Combined effects of retinoic acid and histone deacetylase inhibitors on human neuroblastoma SH-SY5Y cells

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Running Title: Effects of HDACi and RA on SH-SY5Y cells

Abbreviations: CKI, cyclin kinase inhibitor; HDACi, histone deacetylase inhibitors; PARP, poly ADP-ribose polymerase; RA, all-*trans* retinoic acid; TSA, trichostatin A; But, sodium butyrate; SAHA, suberoylanilide hydroxamic acid.

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ABSTRACT

All-*trans* retinoic acid (RA) causes differentiation of neuroblastoma cells, and retinoids have been used in clinical trials in children with advanced neuroblastoma. Combination of RA with histone deacetylase inhibitors (HDACi) could result in improved antitumorigenic activity. We have examined the effect of the HDACi trichostatin A (TSA), sodium butyrate (But), and suberoylanilide hydroxamic acid (SAHA), alone and in combination with RA in human neuroblastoma SH-SY5Y cells. At concentrations that cause sustained increase of histone H3 acetylation, HDACi produced extensive apoptotic cell death as shown by flow cytometry analysis and induction of poly ADP-ribose polymerase (PARP) proteolysis. HDACi inhibited SH-SY5Y cell growth at much larger extent than RA. This compound did not cause apoptosis and did not further increase HDACi-mediated cell death. In contrast, both types of drugs cooperated to inhibit cell growth, although synergistic effects were not found. In surviving cells, HDACi repressed Cyclin D1 expression and increased the cyclin kinase inhibitors (CKI) $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Cyclin D1 was not affected by RA, but this retinoid also increased CKI levels. Induction of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ by HDACi was further enhanced in the presence of RA. This effect appears to be at least partially due to transcriptional stimulation of CKI gene expression, since both types of drugs cooperated to increase CKI mRNA levels and to activate the CKI promoters in transient transfection assays. These results show the strong anti-tumorigenic effects of HDACi in neuroblastoma cells and reinforce the idea that combination therapy could be useful to inhibit tumor growth.

Introduction

Histone deacetylase inhibitors (HDACi) constitute a promising treatment for cancer therapy due to their low toxicity, and first-generation HDACi are currently being tested in phase I/II clinical trials (1,2). There are distinct classes of HDAC inhibitors, including among others short-chain fatty acids such as butyrate and hydroxamic acids such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), that function by binding to the catalytic site of the enzyme (3).

During tumorigenesis, histone hypoacetylation can result in the silencing of genes involved in regulation of cell growth, differentiation, and apoptosis. HDACi increase acetylation of histones and transcription factors (4), which can reverse gene silencing facilitating gene expression (5). However, not all genes are upregulated by treatment with HDACi and the ratio of upregulated to downregulated genes has been found to be close to 1:1 (2). HDACi have been shown to induce G1-phase cell cycle arrest with up-regulation of $p21^{Waf1/Cip1}$ (6-7), which was associated with an Sp1 site in the gene promoter (8-11). These changes can result in cell type specific effects including reduced proliferation and metabolic activity, induction of apoptosis and differentiation (12-15).

Neuroblastoma accounts for more than 15% of cancer-related deaths in early childhood (16). Thus, new drugs are needed for the treatment of this disease. The finding that all-*trans* retinoic acid (RA) causes differentiation of neuroblastoma cells (17), led to clinical trials that demonstrated a substantial clinical benefit for retinoid therapy in advanced neuroblastoma, although 50% of children still relapsed (18).

The effects of RA are mediated by nuclear receptors. In the absence of ligand these receptors act as transcriptional repressors due to the binding of corepressor complexes that contain histone deacetylases (HDACs). Ligand binding allows the release of corepressors and facilitates the ordered recruitment of coactivator complexes, some of

which possess histone acetylase (HAT) activity, that cause transcriptional stimulation (20). Several retinoids are able also to inhibit the activator protein-1 (AP-1) transcription pathway, which is activated upon growth factor signaling (19,20) or even to have non-genomic effects that lead to stimulation of signaling pathways involved in neuronal differentiation (21).

