Journal of Human Genetics (2016) 61, 823–830

www.nature.com/jhg

ORIGINAL ARTICLE

Transcript, methylation and molecular docking analyses of the effects of HDAC inhibitors, SAHA and Dacinostat, on *SMN2* expression in fibroblasts of SMA patients

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Several histone deacetylase inhibitors (HDACis) are known to increase Survival Motor Neuron 2 (SMN2) expression for the therapy of spinal muscular atrophy (SMA). We aimed to compare the effects of suberoylanilide hydroxamic acid (SAHA) and Dacinostat, a novel HDACi, on SMN2 expression and to elucidate their acetylation effects on the methylation of the SMN2. Cell-based assays using type I and type II SMA fibroblasts examined changes in transcript expressions, methylation levels and protein expressions. *In silico* methods analyzed the intermolecular interactions between each compound and HDAC2/HDAC7. SMN2 mRNA transcript levels and SMN protein levels showed notable increases in both cell types, except for Dacinostat exposure on type II cells. However, combined compound exposures showed less pronounced increase in SMN2 transcript and SMN protein level. Acetylation effects of SAHA and Dacinostat promoted demethylation of the SMN2 promoter. The *in silico* analyses revealed identical binding sites for both compounds in HDACs, which could explain the limited effects of the combined exposure. With the exception on the effect of Dacinostat in Type II cells, we have shown that SAHA and Dacinostat increased SMN2 transcript and protein levels and promoted demethylation of the *SMN2* gene.
Journal of Human Genetics (2016) **61,** 823–830; doi:10.1038/jhg.2016.61; published online 2 June 2016

INTRODUCTION

With an incidence of 1/6000 to 1/10 000 live births and a carrier frequency of 1/40 to 1/50, spinal muscular atrophy (SMA) is the second most common cause of an autosomal recessive hereditary disorder after cystic fibrosis.[1] *Survival Motor Neuron 1* (*SMN1*) gene deletion has been detected in 96% of SMA patients, and the remaining patients showed intragenic mutations of the gene. *Survival Motor Neuron 2* (*SMN2*) is a highly homologous copy of *SMN1*.[1,2] Affected patients present variable copy numbers of *SMN2* that are inversely related to SMA severity.[3,4]

Enhancing *SMN2* gene expression using small-molecule compounds has been proposed as a therapeutic strategy for treating SMA.[5] Several histone deacetylase inhibitors (HDACis), such as short chain fatty acids (valproic acid (VPA), phenylbutyrate), hydroxamic acids (LBH589 (Panobinostat), suberoylanilide hydroxamic acid (SAHA), trichostatin A) and benzamides (M344 (*N*-hydroxyl-7-aminoheptanamide), MS-275), have shown promising therapeutic effects on SMA-derived cells (reviewed in Mohseni *et al.*[6]).

Histone acetyltransferases relax chromatin by adding acetyl groups, whereas histone deacetylases (HDACs) neutralize the actions of histone acetyltransferases by removing acetyl groups. HDACis increase gene accessibility to transcriptional machinery by preventing deacetylation, thus maintaining histone acetylation and subsequently activating gene promoters.[7,8]

Dacinostat is a new hydroxamate-based HDACi with potential anticancer activity. Dacinostat is a potent HDACi that is currently in a phase I clinical trial for the treatment of leukemia. Dacinostat is generally more potent than SAHA, another hydroxamic acid, in low nanomolar doses.[9] Dacinostat also shows fewer toxic effects to normal human hematopoietic cells.[10]

Other HDACis have been shown to increase SMN2 transcript by activating the *SMN2* promoter. SAHA, an HDACi, increased full-length SMN2 transcript levels in SMA.[8,11,12] However, there has been no report on the effect of Dacinostat on *SMN2* gene expression. In this study, we compared the effects of SAHA and Dacinostat on *SMN2* expression.

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Received 13 November 2015; revised 25 April 2016; accepted 6 May 2016; published online 2 June 2016

It is known that HDACis can activate gene promoters by facilitating histone acetylation, as mentioned above. However, the *SMN2* promoter is regulated by DNA methylation. Hauke *et al.*[11] showed a direct correlation between the level of *SMN2* methylation and disease severity and provided evidence that hypermethylation promotes *SMN2* silencing. Along this line, other reports have shown that inhibition of HDACs by specific inhibitors can reactivate endogenous genes or reporter constructs that have been silenced by DNA methylation.[13] However, whether the *SMN2* promoter methylation status can be controlled by HDACis remains unclear. To answer this question, we also assessed the methylation status of the *SMN2* gene before and after treatment with SAHA and Dacinostat.

In this study, we performed cell-based assays to assess transcript levels, methylation levels and protein levels as well as *in silico* analyses on the intermolecular interactions between the HDACis and the HDACs.

