtuins

Consistent with our findings from the yeast genetic screen, tenovin-6 decreases purified human SirT1 peptide deacetylase activity in vitro with an IC_{50} of 21 μM and human SirT2 activity with an IC_{50} of 10 μM (Figures 5A and 5B). Tenovin-1 is not sufficiently water soluble to carry out a complete titration in the sirtuin biochemical assays. Nevertheless, it is possible to observe that at a concentration of 10 μM, tenovin-1 inhibits SirT2 deacetylase activity to the same extent as tenovin-6 (data not shown). Inhibition of SirT3 by tenovin-6 in this assay was significantly lower with an IC_{50} of 67 μM (Figure 5C). As a control, the activity of HDAC8 (a class I histone deacetylase) (Holbert and Marmorstein, 2005) is poorly inhibited by tenovin-6 with an IC_{50} above the highest concentration tested (90 μM; Figure 5D). Furthermore, unlike trichostatin A (an inhibitor of class I and II HDACs), tenovins did not inhibit deacetylation of a cell permeable substrate for all classes of HDACs (Biomol Cat. No. KI-104) (data not shown), supporting the view that tenovins are not general inhibitors of HDAC activity. Accordingly, there are no class I or II HDAC-related genes in the hit list from the tenovin-6 yeast genetic screen (Table S3). Tenovin-6 does not inhibit enzymatic assays in general as the activity of a panel of 51 purified kinases was not significantly affected (data not shown). Other assays in which tenovins showed no effect included a DNA replication assay in *Xenopus* oocyte extracts (A.J. Score and J.J. Blow, personal communication) and an in vitro RNA polymerase I transcription assay using human cell extracts (K. Panov and J. Zomerdijk, personal communication).

Figures 5E and 5F are Lineweaver-Burke plots for tenovin-6 against the two substrates of SirT1 in the biochemical assay. These experiments suggest that tenovin-6 inhibition of sirtuin activity is not due to a competition with the substrates.

Validation of SirT1 as a Target for Tenovins in Mammalian Cells

Specific inhibition of SirT1 expression through siRNAs leads to increased tumor cell death with no toxic effect on normal cells in culture (Ford et al., 2005). Although p53 is not essential for tumor cell killing by SirT1 depletion (Ford et al., 2005), p53 function may contribute as it has been shown that SirT1 destabilizes p53 through its ability to catalyze deacetylation of p53 at lysine 382 (Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001) and that acetylation of p53 augments its DNA binding ability (Luo et al., 2004). Accordingly, cells derived from SirT1 deficient mice and cells treated with siRNAs against SirT1 show high levels of hyperacetylated p53 (Cheng et al., 2003; Ford et al., 2005), and as shown here (Figure 6A), a dominant-negative SirT1 (Luo et al., 2001) mutant increases p53-dependent transcriptional activity. Since tenovins activate p53 but do not necessarily require intact p53 to kill cells and also inhibit SirT1 function in vitro, it was reasonable to test whether these compounds increase p53 acetylation in cells and whether SirT1 influences the effects of tenovins on p53. In Figure 6B, we show that tenovin-1 protects p53 from mdm2-mediated degradation but has a significantly reduced effect on p53 levels in cells overexpressing SirT1. Furthermore, tenovin-1 (and tenovin-6, data not shown) rapidly increases the levels of endogenous K382-Ac p53 in cells (Figure 6C). Although the increase in acetylated endogenous p53 by tenovins is fast and dramatic, we could not rule out that at least a proportion of this increase was a consequence of the elevation of total p53 levels. In order to overcome this problem, H1299 (p53 null) cells were transfected with a p53 expression vector (in the absence of ectopic mdm2) and treated with compound. Under these conditions, tenovin-6 and tenovin-1 increase the levels of p53 acetylated at lysine 382 even when total p53 levels remain constant (Figures 6D and 6E). In the

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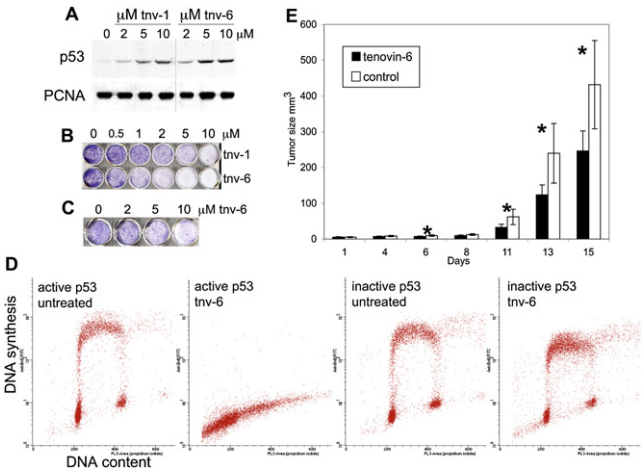

Figure 3. Tenovin-6 Delays Tumor Growth In Vivo

(A) MCF-7 cells were treated with the indicated concentrations of tenovin-1 or tenovin-6 for 6 hr. p53 levels were analyzed and PCNA was detected as a loading control using the monoclonal antibody PC10 (Woods et al., 1991).