Combination therapy of RA with HDACIs may improve efficacy while reducing side effects. An enhanced inhibitory effect on neuroblastoma cell growth when the HDACi M-carboxycinnamic acid bishydroxamide (CBHA) was combined with RA has been found (22). To further investigate the effects of deacetylase inhibition, we compared the potency of the HDACi sodium butyrate, TSA and SAHA alone and in combination with RA to inhibit growth of human SH-SY5Y neuroblastoma cells. This cell line has proven to be a valuable model for studying the effects of RA on neuronal differentiation, including extension of neuritic processes and growth arrest (23,24).

Materials and Methods

Cell proliferation assays

SH-SY5Y cells were grown in RPMI containing 10% fetal calf serum (24). 24 h before the beginning of treatments cells were shifted to medium containing AGX100 resin-charcoal treated serum to eliminate retinoids. Cytotoxicity was determined by colorimetric MTT assay, as recommended by the manufacturer (Roche). Absorbance was read at 550 and 690 nm. Cells were plated in 96-wells plates (7×10^3 cells/well), and assays performed after incubation for 24 and 48 h with RA and HDACi. When the Caspase Family Inhibitor (Fluoromethylketone) Z-Val-Ala-Asp (OMe)-FMK (Z-VAD) was used cells were inoculated at a density of 20×10^3 cells/well. DNA synthesis was determined by [^{3}H]thymidine incorporation in cells inoculated in 6-multiwells plates (6

x 10^5 cells/well). Cells were treated for 24 h with the different compounds and for the last 2 h with 2 μCi/well of [^{3}H]thymidine. Cells were disrupted and incorporated radioactivity was determined in a liquid scintillation Beta Wallac Counter. For counting, cells were inoculated in 6-well plates at 5 x 10^4 cells/well, treated 24 later with the different compounds and counted every two days in Neubauer chambers. Cell morphology was assessed at 200X in a Zeiss inverted phase contrast microscope.

Flow cytometry

Triplicate cultures of SH-SY5Y cells grown in 60 mm Petri dishes were transferred to the medium containing depleted serum and after 24 h incubated with the different compounds for 48 h. Both floating and adherent cells were collected, washed twice with cold PBS, fixed with chilled ethanol 70% and centrifuged. Pellets were incubated for 30 min at 37ºC with 0.1 μg/ml RNAse A and stained with propidium iodide (50 μg/ml) for sorting in a FACScan (Becton-Dickinson) cell sorter. Percentage of cells in subG1, G1, S and G2/M phases was calculated with WinMDI and Cylchred software for Windows.

Western blot

Cell lysates were obtained as previously described (25). Proteins were separated in SDS-PAGE and transferred to PDVF membranes (Immobilon Millipore) that were blocked for 1 h at room temperature with 4% BSA. Incubation with primary antibodies (25,26) was performed overnight at 4ªC, and with the secondary antibody for 1 h at room temperature. Blots were visualized with ECL (Amersham). Antibodies against Cyclin D1, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and poly (ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Inc and used at a 1:2000 dilution. The antibody for acetylated histone H3 in lysines 9 and 14 (Upstate) was used at a 1:5000 dilution. Membranes were stripped, blocked and reprobed with anti human actin antibody (Santa Cruz).

Northern blot

Total RNA from cells grown in 90 mm dishes was isolated with TriReagent (Sigma), following the manufacturer's protocol. Total RNA (15 µg) was run in 1% formaldehyde-MOPS agarose gels and transferred to nylon Nytran-N (Schleicher & Schuell) as described (24-26). Then RNA was linked to membrane using UV-Stratalinker and stained with 0.02% methylene blue to assess equal loading. Probes were labeled by random primer with Ready-To-Go DNA Labeling kit and [α-^{32}P]dCTP (Amersham Pharmacia Biotech). The labeled probes were hybridized with specific probes for Cyclin D1, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ as described (25-26).

Transient transfections

SH-SY5Y cells ($1x10^6$) were plated in 60 mm wells, changed to serum-free medium 24 hours later and transfected with 5 µg of luciferase reporter plasmids containing 2.3 kb of the human $p21^{Waf1/Cip1}$ promoter and 1.9 kb of the human $p27^{Kip1}$ promoter (26). Transfection was obtained by incubation with a mixture of cationic liposomes (1.5 µl/µg DNA) for 6 h. Cells were then treated for 48 h with RA and/or HDACi and activity determined. Each experiment was performed in triplicate and was repeated at least 3 times. Data are mean ± S.D and are expressed as fold induction over the values obtained in the untreated cells.