MATERIALS AND METHODS

Cell culture and compound exposures

Human fibroblasts of a 2-year-old male SMA type I patient (GM09677; Coriell Cell Repository, Camden, NJ, USA) and a 1-year-old male SMA type II patient (GM22592; Coriell Cell Repository) were cultured in 25-cm^2 flasks (TPP, Trasadingen, Switzerland) using high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 1% GlutaMAX (Thermo Scientific, Waltham, MA, USA) and 10% fetal bovine serum (FBS) (JR Scientific Inc., Woodland, CA, USA). The cells were incubated at 37 °C with 10% CO_2. SAHA (Selleck Chem, Houston, TX, USA) and Dacinostat (Selleck Chem) were freshly dissolved in dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO, USA) before use and added to the flasks at concentrations of 1 and 10 µM (individual SAHA exposure), 3.2 and 32 nM (individual Dacinostat exposure) and 8 µM SAHA +25 nM Dacinostat (combination exposure) for 48 h. Compound exposure was performed at 90% of cell confluence. Medium without compound was added to another cultured flask as untreated cells (Mock). All experiments were repeated three times. Cultured cells were harvested by the trypsin-EDTA method.

Table 1 Results of viability assay using different concentrations of Dacinostat and SAHA–Dacinostat

			Viable cells (%)	
Drug	*Concentration*	*Treatment period (h)*	*Type I cells*	*Type II cells*
Dacinostat (nM)	500	24	<50	<50
	320	48	<50	<50
	90	48	<50	<50
	45	48	89	89
		72	82	80
	32	48	97	97
		72	96.7	96
	30	48	97	97
		72	97	97
	25	48	97	97
		72	97	97
SAHA (µM)	30	24	<50	<50
	20	24	<50	<50
	15	48	59	58
	14	48	60	58
	13	48	83	82
	10	48	97	97
		72	97	96
SAHA (µM)–Dacinostat (nM)	10–32	24	81	80
	9–30	48	88	89
		72	84	85
	8–25	48	97	97
		72	92	91

Abbreviation: SAHA, suberoylanilide hydroxamic acid.

SMN2 splicing integrity of GM09677 and GM22592 fibroblasts

Previous reports showed variable splicing patterns beyond exon 7, which is commonly known, in the SMN2 transcripts of different SMA-derived cells.[14–16] These studies described skipping involving exon 5, where one of the probes in this study was located. This phenomenon may cause false results in *in vitro* studies, especially in those that screen substances that would restore the SMN2 exon 7 splicing defect. Various cells were checked except the cells we used in this study. Therefore, we performed long-range RT-PCR analysis of the whole SMN2 transcript in both cells and confirmed the results using direct sequencing. We designed the following primers for this experiment: 5′-TTTGC TATGGCGATGAGC-3′ located in exon 1 and 5′-CCCCACCTCAGTCTTTTA CA-3′ located in exon 8.

Cell viability assay

To determine the maximum concentration of compounds for exposure, we performed cell viability assays using several different concentrations of the compounds (Table 1), either individually or in combination. The trypan blue dye exclusion test[17] was used to determine the cell viability and compound cytotoxicity. The cytotoxic effect of SAHA and Dacinostat was determined using human fibroblasts from both SMA type I and type II patients. The compounds were added to cultured cells that were at 90% confluence, and the cultures were incubated for 24–72 h at 37 °C in 10% CO_2 atmosphere. Cell viability was calculated as the percentage of viable cells in the total cell count.

SMN2 mRNA transcript analysis

Cells were harvested by trypsinization (Life Technology, Woodland, CA, USA), and a small aliquot was used for cell count. The cell pellet was re-suspended in culture media containing no supplements to a concentration of 1 million cells per ml. Cell lysates were prepared using the QuantiGene Sample Processing Kit (Affymetrix, Santa Clara, CA, USA).

SMN2 mRNA expression was quantified using the QuantiGene Plex 2.0 assay (Affymetrix). This assay combines branched DNA (bDNA) signal amplification and multi-analyte profiling bead (xMAP) technologies to enable the detection and quantitation of multiple RNA targets simultaneously. The bDNA assay is a hybridization-based method of target-specific RNA quantitation using labeled DNA probes. Mean fluorescence intensity (MFI) was measured using Luminex200 (Affymetrix).

To quantify overall *SMN2* expression (Overall-SMN2), an mRNA probe was designed against exon 5 (5′-AACAUCAAGCCCAAAUCUGC-3′). Beta-actin mRNA was used as an endogenous reference gene, and SMN2 expression levels were calculated relative to those of β-actin.

To quantify the amount of exon 7 inclusion within the SMN2 transcripts (E7-SMN2), an mRNA probe was designed to bind to the exon 6–exon 7 junction (5′-AUACUGGCUAUUAUAUGGGUUUU-3′).

We mathematically inferred the Δ7-SMN2 transcript level using the following formula: Δ7-SMN2 = (O-SMN2) − (E7-SMN2), where O-SMN2 = MFI of overall SMN2 expression that was normalized against β-actin expression and E7-SMN2 = MFI of SMN2 exon 7 inclusion that was normalized against β-actin.

