

Identification of Novel Isoform-Selective Inhibitors within Class I Histone Deacetylases

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Abstract:

Histone deacetylases (HDAC) represent an expanding family of protein modifying-enzymes that play important roles in cell proliferation, chromosome remodeling and gene transcription. We have previously shown that recombinant human HDAC8 can be expressed in bacteria and retain its catalytic activity. To further explore the catalytic activity of HDACs, we expressed two additional human class I HDACs, HDAC1 and HDAC3, in baculovirus. Recombinant HDAC1 and HDAC3 fusion proteins remained soluble and catalytically active and were purified to near homogeneity. Interestingly, trichostatin (TSA) was found to be a potent inhibitor for all three HDACs (IC_{50} ~0.1-0.3 μ M), while another HDAC inhibitor MS-27-275 preferentially inhibited HDAC1 (IC_{50} ~0.3 μ M) versus HDAC3 (IC_{50} ~ 8 μ M), and had no inhibitory activity toward HDAC8 (IC_{50} >100 μ M). MS-27-275 as well as TSA increased histone H4 acetylation, induced apoptosis in the human colon cancer cell line SW620 and activated the SV40 early promoter. HDAC1 protein was more abundantly expressed in SW620 cells compared to that of HDAC3 and HDAC8. Using purified recombinant HDAC proteins, we identified several novel HDAC inhibitors that preferentially inhibit HDAC1 or HDAC8. These inhibitors displayed distinct properties in inducing histone acetylation and reporter gene expression. These results suggest selective HDAC inhibitors could be identified using recombinantly expressed HDACs and that HDAC1 may be a promising therapeutic target for designing HDAC inhibitors for proliferative diseases such as cancer.

Histone acetylation modifies specific lysine residues on histones and other nuclear and cytoplasmic proteins and play important roles in pivotal cellular functions such as chromosome remodeling, gene transcription and cell proliferation. Histone acetyltransferase (HAT) and histone deacetylase (HDAC) control the addition and removal of acetyl groups on proteins and maintain a dynamic balance of steady-state protein acetylation. In addition to histone, transcription activators (Src-1, p300, ACTR, and PCAF) and repressors (Sin3, pRB, YY1 and NcoR) have been shown to be associated with HAT and HDAC, respectively (Cress and Seto, 2000; Kouzarides, 2000). Several transcriptional activators even contain intrinsic HAT activities (CBP, p300 and PCAF) (Ogryzko et al., 1996; Krumm et al., 1998). Others (p53 and myoD) are acetylated and acetylation regulates their activities (Sartorelli et al., 1999; Luo et al., 2000). Oncogenes such as BRCA-1 and -2 have been shown to be associated with HDACs and HATs (Siddique et al., 1998; Yarden and Brody, 1999). In particular, the viral oncogene E1a has been shown to modulate histone acetyltransferase activity of p300 and PCAF (Chakravarti et al., 1999; Hamamori et al., 1999).

Multiple forms of HDAC have been identified in mammalian cells. In human, at least 11 HDACs have been uncovered. They are classified into two general classes: class I (HDAC1, 2, 3, 8, 11) and class II (HDAC4, 5, 6, 7, 9, 10) (Gray and Ekstrom, 2001; Gao et al., 2002; Kao et al., 2002). Class I enzymes are generally smaller polypeptides of around 500 amino acids while class II HDACs are much larger proteins with approximately 1000 amino acids. Members of class II HDACs (HDAC4 and 5) have been shown to shuttle between the cytoplasm

and nucleus and regulate myogenesis (Lu et al., 2000; McKinsey et al., 2000). In contrast, most of the class I HDACs have been shown to be localized in the nucleus. In addition to these two main classes of HDACs, a third class of HDACs have been described that requires NAD as co-factor (Imai et al., 2000).