Results

Effects of HDACi on histone H3 acetylation

Incubation of SH-SY5Y cells during 6 hours with TSA, But and SAHA caused a dose-dependent increase of histone H3 acetylation. A detectable increase was observed with the lower dose of TSA used, 50 nM, and 100 nM TSA caused an acetylation at least as strong as that produced by 2 mM But or 1 µM SAHA (Fig. 1A). However, the effect of these TSA concentrations on H3 acetylation is transient. Whereas But and

SAHA produced a sustained increase in histone H3 acetylation that was very strong after 48 h, concentrations of TSA 200 nM or lower had little effect at this time point (Fig. 1A). As shown in Fig. 1B, 100 nM TSA caused a stronger acetylation than 2 mM But after 6 h of incubation, but the opposite was true after 24 h. Re-addition of TSA after 24 h caused again a strong increase in the levels of acetylated H3 histone (Fig. 1C), suggesting that this reduction results from a rapid metabolism of TSA in SH-SY5Y cells rather than being secondary to changes in HDAC sensitivity to this compound.

Combined effects of HDACi and RA on SH-SY5Y cell growth and morphology

By using MTT assays to measure viability, the dose- and time-dependent effects observed showed that significant killing of SH-SY5Y cells by HDACi required 48 h. At 24 h (Fig.2A, left panel), the different HDACi had little effect even at high concentrations both in the presence and absence of RA. However, after 48 h concentrations of 100 nM TSA, 1 mM But or 0.5 µM SAHA were ineffective, but higher concentrations (200 and 500 nM TSA; 2 and 5 mM But; and 1 and 2 µM SAHA) caused a strong dose-dependent decrease of cell viability. Whereas RA (1 µM) did not reduce this parameter, the retinoid was able to increase the effect of low HDACi concentrations, although a strong synergistic effect was not found (Fig. 2A, right panel). Direct cell counting also demonstrated a marked effect of HDACi on SH-SY5Y cell viability. Fig.2B shows that whereas control cells proliferate rapidly, at 48 h TSA 200 nM, But 2 mM and SAHA 1 µM caused a strong inhibition of cell proliferation, and that essentially no viable cells remain after 4 days of treatment with the HDACi. An inhibitory effect of HDACi on DNA synthesis was already observed at 24 h (Fig. 2B). Concentrations higher than 200 nM TSA, 2 mM But or 0.5 µM SAHA reduced thymidine incorporation and RA, that only caused a weak dose-dependent inhibition, again potentiated HDACi action.

In SH-SY5Y cells, RA treatment for 48 h induced morphological differentiation with neurite extension (Fig. 3). In contrast, morphology after treatment with HDACi (200 mM TSA, 2 mM But or 1 µM SAHA) was typical of cells undergoing cell death, with neurite shortening and appearance of cells in suspension. This occurred both in the presence and absence of RA (Fig. 3).

Flow cytometry analysis confirmed that HDACi produced SH-SY5Y cell death, manifested by accumulation of subG1 cell debris. Approximately 60% of cells treated with 2 mM But or 1 µM SAHA were in subG1 both in the presence and absence of 1 µM RA after 48 h of incubation (Fig. 4A). These compounds also caused a significant decrease on the number of cells on S-phase. In contrast, 100 nM TSA had little effect on cell death or cell cycle progression, showing that a sustained increase in histone acetylation is required for these HDACi actions.

Proteolysis of PARP after incubation for 48 h with 200 nM TSA, 2 mM But or 1 µM SAHA (Fig. 4B), demonstrated that HDACi caused apoptotic cell death. In contrast, incubation with 1 µM RA for the same time period did not alter cell cycle distribution and did not induce apoptotic SH-SY5Y cell death.

To determine if cell death caused by HDACi is caspase dependent, we first determined PARP proteolysis in the presence and absence of the caspase inhibitor Z-VAD. As shown in Fig.5A the 89 kDa band representing cleavage of the protein was found after 24 h of incubation with 2 mM But or 2 µM SAHA alone, but this band was absent in cells treated with the combination of HDACi and Z-VAD. The effect of the caspase family inhibitor was also examined in MTT assays performed in cells treated with But (2 and 5 mM) and SAHA (1 and 2 µM). Fig. 5B shows that at 24 h incubation these compounds had little effect on cell viability, in agreement with the results shown in Fig.2A. However, after 48 h both inhibitors caused a dose-dependent reduction of

cell viability that was significantly reversed in the presence of Z-VAD, indicating the participation of caspases in HDACi-mediated apoptosis of SH-SY5Y cells.