SMN protein analysis

Cells were pelleted after harvesting by centrifugation at 4 °C and were homogenized in 1 ml lysis buffer containing 0.5 mM protease inhibitors of PIC8340 and 1 mM PMSF. Total protein concentration was measured using the Qubit protein assay kit (Life Technologies).

To determine the amount of SMN protein, the total protein concentration was adjusted to 100 µg ml^{-1}. SMN protein was measured using an SMN ELISA

kit (Enzo Life Science, East Farmingdale, NY, USA) provided by Varioskan Flash instrument, version 4.00.53 (Thermo Scientific).

SMN2 promoter methylation analysis

We investigated SMN2 promoter methylation using Methylation-Specific High-Resolution-Melting analysis (MS-HRM). Primer design for three CpG Islands (CGIs 1,2,4) within the SMN2 promoter region[11] was conducted using the Methyl Primer Express 1 (Applied Biosystems, Carlsbad, CA, USA) CGI Finder and Plotting Tool (www.EBI.ac.uk/emboss) based on the criteria described elsewhere.[18,19]

PCR amplification and MS-HRM analysis were performed using the PIKO Real96 Real Time PCR System (Thermo Scientific). The PCR amplifications and HRM data were performed, monitored and analyzed using PIKO Real96 Software (Thermo Scientific). PCR amplification was performed in a total volume of 10 µl, containing 1× EpiTect HRM PCR Master Mix (Qiagen, Amtsgericht Düsseldorf, Germany), 200 nM of each designed primer and 10 ng of bisulfite-treated DNA template. All experiments were performed at least three times. The final results are reported as the mean methylation percentage ± s.e.m.

Molecular docking simulation

We used two HDACs with X-ray crystal structures available in the Protein Data Bank for the molecular docking simulation: HDAC2, which represents Class I HDACs; and HDAC7, which represents Class II HDACs. Docking simulation[20] was performed on the X-ray crystal structures of HDAC2 (PDB code: 4LXZ,[21] resolution 1.85 Å) and HDAC7 (PDB code: 3C0Z,[22] resolution 2.1) using Autodock 4.2.[23]

The proteins were prepared using one chain; all of the non-polar hydrogens merged. Water molecules and other ligand molecules were removed. Dacinostat was built as a pdb file using the PRODRG2 server.[24] The co-crystal ligand (SAHA) was separated from the protein. Hydrogen atoms and rotatable bonds were added and assigned to the ligand using the AutoDockTool and AutoTors, respectively. Gasteiger charges and Kollman united atom charges were added to the ligand and receptor, respectively, followed by the addition of atomic solvation parameters. The grid calculation was performed using Autogrid4 program, in which a box dimension of 60 × 60 × 60 in x, y and z directions with a grid spacing of 0.375 Å was set. The Lamarckian Genetic Algorithm was used with the following parameters: population size of 50, elitism of 1, mutation rate of 0.02, crossover rate of 0.80, local search rate of 0.06, 250 000 energy evaluations and 100 search runs. The final docked conformations were clustered using a cluster tolerance of 1.0 Å root-mean-square deviation. The conformation of the ligand with the lowest predicted binding free energy selected from the most populated cluster was used in subsequent analysis.

Statistical analysis

Statistical analysis of the data was performed using IBM SPSS statistic 20 (SPSS Inc., Chicago, IL, USA). Analysis of variance was used to compare the data obtained from the mock and the compound-exposed cells. A probability of less than 0.05 ($P<0.05$) was considered as statistically significant. The Student's *t*-test was applied to compare the mean methylation levels in the CGIs of the *SMN2* promoter region.

RESULTS

SMN2 splicing integrity of GM09677 and GM22592 fibroblasts

Our SMN2 splicing assay using long-range RT-PCR could not identify splicing isoforms other than the full-length and Δ7 in GM09677 and GM22592 cells (data available upon request). Although we could not completely rule out the existence of less abundant splicing isoforms, this result may indicate that on the overall outlook, the full-length isoform and the Δ7 isoform remain the two most prominent SMN2 transcripts in the cells used for this study. This result is in accordance with a previous report using SMA fibroblast GM03813, where slight additional skippings were noted in Δ5, Δ5,7, Δ3 and Δ3,7 isoforms.[16] We argue that whether similar additional skippings occur in GM09677 and GM22592 cells, they may not significantly affect our results.

Cell viability assay

Table 1 details the cell viability results at different concentrations of SAHA, Dacinostat and SAHA+Dacinostat. In individual compound exposures, both SAHA and Dacinostat sustain 97% viable cells at concentrations of 1 and 10 µM and also at 3.2 and 32 nM after a 48-h exposure. Exposures at higher dosages of individual compounds resulted in lower viability. Unfortunately, the combination of 10 µM SAHA and 32 nM Dacinostat decreased the cell viability to only 81% (type I cells) and 80% (type II cells) after only 24 h of exposure. Trials of different combinations of maximum SAHA/Dacinostat concentrations could only reach less than 90% cell viability. We obtained 97% cell viability at combined concentrations of 8 µM SAHA+25 nM Dacinostat for a 48-h exposure.