Several HDAC inhibitors, including sodium butyrate and trichostatin (TSA), have been shown to have a wide range of effects in cultured cells and in animals. These effects include specific gene activation, inhibition of cell proliferation and cell cycle arrest, as well as induction of cell differentiation (Kuo and Allis, 1998; Kouzarides, 1999). Importantly, two HDAC inhibitors (MS-27-275) (Saito et al., 1999) and FR901228 (Nakajuma et al., 1998) have demonstrated tumor growth suppression *in vivo*. These results suggest complex regulatory roles for HDACs in many vital cellular functions and potential therapeutic utilities of HDAC inhibitors.

With the identification of 11 human HDACs, it was suggested that these HDACs may play distinct roles in cellular functions. However, little is known about the catalytic properties of individual HDACs and their sensitivity to known inhibitors. We undertake an effort to express three class I human HDACs (HDAC1, 3 and 8), and studied their sensitivity to HDAC inhibitors. Our results demonstrated that soluble and active HDAC1 and 3 could be generated and the recombinant proteins may be utilized to profile HDAC inhibitors. Our data also demonstrated that individual HDACs displayed distinct inhibition profile towards known or novel HDAC inhibitors and inhibition of HDAC1 correlated with the inhibition of cell proliferation and apoptosis. In addition, we examined the

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expression of HDAC1 in several normal and malignant human tissues using immunohistochemistry techniques.

Methods

Materials. Nylon membrane (Biotran) was purchased from ICN Biotechnologies (Costa Mesa, CA). α - 32 P-dATP (3000 Ci/mmol) was purchased from NEN-Dupont (Boston, MA). Oligo-dT agarose was obtained from Pharmacia Biotech (Piscataway, NJ). Routine molecular cloning and sequence analyses was performed as previously described (Sambrook et al., 1989). Reagents for subcloning and sequencing were purchased from Promega Inc. (Madison, WI). PCR reagents were obtained from Perkin-Elmer Inc. (Norwalk, CT). Random priming labeling kits were obtained from Promega Inc. (Madison, WI). TSA and butyrate were purchased from Sigma Inc. (St. Louis, MO). 3 H histone was generated by labeling HEK293 cells with 3 H acetate in the presence of 10 mM butyrate as described (Yoshida et al., 1990). Computer programs used to analyze cDNA and protein sequences were BLASTN, BLASTP, and Lasergene-DNA star programs. Polyclonal antibodies for HDAC3 and 8 were generated in rabbits by using the purified GST-hHDAC3 and 8 as described below. Anti-HDAC1 antibody was obtained from Sigma.

Recombinant expression and purification of hHDAC1 and hHDAC3. All plasmid constructions were generated by standard cloning methods (Sambrook et al., 1989). The DNA sequence of all vector insertions were confirmed on both strands by automated di-deoxy-sequencing (Applied Biosystems).

Full-length Histone Deacetylase I (GenBank NM_004964) was PCR amplified from a human cDNA library (Clontech, CA) from using the following primer set (Forward: 5'-ATTAAATATGGATCCTGGGAACACGATATG-

GCGCAGACGCAGGGCACCCGG -3' and Reverse: 5' AATAAATAACTCG-AGGGAGAGGTCCATTCAGGCC 3'), tailing 5' with *Bam* HI and 3' with *Xho* I as underlined. GST encoding sequence was PCR amplified from GSTHD3pFB using a primer set tailed 5' with *Eco* RI and 3' with *Bam* HI (Forward: 5'-GCGCGCGGAATTCAAAGGCCTACG-3' and Reverse: 5' AATA-TTATAGGATCCTTTGGAGGATGGTCGCCACCACC-3'). The HDCA1 PCR fragment was digested with *Bam* HI and *Xho* I and GST PCR fragment was digested with *Eco*RI and *Bam* HI, and these were ligated together with pFastbac-1 vector (Invitrogen) digested with *Eco*RI and *Xho* I. The 3 way ligation generated GSTHD1pFB, containing an N-terminal GST fusion with HDAC1.