(B) ARN8 cells were treated with the indicated concentrations of tenovin-1 or tenovin-6 for 72 hr. Surviving cells were fixed and stained with Giemsa.

(C) ARN8 cells were treated with the indicated concentrations of tenovin-6 for 2 hr, after which the medium was substituted by fresh medium. Four days after, surviving cells were fixed and stained with Giemsa.

(D) SKNSH-pCMV cells (active p53) or SKNSH-DNp53 cells (inactive p53) were treated with 5 μM tenovin-6 for 48 hr. Cells were pulse-labeled with BrdU. DNA synthesis and DNA content were monitored by measuring BrdU incorporation and propidium iodide staining followed by FACS.

(E) ARN8 melanoma cells were injected into the flank of SCID mice and allowed to develop into tumors. Tenovin-6 (in 20% cyclodextrin) was administered daily by intraperitoneal injection at 50 mg/kg, and tumor growth was measured over a period of 15 days (n = 9). Control animals (n = 9) were treated with 20% cyclodextrin. Growth measurements were averaged between groups and plotted. Error bars correspond to 95% confidence intervals. Outliers were not excluded. Mice receiving tenovin-6 had significantly reduced tumor growth as analyzed by a Mann Whitney U-test (day 6, p = 0.045; day 11, p = 0.0179; days 13 and 15, p = 0.0247).

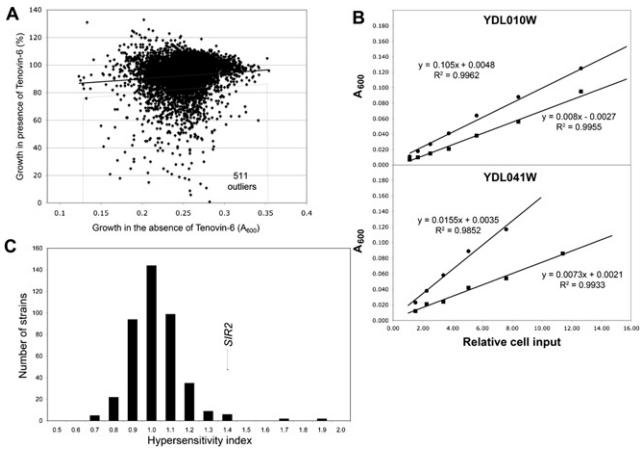

Figure 4. Yeast Genetic Screen to Identify Tenovin-6 Hypersensitive Yeast Strains from within a Genome-wide Heterozygous Gene Deletion Collection

(A) Plot of % control growth at 16.7 μM tenovin-6 versus growth in the absence of compound (OD_{600}) for 6261 heterozygous gene deletion strains showing the positive correlation between growth in the presence of compound and overall growth in its absence. Outlier strains (511) showing potential hypersensitivity were identified as shown.

(B) Examples of data from a secondary screen in which growth of a range of initial cell concentrations plus and minus compound were plotted. YDL010W is an example of a nonhypersensitive strain while YDL041W is the hypersensitive strain heterozygous for a *SIR2* truncation. Graphs show the coefficient of linear correlation (R^2) and best-fit equations giving the slope parameter used to generate the hypersensitivity index.

(C) Histogram showing the distribution of hypersensitivity index values for 408 strains giving reproducible data and indicating the position of the YDL041W heterozygote (*SIR2*).

presence of overexpressed SirT1, the levels of K382-Ac p53 cannot be increased by tenovins (Figure 6E).

Interestingly, when we used a transcriptionally inactive p53 mutant with a normal protein conformation but impaired DNA binding ability (p53R273H), we observed different behavior. First, tenovin-1 does not increase the levels of mutant p53 K382 acetylation (Figure 6E, lower panels, and Figure 6F). Second, SirT1 overexpression slightly diminishes wild-type p53 levels as expected, but has the opposite effect on p53R273H levels (Figure 6E). Furthermore, the proportion of mutant p53R273H acetylated at K382 is significantly higher than the proportion of acetylated wild-type p53 (Figure 6F). This difference in the relative amounts of K382-Ac p53 can also be observed among cell lines with different p53 status (Figure S3). Finally, tenovin-1 does not protect mutant p53 effectively from mdm2-mediated degradation (Figure 6G). This correlation between strength of effects of tenovins and SirT1 on wild-type and mutant p53 further supports the view that tenovins work through the inhibition of SirT1 activity in cells.