Comparison between SAHA and Dacinostat in modulating SMN2 transcript levels

The levels of Overall-SMN2 and E7-SMN2 transcripts were significantly higher in SAHA-treated type I and type II fibroblasts compared with untreated fibroblasts (Table 2). Overall-SMN2 transcript

Table 2 Overall-SMN2, E7-SMN2 and Δ7-SMN2 expression upon 48-h exposure with SAHA and Dacinostat

	Overall-SMN2		*E7-SMN2*		*Δ7-SMN2*	
	MFI ± s.e.m.	*Fold ± s.e.m.*	*MFI ± s.e.m.*	*Fold ± s.e.m.*	*%*[a]	*Fold ± s.e.m.*
Type I cells						
Mock	27.63 ± 1.76		12.7 ± 0.17			
S	47.56 ± 6.66	1.73 ± 0.1[b]	26.96 ± 1.26	2.12 ± 0.07[b]	21.2	1.39 ± 0.95
D	50.30 ± 9.79	1.86 ± 0.45	56.16 ± 0.59	4.43 ± 0.08[b]	44.2	(–2.8)
S+D	45.5 ± 4.61	1.64 ± 0.24[b]	23.66 ± 3.22	1.86 ± 0.23	18.6	1.47 ± 0.35
Type II cells						
Mock	36.4 ± 3.58		10.23 ± 2.71			
S	127.83 ± 3.35	3.53 ± 0.24[b]	62.13 ± 3.13	6.07 ± 0.20[b]	60.7	2.51 ± 0.24
D	49.36 ± 4.37	1.4 ± 0.07	32.4 ± 6.98	3.15 ± 0.97[b]	31.7	0.64 ± 0.09
S+D	29.23 ± 5.2	0.8 ± 0.15	35.28 ± 8.2	3.44 ± 0.99[b]	34.5	(–4.33)

Abbreviations: MFI, mean fluorescence intensity; SAHA, suberoylanilide hydroxamic acid; SMN2, Survival Motor Neuron 2.
[a]Percentage of exon 7 inclusion, on assumption that the baseline MFI value comprises 10% of the normal exon 7 inclusion level. S = 10 µM SAHA; D = 32 nM Dacinostat; S+D = 8 µM SAHA in combination with 25 nM Dacinostat.
[b]Indicate significant fold increase as compared with Mock ($P<0.05$).

increased by 1.73 ± 0.10-fold ($P < 0.043$) and 3.53 ± 0.24-fold ($P < 0.0003$) in type I and type II cells, respectively. E7-SMN2 transcript in type I and type II cells increased by 2.12 ± 0.07-fold ($P < 0.0003$) and 6.07 ± 0.20-fold ($P < 0.0002$), respectively. While E7-SMN2 transcript increased, our results showed that the differences between the Overall-SMN2 and E7-SMN2 that approximate the Δ7-SMN2 transcript level resulted in 1.39- and 2.51-fold increase in levels of the transcripts lacking exon 7 in type I and type II cells, respectively.

Similarly, the levels of Overall-SMN2 and E7-SMN2 transcript were significantly higher in Dacinostat-treated fibroblasts compared with untreated fibroblasts (Table 2). The increase in Overall-SMN2 transcript was 1.86 ± 0.23-fold and 1.4 ± 0.07-fold in type I and type II cells, respectively. E7-SMN2 transcript showed 4.43 ± 0.08-fold and 3.15 ± 0.97-fold increases in type I and type II cells, respectively. Δ7-SMN2 transcript in type I and type II cells seemed to be consistently decreased, although not significantly.

However, our experiments showed that exposure to the combined compounds did not result in a sum of individual compound exposure (Table 2). For Overall-SMN2, our experiments showed only a 1.64 ± 0.24-fold increase in type I cells and a relatively similar increase in transcript level in type II cells. For E7-SMN2, our analysis showed 1.86 ± 0.26-fold and 3.44 ± 0.99-fold increases in type I and type II cells, respectively. The Δ7-SMN2 transcript in type I and II cells decreased, although not significantly.

Comparison between SAHA and Dacinostat in modulating SMN protein levels

Figure 1 illustrates the effects of the compounds on the SMN protein levels. An increase in SMN protein was observed in both cell types after 48 h treatment with Dacinostat and SAHA. In accordance with the increase in SMN2 transcript, SMN protein levels were significantly increased compared with those of untreated cells; 10 μM SAHA treatment of type I and type II cells resulted in 1.98 ± 0.46 ($P < 0.009$) and 1.91 ± 0.09-fold increases ($P < 0.035$), respectively. Similar increases were also noted with 32 nM Dacinostat exposure in type I and type II cells, and the SMN protein levels were elevated 2.54 ± 0.58-fold ($P < 0.008$) and 1.19 ± 0.21-fold ($P = 0.047$), respectively. However, combined compound exposures only induced a slight increase in SMN protein levels, which is insignificant when compared wih individual compound exposures.