H3 acetylation and CKI and cyclin D1 expression in HDACi-treated cells

The effect of 24 h incubation with the various HDACi on histone H3 acetylation paralleled closely regulation of cyclin D1 and CKI levels in SH-SY5Y cells (Fig. 6A). TSA was effective at 200 nM, and at 500 nM that induced a very strong histone H3 acetylation, caused a marked reduction of cyclin D1 and a strong increase of $p21^{Waf1/Cip1}$. But was effective from 0.5 mM, causing a progressive decrease of cyclin D1 and a concomitant induction of the CKI, and the same was true for SAHA between 0.5 and 2 µM. RA did not increase the levels of acetylated H3 and did not reduce cyclin D1 expression. However, an increase in $p21^{Waf1/Cip1}$ expression was also found after treatment with 10 µM RA (Fig. 6A). On the other hand, the combination of SAHA or But with 1 µM RA did not cause a further reduction of cyclin D1 expression, but up-regulation of $p21^{Waf1/Cip1}$ levels by the HDACi was further increased in the presence of the retinoid (Fig. 6B), suggesting that regulation of this CKI plays a more important role than cyclin D1 on the repression of SH-5Y5Y cell growth found with the combined treatment. Since other CKIs could also contribute to the arrest of cell growth observed after HDACi and RA treatment, the levels of $p27^{Kip1}$ were also measured. As shown in Fig. 6C, the levels of this inhibitor were also elevated after the treatments, although less strongly than $p21^{Waf1/Cip1}$.

When the time-course of the effect of But (2 mM) on CKI expression was analyzed, it was found that the induction of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ levels followed similar kinetics. No CKI induction was observed after 1 and 3 h of incubation with the

inhibitor, but a detectable increase was found between 5 and 7 h (Fig.6D). The influence of increasing concentrations of the different HDACi, as well as RA, was then examined after 6 h of treatment (Fig.6E). Whereas at 24 h an increase in CKI expression was only detected at high TSA concentrations (see Fig.6A), at 6 h of incubation the increase in $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ levels were maximal at a TSA concentration as low as 50 nM. These results match again the stimulation of histone H3 acetylation, since the actions of low TSA concentrations were transient and a strong acetylation after 24 h was only obtained at high concentrations of this inhibitor. This contrasts with the effects of butyrate that caused a sustained induction of CKI and histone acetylation, at all concentrations used. On the other hand, RA was also effective to increase the CKIs after 6 h of treatment even at 0.1 µM.

In agreement with previous reports indicating that HDACi can induce $p21^{Waf1/Cip1}$ gene transcription, treatment with these compounds at concentrations that caused H3 acetylation also increased the levels of $p21^{Waf1/Cip1}$ transcripts in SH-SY5Y cells (Fig. 6A). In addition, these levels were further induced in the presence of RA. $p27^{Kip1}$ mRNA levels also increased after incubation with But 2 mM and SAHA 1 µM, and RA further potentiated this increase. Cooperation of the retinoid with HDACi to stimulate activity of a luciferase reporter plasmid containing the $p21^{Waf1/Cip1}$ promoter in transient transfection assays was also found (Fig. 6B). RA increased by 2 fold promoter activity, and the various HDACi were able to further induce this stimulation. TSA 200 nM was less potent that But 2 mM or SAHA 1 µM for this induction. Induction of $p27^{Kip1}$ promoter activity was also found after the treatments. RA was able to induce by 3-4 fold reporter activity and to cooperate with the HDACi to stimulate the promoter (Fig. 6C).

Discussion

We have compared the effect of the HDACi sodium butyrate (But) with that caused by two hydroxamic acid derivatives, TSA and SAHA, on human SH-SY5Y neuroblastoma cells. It has been shown that TSA at nanomolar concentrations causes a strong accumulation of acetylated histones in cells and blocks HDAC activity in vitro (27). Our work confirms that low concentrations of TSA cause a strong increase in histone H3 acetylation in SH-SY5Y cells. However, the effect of this inhibitor is transient when used at doses lower than 200 nM. This could result from a short-life of this compound in these cells, since re-addition of TSA again increases acetylation. The effects of But, at millimolar concentrations, or SAHA, a second generation HDACi (28) at micromolar concentrations, are more stable being able to increase acetylation even after 48 hours. Interestingly, SAHA has been successful in phase II trials for the treatment of cutaneous T cell lymphoma (29), and is the first HDACi approved by the FDA to enter the clinical oncology market.