DNA-damaging agents are known to increase p53 activity and promote acetylation of p53 (Appella and Anderson, 2001). Hence, it could be argued that the effect of tenovins could be at least partially mediated by DNA injury. However, unlike etoposide and other DNA-damaging compounds, tenovin-1 does not score in comet assays (Figure S4A). Furthermore, tenovin-1 does not increase the levels of p53 phosphorylated at serine 15 or the levels of phosphorylated histone H2AX (Figure S4B), both of which are established indicators of the activation of the DNA damage response (Meek, 1994; Sedelnikova et al., 2003). This, together with the mild and reversible effects on normal fibroblasts, suggests that tenovins are potentially safer than many of the currently used highly genotoxic cancer therapeutics.

The base line levels of p19ARF tumor suppressor are significantly lowered in mouse embryonic fibroblasts from SirT1-deficient mice, and this is reversed upon reintroduction of SirT1 expression (Chua et al., 2005). Consistent with this interesting observation, overexpression of a dominant-negative form of SirT1 correlates with decreased human ARF protein (p14ARF) levels in nucleoli (data not shown). Showing that tenovin treatment and SirT1 depletion have similar effects and therefore strengthening that tenovins act through inhibition of SirT1 in cells, endogenous p14ARF expression is lowered after tenovin-1 treatment (Figure 6H). It should be noted that p14ARF is a potent inhibitor of mdm2's activity leading to increased levels of active p53 (Sherr, 2006). Hence, the negative effect of tenovins on p14ARF could buffer the p53 response to tenovins in normal cells (as these have functional p53 and p14ARF) but is irrelevant in the p53-wild-type tumor cells, including BL2, ARN8 (A375-derived), and MCF7, which are known to be p14ARF deficient (Lindstrom et al., 2001; Stott et al., 1998).

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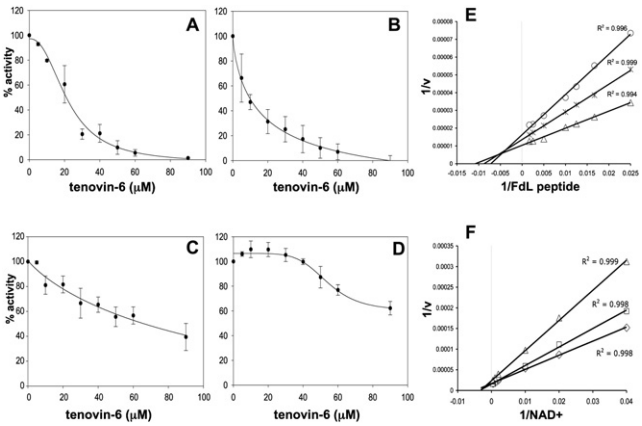

Figure 5. Tenovin-6 Inhibits the Protein Deacetylase Activities of Purified Sirtuins SirT1 and SirT2

(A–D) Increasing concentrations of tenovin-6 were added to purified human SirT1 (A), SirT2 (B), SirT3 (C), or HDAC8 (D) reaction mixtures. Values correspond to the average enzyme activity of three independent experiments ± SD. Estimated IC_{50} values for SirT1, SirT2, and SirT3 in the assay conditions are 21, 10, and 67 μM, respectively.

(E) Analysis of tenovin-6's ability to compete for binding sites with the SirT1 FdL acetylated peptide substrate. In vitro SirT1 inhibition assay was carried out with tenovin-6 at 0, 50, and 75 μM with varying FdL concentrations and a constant NAD+ concentration of 1 mM. All assays contained the same amount of DMSO (0.25%). The data is presented as a Lineweaver-Burke plot (where the x axis intercept is $-1/K_m$ and the y axis intercept is $1/V_{max}$). Trend lines were then added to create a straight line. The trend lines for 0 μM (triangles), 50 μM (asterisks), and 75 μM (circles) tenovin-6 all have an R^2 values above 0.99. Data points are the average of triplicate experiments.

(F) Analysis of tenovin-6's ability to compete for binding sites with SirT1 cosubstrate NAD+. In vitro SirT1 inhibition assay was carried out with tenovin-6 at 0, 25, and 50 μM with varying NAD^+ concentrations and a constant FdL acetylated peptide concentration of 200 μM. All assays contained the same amount of DMSO (0.25%). The data are presented as a Lineweaver-Burke plot. The trendlines for 0 μM (diamonds), 25 μM (squares), and 50 μM (triangles) tenovin-6 all have an R^2 value above 0.99. Data points are the average of triplicate experiments.