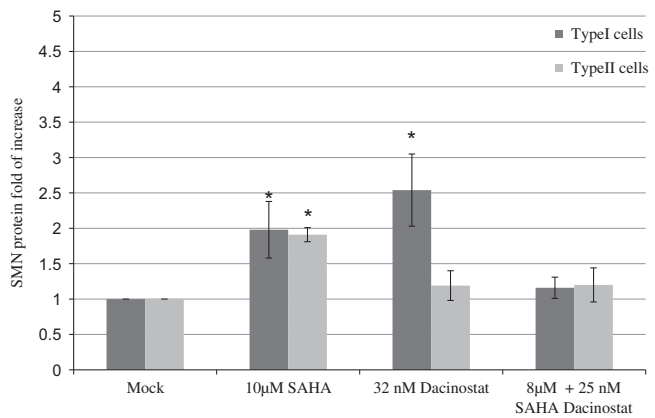

Figure 1 Effect of Dacinostat and SAHA in maximum dosages on SMN protein expression. The effects are shown as fold increase ± s.e.m. compared with Mock. *Indicates a significant fold increase compared with Mock.

SMN2 promoter methylation analysis

Table 3 shows the methylation levels of three CGI sites after exposure to SAHA and Dacinostat. The *SMN2* promoter of SAHA-treated type I and II cells was significantly demethylated at all the three sites after 48 h of exposure when compared with those of the untreated cells. Methylation decreases were also observed at all three CGIs of the *SMN2* promoter in Dacinostat-treated type I and II cells when compared with those of the untreated cells after 48 h. A reduction in methylation was also observed after combined compound exposure, although to a lesser extent than after individual exposures.

Docking simulation analysis

Our results showed that the combination of SAHA and Dacinostat exerted a less prominent effect on the increase of SMN2 transcript and protein levels compared with the individual treatment with the drugs. To clarify the mutual cancellation effect in the combination treatment of SAHA and Dacinostat, we performed docking simulation analysis of the drugs and HDAC2/HDAC7.

Figure 2 illustrates the intermolecular interactions between the individual compounds and HDAC2 and HDAC7. The intermolecular interactions of SAHA co-crystallized with HDAC2 were studied and analyzed as shown in Figure 2a. Five critical H-bonds were formed between SAHA and Tyr308, Asp181, His145, His146 and Asp104. Phe155 and Pro34 were mainly expected to contribute in hydrophobic interactions. The estimated free energy of binding calculated by Autodock 4.2 for SAHA was -6.24 kcal mol^{-1}. Dacinostat showed a lower free energy of binding at -8.44 kcal mol^{-1}, which indicates a higher affinity to HDAC2 than SAHA. The results showed that Dacinostat formed H-bonds with the same residues of the binding sites as SAHA (that is, Tyr308, Asp181, His145, His146 and Asp104), as shown in Figure 2b. Aromatic interactions were observed between the center ring of Dacinostat and Phe155, while Pro34 participated in a hydrophobic interaction.

The intermolecular interactions between SAHA and HDAC7 (PDB code: 3C0Z) showed five important H-bonds with Asp801, Asp707, His669, His670 and His626, as shown in Figure 2c, with a free energy of binding of -6.56 kcal mol^{-1}. This result indicates that SAHA has a similar affinity toward HDAC2 and HDAC7. Dacinostat showed a lower affinity toward HDAC7 than HDAC2, with a free energy of binding of -7.34 kcal mol^{-1}; however, this affinity is still higher than that of SAHA. Dacinostat also formed the same H-bonds with HDAC7

Table 3 SMN2 promoter methylation level upon exposure with SAHA and Dacinostat

	CGI1	*CGI2*	*CGI4*
Type I cells			
Mock	59.31 ± 0.61	58.95 ± 1.61	65.77 ± 2.02
S	52.78 ± 0.43[a]	48.81 ± 1.01[a]	53.87 ± 1.32[a]
D	52.32 ± 0.99[a]	48.33 ± 2.41[a]	57.83 ± 1.36
S+D	50.40 ± 2.32[a]	53.83 ± 3.16	61.93 ± 0.76
Type II cells			
Mock	64.69 ± 2.18	68.48 ± 4.44	69.41 ± 2.07
S	47.94 ± 0.85[a]	56.32 ± 1.8[a]	61.17 ± 4.13[a]
D	49.17 ± 2.89[a]	56.67 ± 1.04[a]	51.62 ± 1.56[a]
S+D	48.93 ± 4.54[a]	56.68 ± 1.04[a]	62.56 ± 0.60

Abbreviations: CGI, CpG Island; SAHA, suberoylanilide hydroxamic acid; SMN2, Survival Motor Neuron 2.
[a]Indicate significant difference as compared with Mock ($P < 0.05$). Methylation levels were expressed as mean of methylation percentage ± s.e.m. S = 10 μM SAHA; D = 32 nM Dacinostat; S+D = 8 μM SAHA in combination with 25 nM Dacinostat.