The concentrations of HDACi that produced long-standing histone hyperacetylation also caused strong growth inhibition. Inhibition is consequence of increase in apoptotic cell death, as indicated by the appearance of cells in subG1, and the induction of PARP proteolysis, a late apoptotic event. HDACi-induced apoptosis appears to be at least partially caspase-dependent, since cell death was attenuated in the presence of a caspase family inhibitor. Besides inducing apoptosis, in the surviving cells HDACi also caused a significant decrease of the number of cells in G1 and S-phases. Since Cyclin D1 is essential for G1 entry, this reduction could be related to the decrease of this protein found in HDACi-treated cells. It has been shown that the HDACi BL1521A reduces Cyclin D1 transcripts in neuroblastoma cells (30) and we also observed this reduction in SH-SY5Y cells treated with But, TSA or SAHA. However, the possibility that HDACi

could also alter Cyclin D1 stability in agreement with the very recent finding that TSA induces ubiquitin-dependent degradation of Cyclin D1 in breast cancer cells (31) cannot be excluded.

Cyclin dependent kinase (CDK) activity is counteracted by cyclin kinase inhibitors (CKI). The WAF/KIP family of CKIs that includes among others $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, inhibits kinase activity of G1/S CDKs (32). In agreement with observations in other cells (6-11,33,34), HDACi caused an important increase of $p21^{Waf1/Cip1}$ in SH-SY5Y cells. It has been suggested that this induction is a critical effector of HDACi for cell cycle arrest and that cells lacking this CKI are insensitive to these compounds (35). However, butyrate can induce G1 arrest in mouse embryonic fibroblasts lacking $p21^{Waf1/Cip1}$, suggesting that Cyclin D1 could be the key factor in this inhibition (36). In any case, growth arrest promoted by HDACi in SH-SY5Y cells was accompanied by both Cyclin D1 reduction and $p21^{Waf1/Cip1}$ induction. This CKI was not the only inhibitor induced by these agents, since the levels of $p27^{Kip1}$ were also increased upon incubation with HDACi. The increase in $p27^{Kip1}$ most likely contributes to the inhibition of growth by HDACi in SH-SY5Y cells. In contrast with $p21^{Waf1/Cip1}$ that appears to be a common target for different HDACi, induction of $p27^{Kip1}$ appears to be more selective. For instance the HDACi TSA and valproic acid, but not butyrate or BL1521, have been found to induce this protein in other neuroblastoma cell lines (7,33,34).

The influence of HDACi on $p21^{Waf1/Cip1}$ levels is due to transcriptional stimulation. Most likely Sp1 binding motifs that mediate $p21^{Waf1/Cip1}$ induction in other cell types (9-11) also mediate induction in SH-SY5Y cells. An increase in the amount of

acetylated histone H3 associated to the $p21^{Waf1/Cip1}$ promoter (8) as well as a reduction in HDAC1 binding (37) has been obtained by chromatin immunoprecipitation assays after treatment of other cells with HDACi. This should also occur in SH-5Y5Y cells in which an increase in H3 acetylation is found. Our data also demonstrate that HDACi increase $p27^{Kip1}$ mRNA levels and stimulate $p27^{Kip1}$ promoter activity in transfection assays. Although the promoter elements that mediate $p27^{Kip1}$ gene transcription by HDACi have not been yet defined, most likely increased association of acetylated histones with the promoter is involved in this stimulation.

Retinoids have been used in clinical trials in patients with neuroblastoma (16-18), although their therapeutic use is limited by their toxicity. Our data show that the effect of RA in SH-SY5Y cell growth is much weaker than that of HDACi. In contrast with HDACi, treatment of SH-SY5Y cells with RA did not cause apoptosis. This could be related to increased expression of Bcl-2 (38), an effective apoptosis suppressor. Also, contrary to that found with HDACi, RA did not reduce Cyclin D1. However, in agreement with previous results in neuroblastoma cells (24,39), RA caused CKI induction. Our data show that the increase in $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ appears to be at least partially due to transcriptional stimulation since RA increased CKI transcripts and caused promoter activation in reporter assays. In addition, it has been recently reported that RA induces $p27^{Kip1}$ phosphorylation that leads to its stabilization and accumulation into the nuclear compartment (40). The promoter elements responsible for induction of $p27^{Kip1}$ gene expression by RA are still unknown, but the increase of $p21^{Waf1/Cip1}$ promoter activity by RA could depend on a promoter response element that mediates $p21^{Waf1/Cip1}$ induction by RA in leukemia cells (41).