Validation of SirT2 as a Target for Tenovins in Mammalian Cells

We have also observed that tenovin-1 (and tenovin-6, data not shown) increases acetylation levels of histone H4 at lysine 16 (Figure 7A), an established substrate for SirT1 and SirT2 (Vaquero et al., 2004, 2006). The observation that tenovins induce a global increase in K16-Ac H4 (Vaquero et al., 2006) indicated that tenovins could also influence SirT2 activity in cells.

SirT2 also promotes deacetylation of α-tubulin at lysine 40 (North et al., 2003). Strong evidence that SirT2 is a target for tenovins in mammalian cells comes from the observation that tenovins-1 and -6 clearly increase acetylated α-tubulin levels (Figure 7B and 7C). Furthermore, SirT2 overexpression significantly weakens the effect of tenovins on α-tubulin acetylation (Figure 7C).

The results presented in Figure 7 together with the correlation between the activities of different tenovin derivatives in the SirT2 biochemical assay and their ability to increase acetylated α-tubulin in cells (Table 1; Table S1) strongly support that tenovins inhibit SirT2 protein deacetylase activity in cells.

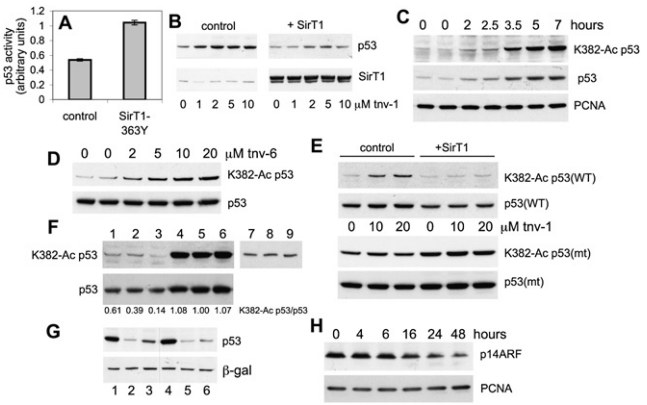

Figure 6. SirT1-Related Effects of Tenovins in Mammalian Cells

(A) MCF7 cells were transfected with the RGC-ΔFos-*LacZ* p53-dependent reporter construct as well as a control vector or an expression vector for the SirT1-363Y dominant negative mutant. All samples were also transfected with a control plasmid expressing luciferase under the control of the SV promoter. β-galactosidase activity was measured 32 hr after transfection and values were normalized using the luciferase readings. Values correspond to three independent experiments ± SD.

(B) H1299 cells (p53 null) were transfected with vectors expressing p53 and mdm2 in the absence or presence of a vector expressing SirT1 (pCMV-SirT1). Cells were treated with increasing concentrations of tenovin-1 for 6 hr, and the levels of p53 and SirT1 were analyzed by western blot using DO1 and antibody 2G1-F7 (Cat. No. 05-707, Upstate), respectively. Note that pCMV-SirT1 encodes SirT1 isoform-1. Endogenous SirT1 isoform-1 was also detected in lanes 1 through 5 upon longer exposure of the blots. The band below ectopic SirT1 could correspond to a SirT1 isoform.

(C) MCF-7 cells were treated with 10 μM tenovin-1 for the indicated times and analyzed by western blotting using an antibody against K382-acetylated p53 (Cat. No. 614202, BioLegend) or the DO1 antibody against the N terminus of p53. PCNA was detected as a loading control.

(D) H1299 cells transfected with a vector for p53 were treated for 6 hr with the indicated concentrations of tenovin-6. K382-acetylated p53 and total p53 were detected as above.

(E) H1299 cells were transfected with a vector for p53 expression (upper panels) or p53R273H (lower panels) in the absence or presence of pCMV-SirT1. Cells were treated for 6 hr with the indicated concentrations of tenovin-1. K382-acetylated p53 and total p53 were detected.

(F) H1299 cells were transfected with a vector for wild-type p53 expression (lanes 1, 2, and 3) or p53R273H (lanes 4, 5, and 6). Cells were left untreated (lanes 3 and 4) or treated with 10 μM (lanes 2 and 5) or 20 μM (lanes 1 and 6) tenovin-6 for 6 hr. K382-acetylated p53 and total p53 were detected, and the ratio between the amount of K382-acetylated p53 and the total amount of p53 in each lane was calculated. Note that these ratios are relative and do not correspond to the actual fraction of acetylated p53 in cells. Lanes 7, 8, and 9 correspond to loading 1/10 of the amount of protein in samples in lanes 4, 5, and 6, respectively.