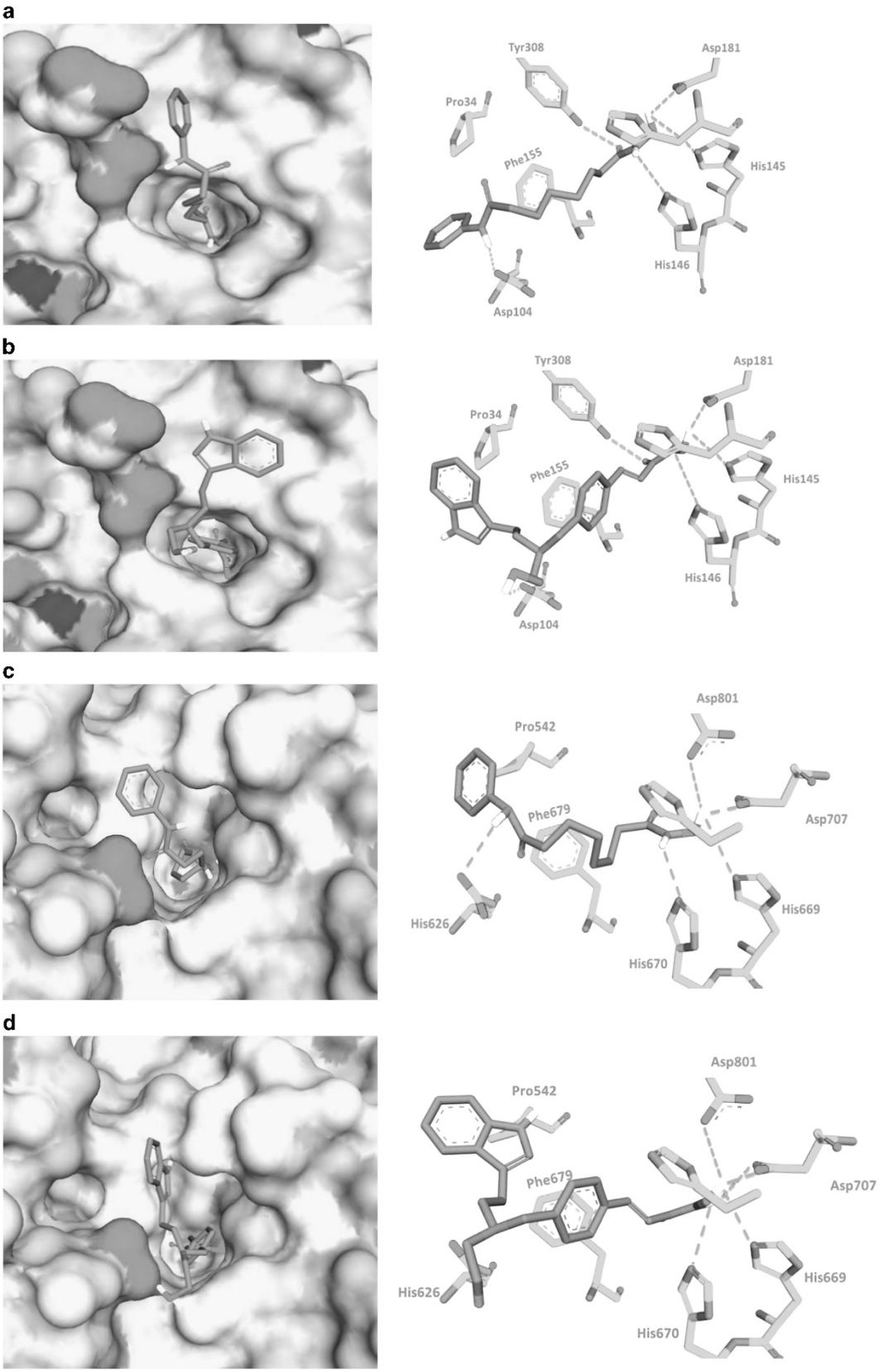

Figure 2 Molecular surface (left) and solid ribbon (right) representations of intermolecular interactions between (**a**) SAHA and HDAC2, (**b**) Dacinostat and HDAC2, (**c**) SAHA and HDAC7, (**d**) Dacinostat and HDAC7. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

as SAHA, as shown in Figure 2d. It is expected that Phe679 may contribute in an aromatic interaction with the center ring of Dacinostat.

DISCUSSION

Although almost all studies on the therapeutic effects of Dacinostat have been focusing on cancer,[10,25–30] our study provided the first account for the potential therapeutic effect of Dacinostat on SMA. In this study, we compared the novel hydroxamic acid, Dacinostat, with another previously studied hydroxamic acid, SAHA, and compared the two compounds with regard to their potential therapeutic effects on SMA.