It has been proposed that the combination of retinoids with other drugs could result in improved antitumorigenic activity, allowing the use of low retinoid concentrations and reducing its side effects (19). The HDACi CBHA and RA have been described to inhibit neuroblastoma cell growth in significantly lower concentrations when used together than when used individually (23). Our results show a clear cooperation of RA with other HDACi to increase $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ levels in SH-SY5Y cells that might be involved in the stronger reduction in cell proliferation found when both agents were used together. This cooperation was also observed for mRNA induction and promoter activation, confirming their transcriptional effect on CKI gene expression. In contrast, RA did not increase HDACi-mediated cell death and did not further reduce cyclin D1 expression. This could explain why strong synergistic effects of both types of drugs on SH-SY5Y growth arrest were not found.

Our results as well as the finding that HDACi can suppress growth in human-mouse neuroblastoma xenografts (13,42), suggest that HDACi that cause a sustained increase of histone acetylation could be promising therapeutic agents for neuroblastoma treatment. Their anti-tumorigenic effects could be stronger than those of retinoids and both types of drugs could cooperate to inhibit neuroblastoma growth.

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References

1. Marks P, Rifkind RA, Richon VM, et al. Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer 2004;1:194-202.

2. Minucci S, Pelicci PG Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 2006;6:38-51.

3. Finnin MS, Donigian JR, Cohen A, et al. Structures of a histone deacetylase homologue bound to the inhibitors TSA and SAHA. Nature 1999;401:188-93.

4. Bannister AJ, Miska EA. Regulation of gene expression by transcription factor acetylation. Cell Mol Life Sci 2000;57:1184-92.

5. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov 2002;1:287-99.

6. Sambucetti LC, Fischer DD, Zabludoff S, et al. Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. J Biol Chem 1999;274:34940-47.

7. Ouwehand K, de Ruijter AJ, van Bree C, et al. The histone deacetylase inhibitor, BL1521 induced a G1-phase arrest in neuroblastoma cells through alteration of cell cycle genes. FEBS lett 2005;579:1523–28.

8. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci U S A. 2000;97:10014–19.

9. Nakano K, Mizuno T, Sowa Y, et al. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J Biol Chem 1997;272:22199-06.

10. Huang L, Sowa Y, Sakai T, Pardee AB Activation of the p21WAF1/CIP1 promoter independent of p53 by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) through the Sp1 sites. Oncogene 2000;19:5712-19.

11. Xiao H, Hasegawa T, Isobe K. p300 collaborates with Sp1 and Sp3 in p21(waf1/cip1) promoter activation induced by histone deacetylase inhibitor. J Biol Chem 2000;275:1371-76.

12. Glick RD, Swendeman SL, Coffey DC et al. Hybrid polar histone deacetylase inhibitor induces apoptosis and CD95/CD95 ligand expression in human neuroblastoma. Cancer Res 1999;59:4392-99.

13. Jaboin J, Wild J, Hamidi H, et al. MS-27-275, an inhibitor of histone deacetylase, has marked in vitro and in vivo antitumor activity against pediatric solid tumors. Cancer Res 2002;62:6108-15.

14. Subramanian C, Opipari AW Jr, Bian X, et al. Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci. U S A 2005;102:4842-47.

15 Peart MJ, Smyth GK, van Laar RK, et al. Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proc Nat. Acad Sci U S A 2005;102:3697-02.

16. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma Nat Rev Cancer 2003;3:203-16.

17. Thiele CJ, Reynolds CP, Israel MA. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 1985;313:404-06.

18. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med 1999;341:1165-73.

19. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nature

Rev Cancer 2001;1:181-93.

20. Aranda A, Pascual A. Nuclear hormone receptors and gene expression Physiol Rev 2001;81:1269-04.

21. Cañón E, Scsucova S, Aranda A. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell 2004;15:5583-92.

22. Coffey DC, Kutko MC, Glick,RD, et al. Histone deacetylase inhibitors and retinoic acids inhibit growth of human neuroblastoma in vitro. Med Pediatr Oncol 2000;35:577-81.