(G) H1299 cells were transfected with a vector for wild-type p53 expression (lanes 1 through 3) or p53R273H (lanes 4 through 6) in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5, and 6) of ectopic mdm2. In lanes 3 and 6, cells were treated for 6 hr with 10 μM tenovin-1. Total p53 was detected with DO1 antibody. β-gal expression was used as a transfection efficiency and loading control.

(H) H1299 cells were treated with 10 μM tenovin-1 for the indicated times. Endogenous p14ARF was detected using a mouse monoclonal antibody (Ab-3 14P03, Neomarkers). PCNA was detected as a loading control.

DISCUSSION

This work describes an effective approach for the discovery of small molecules with potential therapeutic relevance consisting of the following steps: (1) identification of bioactive compounds

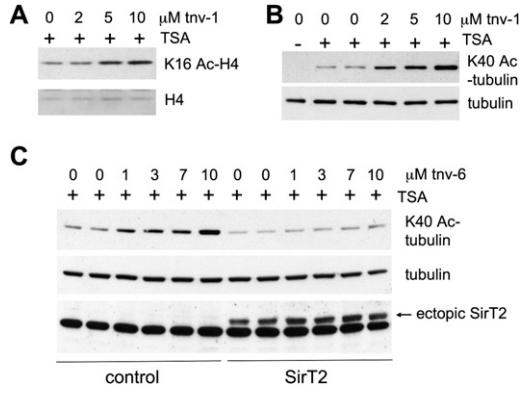

Figure 7. SirT2-Related Effects of Tenovins in Mammalian Cells

(A) H1299 cells were treated with 40 nM trichostatin A (TSA) (Cat. No. T8552, Sigma) to deplete nonsirtuin histone deacetylase activities. Where indicated, tenovin-1 was also added. After 16 hr, samples were analyzed using antibodies against K16 acetylated histone H4 (Cat. No. 07-329, Upstate) and total H4 (Cat. No. 07-108, Upstate).

(B) H1299 cells were treated for 16 hr as indicated. Samples were analyzed with antibodies to K40 acetylated α-tubulin (Cat. No. T6793, Sigma) or total α-tubulin (Cat. No. T9026, Sigma).

(C) H1299 cells were permanently transfected with a control vector (pcDNA3-Neo) (lanes 1–6) or a vector for SirT2 overexpression (pcDNA3Neo-SirT2) (lanes 7–12) and treated for 16 hr with 40 nM trichostatin A in the absence or presence of 1, 3, 7, or 10 μM tenovin-6. Samples were analyzed using antibodies against K40-acetylated α-tubulin, total α -tubulin, and SirT2 (Cat. No. 2313, Cell Signaling). Note that pcDNA3-SirT2 encodes SirT2 isoform-1, which is slightly larger than isoform-2. Endogenous SirT2 isoform-1 could also be detected in lanes 1 through 6 upon longer exposure of the blots. The band below the ectopic SirT2 may correspond to SirT2 isoform-2, but this was not confirmed.

that are not general cytotoxics by screening a library of compounds for their ability to increase the synthesis of a p53-dependent reporter in mammalian cells; (2) prioritization of the hits through an ordered series of secondary assays, including testing their effect on the cell cycle and their ability to increase p53 levels early after treatment; (3) improvement of the water solubility properties of the selected hit compound following the generation of the required SAR data; (4) examination of the hit's activity in vivo; (5) identification of its putative cellular target through a genetic screen; (6) testing the activity of the compound in biochemical assays; and (7) validation of the compound's mechanism of action in cultured cells. Our results also highlight a major advantage of suitable mammalian cell-based screens over biochemical screens, which is the up-front identification of selective compounds that are bioactive at low concentrations.

Here, we describe our first attempt to characterize a hit compound from the primary screen. The ability of tenovins-1 and -6 to delay growth of tumors derived from a highly aggressive melanoma cell line without significant general toxicity showed that these compounds are active in vivo as single agents and suggested their potential value as lead compounds for further medicinal chemistry studies. It was clear, however, that such optimization studies would be significantly aided by the elucidation of the molecular targets for tenovins. Using a yeast-based genetic assay, we identified the NAD^+-dependent deacetylase Sir2p as a possible target for tenovin-6. The observation that the yeast heterozygous knockouts for *ESC2* and *ISW1* were also hypersensitive to tenovin-6 (Table S3) strengthened this possibility. Esc2p is a SUMO-like protein that interacts with Sir2p (Cuperus and Shore, 2002; Novatchkova et al., 2005), and Isw1p is an ATP-dependent chromatin remodeller that interacts with Esc8p, another Sir2p-interacting factor (Cuperus and Shore, 2002). It is possible that the list of 16 yeast genes obtained from the genetic target identification screen (Table S3) may help to identify novel sirtuin interacting proteins.