Histone Deacetylase-3 (GenBankU75697) was cloned from Jurkat cell (ATTC CRL-8130) polyA+ RNA by RT-PCR using Superscript II reverse transcriptase for oligo-dt primed 1st cDNA synthesis according to the manufacturer's instructions (Life Technologies cat No 18089-011) followed by PCR using the oligonucleotide primer set. (Forward: 5'-CATATGGCCAAGACC-GTGGCCTATTTC-3' and Reverse: 5'-ATCAATGGTGATGGTGATGGTGGC-TGCCGCGGCCTTCAATCTCCACATCGCTTTCCTTGTC-3'), incorporating an *Nde* I restriction site 5' (underlined) and sequence encoding Factor Xa protease site (IEGR) followed by a 6 His tag and stop sequence at the 3' end. PCR was performed for 25 cycles (2 minutes 92°C, 3 minutes 55°C, and 3 minutes 72°C) on 5ul RT-PCR reaction using expand high fidelity polymerase (Roche, Indianapolis IN). The resultant PCR product was TA subcloned into PCR-2.1 vector (Invitrogen, Carlsbad CA). Site-directed mutagenesis was used to

introduce a silent change in HDAC3 coding sequence using the QuickChange Mutagenesis Kit (Stratagene, La Jolla CA) in order to eliminate the internal *Nde* I for subcloning purposes. The following mutagenesis primer set was used containing the overlapping base change as underlined: (Forward: 5'-AATGTTGCCCGCTGCTGGACCTATGAGACA-TCGCTGCTGGTAGAA-3; and Reverse: 5'-CTTCTACCAGCAGCGATG-TCTCATAGGTCCAGCAGC-GGGCAACATT-3').

Full-length HDAC3 was transferred on an *Xba* I- *Xho* I fragment and ligated between the same sites in pFastbac-1 (Invitrogen). The resultant plasmid, HD3pFB, was further modified by adding an N-terminal glutathione S-transferase (GST) tag (GenBank M14654). The GST encoding sequence was PCR amplified from pGEX2.1 using the following primer set tailed 5' with *Xba* I and 3' with *Nde* I as underlined (Forward: 5'- TCTAGAAATGTCCCCTATACT-AGGTTATTGG-3' and Reverse: 5'- CATATGGTGTTCCCATTTGGAGGAT-GGTCGCCACCA-CC-3'). The resultant PCR product was subcloned into pCR2.1 from where it was excised on an *Xba* I- *Nde* I fragment and ligated between the same sites of HD3pFB to generate GSTHD3pFB.

For purification of recombinant HDACs, two liters of baculovirus-infected Sf9 cell pastes of HDAC1 or HDAC3 with GST fusion at the N-terminus were lysed in 100 ml of lysis buffer (25 mM Tris, pH 7.8, 3 mM MgCl₂, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100 and protease inhibitor cocktail) and centrifuged at 20,000 x g for 1 hour. The cell supernatant was allowed to bind to 8 ml of Glutathione Sepharose 4B resin at 4° C for 1 hour before loading onto a column.

The column was washed with buffer A (25 mM Tris, pH 7.8, 3 mM MgCl₂, 150 mM NaCl, 10% glycerol) with 0.5% Triton X-100 extensively. The GST fusion protein was eluted with buffer A containing 10 mM reduced glutathione. The purified protein was dialyzed into final buffer A and kept at -70° C before activity assay. The HDAC1 was further purified using MonoQ column and eluted with linear gradient of 150 mM NaCl to 1 M NaCl. Purified HDAC1 was kept at -70° C before use.

HDAC activity assay HD assays were performed essentially as described (Hu et al., 2000). The 200 ul HDAC assay reaction contained 10 mM Tris pH 8, 150 mM NaCl, 1 mM MgCl₂ (HDAC assay buffer), 5000-10,000 cpm of H³-histone or H4 peptide (SGRGKGGKGLGK(³H-acetylated)GGAKRHRC), and varying amounts of purified HDACs. Assays were performed at 25° C. After one hour of incubation, 50 ul of stop mixture (1 M HCl and 0.16 M acetic acid) was added to the sample and 1 ml of ethyl acetate was added to extract released tritiated acetate. 800 ul of the ethyl acetate was counted in a liquid scintillation counter (Beckman Inc.). ³H-labeled histone substrate was made using HEK293 cells and labeled with ³H-acetate in the presence of 10 mM butyrate and labeled histone was isolated as described (Hu et al., 2000). ³H-labeled, acetylated H4 peptide was custom synthesized by Amersham Inc (Piscataway, NJ).