23. Liu Y, Encinas M, Comella JX, et al. Basic Helix-Loop-Helix proteins bind to TrkB and p21Cip1 promoters linking differentiation and cell cycle arrest in neuroblastoma cells. Mol Cell Biol 2004;24:2662-72.

24. Pérez-Juste G, Aranda A. Differentiation of neuroblastoma cells by phorbol esters and insulin-like growth factor-1 is associated with induction of retinoic acid receptor gene β expression. Oncogene 2000;18:5393-02.

25. Pérez-Juste G, Aranda A. The cyclin-dependent kinase inhibitor $p27^{kip1}$ is involved in thyroid hormone-mediated neuronal differentiation. J Biol Chem 1999;274: 5026-31

26. García-Silva S, Aranda A. The Thyroid Hormone Receptor is a Suppressor of ras–Mediated Transcription, Proliferation and Transformation. Mol Cell Biol 2004;24: 7514-23

27. Yoshida M, Kijima M, Akita M, Beppu T Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A J Biol Chem 1990;265:17174-79

28. Richon VM, Emiliani S, Verdin E, et al. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A 1998;95:3003-07.

29. Duvic M, Talpur R, Ni X, Zhang C, Hazarika P, Kelly C, Chiao JH, Reilly JF, Ricker JL, Richon VN, Frankel S. Phase II Trial of Oral Vorinostat (Suberoylanilide Hydroxamic Acid, SAHA) for Refractory Cutaneous T-cell Lymphoma (CTCL). Blood. 2006 Sep 7; [Epub ahead of print]

30. de Ruijter AJ, Meinsma RJ, Bosma P, et al. Gene expression profiling in response to the histone deacetylase inhibitor BL1521 in neuroblastoma. Exp Cell Res 2005;309:451-67.

31. Alao JP, Stavropoulou AV, Lam EW, et al. Histone deacetylase inhibitor, trichostatin A induces ubiquitin-dependent cyclin D1 degradation in MCF-7 breast cancer cells. Mol Cancer 2006; 20:5-8.

32. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative inhibitors of G1-phase progression. Genes & Dev 1999;13:151-12.

33. Rocchi P, Tonelli R, Camerin C, Purgato S, Fronza R, Bianucci F, Guerra F, Pession A, Ferreri AM. p21Waf1/Cip1 is a common target induced by short-chain fatty acid HDAC inhibitors (valproic acid, tributyrin and sodium butyrate) in neuroblastoma cells. Oncol Rep 2005;13:1139-44.

34. Wang ZM, Hu J, Zhou D, Xu ZY, Panasci LC, Chen ZP. Trichostatin A inhibits proliferation and induces expression of p21WAF and p27 in human brain tumor cell lines. Ai Zheng 2002;21:1100-5

35. Archer SY, Meng S, Shei A, Hodin RA. p21WAF1 is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc Natl Acad Sci U S A 1998;95:6791-96.

36. Vaziri C, Stice L, Faller DV Butyrate-induced G1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. Cell Growth Differ 1998;9:465-74.

37. Gui CY, Ngo L, Xu WS, et al. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. Proc Natl Acad Sci U S A 2004;, 1241-46.

38. Lasorella A, Iavarone A, Israel MA. Differentiation of neuroblastoma enhances Bcl2 expression and induces alterations of apoptosis and drug resistance. Cancer Res 1995;55-4711-16.

39. Wainwright LJ, Lasorella A, Iavarone A. Distinct mechanisms of cell cycle arrest control the decision between differentiation and senescence in human neuroblastoma cells. *Proc Natl Acad Sci USA* 2001;**98**:9396–400.

40. Borriello A, Cucciolla V, Criscuolo M, et al. Retinoic Acid Induces p27Kip1 Nuclear Accumulation by Modulating Its Phosphorylation *Cancer Res* 2006;**66**: 4240-48.

41. Liu M, Iavarone A and Freedman LP. Transcriptional activation of the human p21WAF1/CIP1 gene by retinoic acid receptor: correlation with retinoid induction of U937 cell differentiation. *J Biol Chem* 1996;**271**:31723-28.

42. Coffey DC, Kutko MC, Glick RD, et al. The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts in vivo, alone and synergistically with all-trans retinoic acid. *Cancer Res* 2001;**61:**3591-94.