Consistent with a central role for the sirtuins as targets for tenovins, these compounds decrease the protein deacetylase activities of purified human SirT1 and SirT2. We have also shown that tenovins affect acetylation of SirT1 and SirT2 substrates in cells and are the only sirtuin inhibitors for which overexpression of SirT1 or SirT2 has been shown to impair their effects. This, together with the correlation between the inhibitory activities of different tenovin derivatives in biochemical assays and cellular assays (Table 1; Table S1), supports the conclusion that SirT1 and SirT2 are important targets for the tenovins in mammalian cells.

Whether discovered through biochemical screens or through cell-based screens, testing a hit compound in other assays is necessary to assess its level of selectivity. In this regard, tenovin-6 does not have an effect on a broad variety of biochemical reactions. We have also observed a degree of selectivity with regards to the effect of tenovins on different sirtuins. Tenovin-6 is less effective as a SirT3 inhibitor in vitro than for SirT1 and SirT2, despite the high level of sequence similarity between these three class-I sirtuins (Michan and Sinclair, 2007). Whether high concentrations of tenovins could also affect this SirT3 in cells will require developing reagents that enable detection of the acetylation status of SirT3 substrates. An advance in this regard is the recent identification of acetyl-CoA synthetase 2 as being susceptible to deacetylation by SirT3 at a specific lysine (Hallows et al., 2006; Schwer et al., 2006).

SirT5 belongs to class-III sirtuins (Michan and Sinclair, 2007). Although it shows some protein deacetylase activity in vitro, this activity is very low (Haigis and Guarente, 2006; Michan and Sinclair, 2007). SirT5 substrates remain unknown, and according to published work, SirT5 has no effect on p53 (Luo et al., 2001). The other human sirtuins (SirT4, SirT6, and SirT7) are less related in sequence and do not show protein deacetylase activities. SirT4 and SirT6 instead act as ADP-ribosyl-transferases. However, none of the hits from the yeast genetic screen are known to exhibit or be related to this type of enzymatic activity. There is no enzymatic activity described for SirT7. Nevertheless, once the exact binding site for tenovins in SirT1 and/or SirT2 has been defined and if this site involves residues conserved among sirtuins, it will be interesting to test the effect of tenovins on other members of the family.

SirT1 and SirT2 catalyze the reaction between an acetylated lysine with NAD^+ leading to the production of deacetylated lysine, 2′-O-acetyl-ADP-ribose and nicotinamide (Jackson and Denu, 2002). One possibility is that tenovins mimic the effect of the byproduct of the sirtuin reaction, nicotinamide, which acts as a physiological noncompetitive inhibitor of sirtuin function (Borra et al., 2004; Grubisha et al., 2005). In fact, the Lineweaver-Burke plots for inhibition of SirT1 by tenovin-6 also indicate a noncompetitive mode of inhibition. Hence, it could be argued that tenovins also alter the activity of other enzymes modulated by nicotinamide. Although none of the deletions in yeast that

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confer hypersensitivity to tenovin-6 involve proteins known to be modulated by NAD^+ or nicotinamide (Table S3), this possibility cannot be excluded.