For reversibility assays, 10 ug of purified HDAC1 was incubated with glutathione-Sepharose beads (Pharmacia Biotech, Piscataway, NJ) for 2 hours at 4°C in 200 ul of HDAC assay buffer. After incubation, the glutathione-Sepharose beads were spun down, washed and re-suspended in 200 ul HDAC assay buffer

containing DMSO vehicle or 1 μ M HDAC inhibitors (TSA or MS-27-275). After incubation at 4°C for 1 hour, beads were re-centrifuged, washed and re-suspended in 200 μ l of HDAC assay buffer. HDAC activity was assayed as described above.

RNA isolation, Western and Northern analysis. RNA isolation and northern blot analysis were performed as previously described (Hu et al., 2000).

Densitometry Analysis. Analysis was performed using ImageJ (v1.30) software package from NIH ([HTTP://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). Autoradiogram was scanned as JPEG image and area under the curve (AUC) was measured for each band and quantified.

Apoptosis Assay Apoptosis analysis. SW620 human colon carcinoma cell line was cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10 % fetal bovine serum. 2×10^4 cells were seeded in each well of 24-well culture plates. Cells were starved overnight in DMEM without serum before addition of TSA and other agents. After overnight treatment, cell apoptosis was determined using the Cell Death Detection ELISA Plus kit (Roche Diagonostic Corporation, Indianapolis, IN) following the manufacturer's protocol. Briefly, 1 ml of lysis buffer for each well was added after removing the medium, after 30 min incubation at room temperature, 20 μ l supernatant was transferred into each well of the streptavidin coated MTP (96 well multiple titer plate). Then 80 μ l immunoreagent-mix was added to each well and incubated for 2 hr at room temperature. Solution was removed and each well was rinsed three times with 250 μ l incubation buffer, then 100 μ l substrate solution was added into each well of the MTP-modules and incubated on a plate shaker at 250 rpm for approximately

15 min or until the colour development was sufficient for a photometric analysis. The optical density at 405 nm was measured. Samples were triplicated and the average value of each sample was interpreted against the positive and negative control samples.

SV40 promoter Reporter Assays 2 ug of SV40 promoter construct (pGL3-promoter luciferase reporter vector, Promega, Madison, MI) was transiently transfected into Rat-2 cells. The SV40 promoter region in pGL3 (nucleotide 44-244) contains transcription initiation sites as well as multiple Sp1 binding sites. This promoter region corresponds to bases 5180-5240 of the SV40 viral genome (Genbank accession # J02400). Empty vector DNA were used to normalized the amount of transfected DNA to 20 ug/100 mm cell culture dish. Our previous experiments demonstrated that this construct, when transiently transfected into Rat-2 cells, can be activated by TSA (Hu et al., 2000). Transfections were performed as described (Hu et al., 2000). 48 hours after transfection, cells were trypsinized and re-seeded into a 96 well dish at approximately 10,000 cells/well. After overnight incubation, the luciferase reporter activity was measured on a Top Counter (TopCounter NXT, Packard, Meriden, CT) using a Luclite luciferase assay kit (Packard, Meriden, CT). When cells were treated with TSA or MS-27-275 at various concentrations, an additional 24 hour incubation was allowed before luciferase activities were measured.