Figure Legends

Figure 1. Effect of HDACi on histone H3 acetylation. **A,** Western blot analysis of acetylated histone H3 (Ac His H3) from control SH-SY5Y cells (C) and cells treated with the indicated concentrations of TSA, butyrate (But) and SAHA for 6 h (upper panels) and 48 h (lower panels). In **B,** cells were treated for 6 and 24 h with 100 nM TSA or 2 mM But. In **C**, cells pretreated for 24 h with 100 nM TSA or 2 mM But were incubated for an additional 6 h period with the HDACi. Actin levels were used as a loading control.

Figure 2. Effect of HDACi and RA on SH-SY5Y cell proliferation. **A**, MTT assays in cells treated for 24 h (left panel) and 48 h (right panel) with 1µM RA in presence and absence of increasing concentrations of TSA (100, 200 and 500 nM), But (1, 2 and 5 mM) and SAHA (0.5, 1 and 2 µM). The first bar indicates the values obtained in the untreated cells and the second bar cells reated with RA alone. **B**, Growth curve of control (C) SH-SY5Y cells, and cells treated with 200 nM TSA, 2 mM But and 1 µM SAHA. **C**, [^{3}H]thymidine incorporation in cells incubated for 48 h with 0, 0.1, 1 and 10 µM RA alone or in combination with TSA (200 and 500 nM), But (2 and 5 mM) and SAHA (1 and 2 µM).

Figure 3. Influence of HDACi and RA on cell morphology. Photomicrographs at 200X of SH-SY5Y cells treated for 48 h with RA (1 µM), TSA (200 nM), Butyrate (2 mM) or SAHA (1 µM), as indicated.

Figure 4. HDACi induce SH-SY5Y cell death by apoptosis. **A**, Cell cycle analysis by flow cytometry after 48 h treatment with TSA, But or SAHA in the presence and absence of RA. The percentage of cells in each phase was obtained from histogram analysis with WinMDI software. Data represent the mean ± S.D of three independent cultures. **B**, Induction of PARP proteolysis by HDACi in cells treated for the same time period with the indicated concentrations of HDACi and/or RA. Two bands are detected in the Western blot of HADCi treated cells: the 113 kDa PARP band and the 89 kDa band representing proteolytic processing. The blot was reprobed with an actin antibody used as a loading control.

Figure 5. Caspases are involved in HDACi-mediated cell death. **A**, PARP proteolysis was determined by Western blot in SH-SY5Y cells treated for 24 h with ???But…alone or in combination with the caspase family inhibitor Z-VAD (20 µM). **B,** MTT assays after 24 and 48 h incubation with But (1 and 2 mM) and SAHA (1 and 2 µM), in the presence and absence of the same concentration of Z-VAD.

Figure 5 Effect of HDACi and RA on expression of Cyclin D1 and CKIs. **A**, Levels of Cyclin D1, $p21^{Waf1/Cip1}$, acetylated histone H3 (Ac His H3) and actin in cells treated for 24 h with the compounds indicated. C: control cells. **B**, Levels of the same proteins in cells incubated with the indicated concentrations of But and SAHA alone and in combination with 1 µM RA for 48 h. **C,** Influence of 24 h incubation with the different HDACi and RA on $p27^{Kip1}$ and actin levels. **D**, Time course of CKI induction. Western blots of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and actin in cells treated for 1, 3, 5 and 7 h with 2 mM But. **E,** Levels of the same proteins in cells incubated with increasing concentrations of HDACi and RA for 6 h.

Figure 6. Transcriptional stimulation of CKI gene expression by HDACi and RA. **A**, Cyclin D1, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ mRNA levels in SH-SY5Y control cells (C) and cells treated for 48 h with TSA, But and SAHA alone or in combination with RA. The 18S ribosomal RNA detected by methylene blue staining was used as a loading control. **B**, Transient transfection assays with a reporter plasmid containing the 5´-flanking region of the $p21^{Waf1/Cip1}$ gene. Luciferase activity was determined in cells treated for 48 h with 200 nM TSA, 2 mM But or 1 µM SAHA in the presence (dark bars) and absence (light bars) of 1 µM RA. Data are mean ± S.D and are expressed as fold induction with respect to the levels obtained in the control cells. In panel **C**, luciferase activity was determined after the same treatments in cells transfected with a plasmid containing the $p27^{Kip1}$ promoter.