Compounds identified through primary biochemical screens are in general significantly more potent in the relevant in vitro primary assay than when added to cells. This is reasonable, considering that these compounds have not been selected for their solubility, stability (in culture medium or in cells), permeability, localization to a particular cellular compartment, or accumulation to high concentrations inside the cell. However, the same situation is not necessarily expected for compounds selected via a cell-based assay. An important aspect of this work is that our mammalian cell-based screen has led to the identification of sirtuin inhibitors that are active in the one digit micromolar range in mammalian cells. Below we summarize the published studies on other sirtuin inhibitors that have been tested in cells focusing on the relevance to cancer research. Several inhibitors of sirtuin deacetylase activity described in the literature are nonspecific (e.g., nicotinamide, suramin, dihydrocoumarin), are of low potency in mammalian cells, have poor water solubility (Grubisha et al., 2005), or have not been characterized in detail for sirtuin-related effects in cells. Other compounds like sirtinol, which was discovered using a yeast phenotypic assay (Grozinger et al., 2001), have been shown to be valuable in cell biology experiments. In this way, sirtinol affects the acetylation status of p53 and histones H3 and H4 in cells at concentrations above 30 μM after incubation times of 24 hr and above (Ota et al., 2007). Splitomycin (Bedalov et al., 2001) undergoes rapid hydrolysis at neutral pH, limiting its use in cell culture conditions. A splitomycin-related compound, cambinol (Heltweg et al., 2006), inhibits SirT1 and SirT2 deacetylase activities in vitro with IC_{50} values in the 55–60 μM range. p53 levels or K382 acetylation of p53 are not increased by cambinol as a single agent (Heltweg et al., 2006). We have confirmed this observation using concentrations of cambinol up to 200 μM (data not shown). The effect of cambinol on p53 requires concentrations of 50 and 100 μM and the addition of a DNA damaging compound (Heltweg et al., 2006). Cambinol also increases acetylated α-tubulin levels but again at concentrations in the 100 μM range. One remarkable feature of cambinol is that it is tolerated as a single agent by epithelial cancer cells, whereas it is highly toxic to Burkitt lymphoma cells in a way that is dependent on Bcl-6 expression (Heltweg et al., 2006). Furthermore, cambinol (100 mg/kg) decreases growth of xenograft tumors derived from a Bcl-6 expressing Burkitt lymphoma cell line (Heltweg et al., 2006). It will be interesting to test whether this Bcl-6-related-enhanced cytotoxicity also occurs with tenovins. Another compound, EX-527, is a very potent SirT1 inhibitor in biochemical assays (Solomon et al., 2006), and a series of compounds structurally similar to EX-527 lead to reduced TNF-α and stimulated adipocyte differentiation (Nayagam et al., 2006). EX-527 clearly increases p53 levels and K382 acetylated p53 at a concentration of 1 μM, but only when it is combined with DNA-damaging agents. Confirming the lack of effect on p53 as a single agent, we have not observed an effect of EX-527 on p53 at concentrations up to 100 μM (data not shown). A recent paper describes the potential use of a SirT2 inhibitor for the treatment of Parkinson's disease (Outeiro et al., 2007). While this application of a sirtuin inhibitor is remarkably interesting, it is unlikely that the compound described in this work (AGK2) is of relevance to cancer research. A compelling argument against AGK2's utility for the treatment of cancer derives from the demonstration by the authors of this paper that AGK2 is nontoxic to tumor cells. As expected for a SirT2 inhibitor, AGK2 increases the levels of acetylated tubulin at concentrations above 10 μM. In summary, a systematic comparison of all sirtuin inhibitors using the same experimental conditions is necessary to evaluate their use in therapy as well as their value as biological tools for the understanding of the cellular processes regulated by SirT1 and/or SirT2. In any case, there are obvious advantages of having several sirtuin inhibitors available. Observing similar effects with several of these compounds may be an effective way to support the involvement of SirT1 and/or SirT2 in a given process. Furthermore, it is possible that different sirtuin inhibitors synergize with certain combinations showing improved therapeutic value.

We are now in the process of analyzing whether any of the other p53 activators discovered through our primary screen are also sirtuin inhibitors. Like tenovins, these compounds could be used for the elucidation of cellular processes modulated by this important group of enzymes (reverse chemical genetics) (Peterson and Mitchison, 2002; Schreiber, 2003) and as lead compounds for the development of treatments for cancer and other hyperproliferative diseases. Inhibition of sirtuins may also be of interest in the study of the aging processes (Longo and Kennedy, 2006). A remarkable finding in this regard is that mouse embryonic fibroblasts derived from SirT1 knockout mice have an extended lifespan (Chua et al., 2005). Exemplifying the utility of small molecules to understand cellular events, our findings with tenovins have led us to complete previous experiments by showing that SirT1 inhibition by transient transfection of a SirT1 mutant with dominant-negative activity leads to increased p53 transcriptional activity. This had not been addressed directly in the literature. Published experiments in this regard involved introducing the expression of the SirT1-363Y dominant negative mutant in cells and selecting surviving and proliferating cells (Luo et al., 2001; Vaziri et al., 2001). Cells where p53 activity has been increased are not likely to constitute a significant proportion of the selected cells. Additionally, we present evidence suggesting that unlike wild-type p53, mutant p53 is highly acetylated at lysine 382. This finding may be crucial in understanding the underlying causes for mutant p53 accumulation in tumors.

Aside from tenovins, so far we have only tested one other optimized hit compound from our screen in animal models. The in vivo activity of this second compound, which is unrelated in structure and target to the tenovins (N.J.W. and S.L., unpublished data), further highlights the efficacy of our general drug discovery approach.