SAHA and Dacinostat for elevating SMN2 expression

Our results were in accordance with those of previous studies showing that SAHA increases *SMN2* gene expression.[8,11,12] While the fold increase ratio (Overall-SMN2: E7-SMN2) between the two mechanisms was approximately 1:1 in type I cells, we observed that the fold increase ratio was approximately 1:2 in type II cells. In addition, given that Δ7-SMN2 expression seems to also increase in both cell types, SAHA may have worked by increasing overall *SMN2* expression as well as exon 7 inclusion. Similarly, Evans *et al.*[31] showed that SAHA increased both overall gene expression and exon 7 inclusion in luciferase reporter cells.

For Dacinostat on type I cells, we found a significant fold increase in exon 7 inclusion and a less than twofold increase in overall *SMN2* expression, which translated into an approximate threefold increase in SMN protein. In type II cells, however, we noted a limited response to Dacinostat exposure, especially on overall SMN2 transcript and SMN protein expression, even though there was a significant fold increase in exon 7 inclusion. The fold protein increase in type II cells was approximately half of that in type I, which is a slight and insignificant result.

Although Dacinostat and SAHA are from the same HDACi class and target identical sites in HDAC2/HDAC7, they are not completely identical. Moreover, the two cell types that we used are also not entirely identical either, apart from difference in *SMN2* copy number. A previous report noted that the responses of SMA fibroblasts and lymphoblasts to treatments with valproate, hydroxyurea and phenylbutyrate were heterogeneous. The report also revealed both intra-patient and inter-patient variability, suggesting that tissue type and individual factors may affect the response to these compounds.[32] Therefore, it could be postulated that the type II cells used in this study might carry some polymorphisms or other biological variations, leading to lower SMN2 expression and SMN protein response to Dacinostat.

In addition, it is notable that the expression levels of E7-SMN2 improperly exceed those of overall-SMN2 in type I cells treated with Dacinostat or type II cells treated with both SAHA and Dacinostat (Table 2). Although expressions of both isoforms should be inter-related, they were investigated using different probes involving different set of chemical reactions. Therefore, the discrepancy might have been caused by variable efficiency of the probes. However, we have shown that at the baseline, the expression levels of E7-SMN2 in untreated type I and type II cells were less than those of Overall-SMN2. Nevertheless, our results showed that, even though E7-SMN2 expressions were higher than Overall-SMN2, their differences were not significant (that is, $P = 0.36$ for Dacinostat in type I cells and $P = 0.34$ for SAHA+Dacinostat in type II cells).

One might argue that we should have employed a more widely used RT-PCR analysis in order to avoid the discrepancy in probe efficiency. The RT-PCR analysis would employ primers located at exon 7 and exon 8 for detecting full-length SMN2 transcripts and at exon 5 and exon 6–exon 8 junction for detecting Δ7-SMN2 transcripts. However, in this regard, the presence and quantification of Overall-SMN2 expression could only be postulated from calculating the sum of both expressions. Besides, involvements of primers at different locations would not rule out the possibility of differential efficiency as we also experienced. In our study, we aimed at detecting primary signals from Overall-SMN2 expression because of our hypothesis that HDACis might also work on increasing an overall gene expression. Besides, our methodology provided primary quantification of the transcript amount where the probes bound directly to the mRNA and thus, circumvented the need of cDNA synthesis.

We noted that the fold increase ratio (Overall-SMN2 to E7-SMN2) between the two mechanisms for Dacinostat is consistently higher than 1:2 in both type I and type II cells. In addition, our calculations for Δ7-SMN2 expression seem to show a consistent decrease. These results might indicate a greater effect of Dacinostat on exon 7 inclusion than on overall SMN2 expression.

Considering that there should be a strong correlation between SMN protein expression and transcript levels, it is notable that the resulting protein levels in our study were not totally consistent with mRNA expression. Although the increase in overall and E7-SMN2 expression was higher in type II cells after SAHA exposure compared with that of type I cells, SMN protein levels were not significantly different between the two cell types. A previous report showed a discrepancy between the different VPA concentrations required for the maximal FL-SMN2 RNA levels and the maximal SMN protein levels within one cell line, suggesting that VPA stimulates transcription and translation of SMN2 differently.[33] Furthermore, another report suggested that there may be a significant modulation of SMN at the post-transcriptional level. This regulation could be different for different cell types and tissues and may also vary by age.[34] Indeed, it has been indicated that the SMN protein level is unrelated to the SMN mRNA level.[35]

Our molecular docking analyses revealed that Dacinostat showed a higher affinity toward HDAC2/HDAC7 compared with SAHA. This *in silico* prediction was evident in type I cells, where Dacinostat showed consistently higher effects toward Overall-SMN2 and E7-SMN2 expressions.