EXPERIMENTAL PROCEDURES

Reagents

A 30,000 compound DiverSet was purchased from Chembridge. Stock solutions were at 2 mM in DMSO. Tenovin synthesis will be described elsewhere (A.M. and N.J.W., unpublished data). Antibody sources are specified in the figure legends.

Cell-Based Compound Screen

p53-reporter assay (primary screen): T22-ΔFos-RGC *lacZ* murine cells were seeded in 96-well plates and incubated for 18 hr in the presence of each

compound at 10 μM. Cells were lyzed and β-galactosidase activity measured in a colorimetric assay as described (Berkson et al., 2005). The robustness of the assay was measured by calculating the average reading of 720 wells treated with 5 ng/ml actinomycin D (1.355) and the corresponding 95% confidence interval (1.332–1.378). For a detailed description on the primary screening and hit selection procedures, see the Supplemental Data.

Cell Lines and Cell Viability Assays

HCT116 and HCT116 $p53^{-/-}$ cells were a gift from B. Vogelstein. EW36 and BL2 were provided by K. Wiman (Lindstrom et al., 2001). NTera2D and NTera2D-DNp53 cells were obtained from M. Saville (Stevenson et al., 2007). ARN8 cells derive from the A375 cells (Blaydes and Hupp, 1998). NHDF fibroblasts were bought from Promocell. SKNH-pCMV and SKNSH-DNp53 were previously described (Smart et al., 1999). All other cell lines were obtained from the ATCC. Cell viability was determined by trypan blue exclusion, Giemsa staining, or MTT assays as described (Smart et al., 1999). Annexin-V/propidium iodide labeling was performed following recommendations by manufacturers (Biovision, K101-25) and quantified by Flow Cytometry. Cell-cycle distribution was carried out by BrdU labeling and FACS as described (Smart et al., 1999).

Tumor Xenograft Studies

Female SCID mice (Harlan) were injected subcutaneously with 1×10^6 ARN8 cells suspended in matrigel (BD Biosiences). Tumors were allowed to reach a size of approximately 10 mm^3. Tenovin-6 was administered daily at 50 mg/kg by intraperitoneal injection. Control animals were treated with vehicle solution containing cyclodextrin 20% (w/v) (Cat. No. C0926 Sigma) and DMSO 10% (v/v). Tumor diameters were measured using calipers, and volumes were calculated using the equation $V = \pi 4/3[(d1 + d2)/4]^3$. Median values of tumor size were calculated for each time point as well as the corresponding 95% confidence intervals. Comparison of control and drug-treated tumor size distributions were made by Mann-Whitney U-test. An alpha-level of 0.05 was considered appropriate for determination of statistical significance. All animal experiments were preformed under Project License number 60/3045 and in accordance with the United Kingdom Coordinating Committee on Cancer Research guidelines and regulations.

Target Identification by a Yeast Genetic Screen

A collection of 6261 yeast strains, each heterozygous for the deletion of a single open reading frame (ORF), was obtained from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) and screened in a two-step assay. For a description on the methodology used and the criteria followed for hit selection, see the Supplemental Data.

In Vitro Deacetylation Assays

Assays were carried out using purified components in the Fluor de Lys Fluorescent Assay Systems (Biomol kits AK555, AK556, AK557, and AK518). Relevant FdL substrates were used at 7 μM and NAD^+ at 1 mM. Tenovins were solubilized in DMSO with the final DSMO concentration in the reaction being less than 0.25%. For SirT1 and HDAC8, one unit of enzyme was used per reaction, and for SirT2 and SirT3, we used five units per reaction. Reactions were carried out at 37°C for 1 hr. Conditions for the acquisition of data for the Lineweaver-Burke plots are specified in the legend for Figure 5.

Transfections

Human wild-type p53, p53R273H, and mdm2 expression vectors are described (Xirodimas et al., 2001). pCMV-SirT1 vector for SirT1 isoform 1 was obtained from Origene (Cat. No. SC127917). SirT1-363Y was expressed using pBabe SirT1-363Y, a kind gift from W. Gu (Luo et al., 2001). pcDNA3-SirT2 was obtained by inserting the human SirT2 isoform 1 coding sequence (aa 1–389) from pCMV-SirT2 (Cat. No. SC127915, Origene) into pcDNA3. H1299 cells were transfected with pcDNA3 or pcDNA3-SirT2, and neomycin resistant cells were selected with G418 (1 mg/ml). Transient transfections for western blotting were performed using the calcium phosphate precipitation protocol as described (Xirodimas et al., 2001). Transfections for the analysis of p53 transcription factor activity were performed using Fugene-6 as recommended (Roche).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, four supplemental figures, and three supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/5/■■■/DC1/.

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