Indications that both compounds act on splicing modulation suggest that they may also target splicing machinery, as previously evident for VPA, which acts by targeting SF2/ASF and hnRNPA1.[36] Indeed, Evan *et al.*[31] suggested that silencing of HDACs 5 and 6 promoted the inclusion and recognition of exon 7. These results suggested that the effect of hydroxamate-based HDACis on splicing might result not only from the inhibition of specific components in the splicing machinery but also by silencing specific HDACs that have putative roles in splicing, which may explain the effects of hydroxamate-based HDACis or even HDACis in general on exon 7 inclusion.

Combination of SAHA–Dacinostat on SMN2 expression

Our results consistently showed that combined exposure to both compounds resulted in a lower effect than we expected. It is most likely that multiple factors might have contributed, because according to our data in Figure 1 the effect of the simultaneous treatment of SAHA and Dacinostat showed much less effect than either a single treatment of SAHA or Dacinostat.

We could propose at least three reasons why this happened. The first is related to the compounds' concentrations. It is reasonable to argue whether the effects of a combined treatment should have been

higher than those of the individual treatments at the same concentrations. The combined treatments did not show an increased effect likely because of the suboptimal concentrations compared with individual treatments.

The second and third plausible reasons are related to our results of the molecular docking simulation. Our docking simulation analysis data showed that both drugs made H-bonds with identical amino acids in HDAC2 and HDAC7. The simultaneous existence of both drugs might have led to competitive binding to the same sites, which led to the second plausible reason. Presence of another compound with the same binding sites would displace the compound which is already bound to the sites, as described previously through drug displacement assay showing interference of Tranilast, an antiallergic drug, to the binding between Warfarin and Human Serum Albumin at Sudlow's site I due to its presence at the same binding sites.[37] Similar situation might have occurred when SAHA and Dacinostat were combined, leading to inhibition of effective binding of both drugs to HDAC2 and HDAC7, resulting in no activation of the *SMN2* expression.

The third plausible reason argues that irreversible structural changes might have happened to the binding site right after binding of any of the HDACis. It has previously been indicated that ligand bound introduces, in general, small structural perturbations at the binding site of the protein, which can lead to important alterations in the recognition pattern of the protein.[38] We postulate that this change would prevent binding of other HDACi molecules after displacement of the first bound HDACi. Therefore, let alone the suboptimal concentrations of the drugs, combination of displacement event and irreversible structural changes may be the reason to lower HDACi effect than that of the individual exposure, leading to considerable restoration of HDAC activity.

We therefore postulate that the increase in SMN2 expression was prevented by the restoration of HDACs activity due to combination of the drugs in our study where multiple mechanisms might have taken place.

Histone acetylation of the SMN2 promoter decreases methylation levels

Exposure to HDAC inhibitors in our study aimed at promoting histone acetylation in the SMN2 promoter. Our data in Table 3 showed that for single compound treatment, methylation levels in compound-treated cells were significantly lower than those in Mock cells in almost all CGIs of both cell types. Thus, our data showed almost consistent demethylation in both types of cells after exposure to SAHA and Dacinostat, which supports the effects of these compounds on the increase in overall SMN2 expression. These results provide evidence that acetylation of the SMN2 promoter with HDACis decreases the methylation level of the gene.

The demethylation effects of Dacinostat are not obviously different from those of SAHA. However, Table 3 also showed that methylation levels resulted from exposure of combined compounds were similar or higher than single compound, but never exceeded those of Mock. This suggested limited effect of combined compound exposure, which is consistent with our findings on the limited increase in SMN2 expression resulted from combined compound exposure.

A number of chromatin modifying enzymes have been shown to recruit DNMTs (DNA methyltransferases) to specific genes and thus target DNA methylation.[39,40] A previous study that exposed cell lines from the human bladder and breast carcinomas to Trichostatin A, an HDACi, demonstrated a reversible crosstalk between histone acetylation and DNA demethylation. This study reported that an increase in histone acetylation by trichostatin A was associated with a significant decrease in global methylation and that there is gene selectivity in the induction of acetylation and demethylation.[41] Furthermore, a previous study reported that HDACis reversed CpG methylation through the inhibition of MAP Kinase I and the subsequent downregulation of DNMT1.[42]

In conclusion, we have shown that SAHA and Dacinostat increase SMN2 transcript and protein levels and promote demethylation within the *SMN2* promoter of type I cells. In type II cells, however, while we observed a significant effect resulted from SAHA exposure, we noted a limited response to Dacinostat exposure, especially on overall SMN2 transcript and SMN protein expression; even though there was a significant fold increase in exon 7 inclusion. Dacinostat was shown to exert a stronger effect toward exon 7 inclusion than SAHA, which may suggest therapeutic potential of Dacinostat for treating SMA. In addition, acetylation of the *SMN2* promoter was shown to induce demethylation of the gene. We also suggested that combined treatment with SAHA and Dacinostat, compounds from the same HDAC inhibitor class, might have led to considerable restoration of HDAC activity which resulted in a decreased effect.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was funded by the Universiti Sains Malaysia Research University Grant No. 1001/PPSP/812072 for Dr Teguh Haryo Sasongko. JM was a recipient of a USM Graduate Assistant Scheme for his PhD study.

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