Results

Expression of recombinant human HDAC1 and HDAC3 We previously have successfully expressed hHDAC8 in *E. coli* using histag fusion (PET-30) vectors (Hu et al., 2000). However, when similar constructs were made to express HDAC1 and HDAC3, the proteins were insoluble and inactive. Thus a baculovirus expression system was chosen for expression of hHDAC1 and hHDAC3. GST-fusion expression vectors were constructed as outlined in Fig. 1. Western blot was used to assess the expression level and solubility (data not shown). Approximately 50% of the GST-HDAC1 and GST-HDAC3 were soluble. HDAC1 was purified using GST affinity chromatography and monoQ HPLC. HDAC3 was purified using GST affinity chromatography alone. Both purified proteins remained soluble and over 80% purity was achieved for both HDACs (Fig. 2). Purified GST-hHDAC1 and GST-hHDAC3 corresponded to ~100 kDa and ~85 kDa, respectively. A small amount of cleaved GST (29 kDa) was visible on the stained gel (star *, Fig.2). This could be due to cleavage by a presumed endogenous protease in Sf9 cells. We attempted to cleave the GST moiety from both fusion proteins, however cleavage resulted rapid aggregation and precipitation of both HDACs. Thus purified GST-HDAC1 and GST-HDAC3 was used for further enzyme assays and analysis of inhibitors without further manipulation. As described previously (Hu et al., 2000), hHDAC8 was expressed as histag fusion protein in *E. coli* and was purified to near homogeneity via NTA-Ni affinity chromatography (Fig. 2).

Enzymatic activity of recombinant hHDAC1, hHDAC3 and hHDAC8 was examined using a conventional HDAC assay. Both recombinant hHDAC1 and hHDAC3 displayed concentration-dependent HDAC activity towards ³H-labeled acetylated-histone H4 peptide. This activity was completely suppressed by 1 μ M TSA (Fig. 3A). Furthermore, when metabolically ³H-labeled, acetylated histones were incubated with all three hHDACs and resolved in a SDS-PAGE, significant reduction in ³H-radioactivity was observed, indicating active deacetylase activity (Fig.3B). Densitometry measurement indicated that reduction of ³H radioactivity after incubation were approximately 65% for 1 μ g of HDAC1, 44% for 1 μ g of HDAC3, and 40% for 1 μ g HDAC8 after 1 hour incubation (Fig.3B, lower panel). These data indicated that purified HDACs were active toward acetylated histone peptide as well as isolated histones.

Differential inhibition of hHDAC1, hHDAC3 and hHDAC8 by TSA and MS-27-275. To assess the sensitivity of recombinant human HDAC1, 3 and 8 toward HDAC inhibitors, we used two known HDAC inhibitors TSA and MS-27-275. TSA inhibited all three HDACs with similar IC₅₀s (100 nM-300 nM) (Fig. 4A), however, MS-27-275 displayed differential inhibition of the three HDACs (Fig. 4B). Recombinant hHDAC1 was highly sensitive to MS-27-275, with an IC₅₀ ~300 nM, similar to that of TSA. hHDAC3 is moderately sensitive to MS-27-275, with IC₅₀ ~8 μ M, while hHDAC8 was insensitive to MS-27-275, with only ~30% inhibition observed at 100 μ M concentration.

To test if the inhibition of HDAC1 is reversible, HDAC1 was first bound to GST beads and incubated with 1 μ M TSA or MS-27-275. Subsequently the beads were washed and assayed for HDAC activity. As shown in Fig. 4C, washing eliminated the inhibitory effect of both TSA and MS-27-275, suggesting that both compounds are reversible inhibitors.

Effects of TSA and MS-27-275 on SW620 colon cancer cells. Since TSA and MS-27-275 displayed different profiles with regards to inhibition of individual HDACs, we compared the effect of both inhibitors on SW620 human colon cancer cells. Northern and western blot analysis (Fig. 5A and B) demonstrated that HDAC1, 3 and 8 were all expressed in these cells. The level of expression of HDAC1 (mRNA and protein) was significantly higher as compared with HDAC3 and 8, and addition of TSA did not affect the expression of HDAC1. In contrast, TSA treatment of SW620 cells modestly induced HDAC3 mRNA (~1.3 fold) and protein (~1.5 fold) (Fig.5A and B), consistent with a previous report (Dangond and Gullans, 1998). When SW620 cells were treated with TSA and MS-27-275, both compounds dose dependently induced histone H4 acetylation (Fig. 6A) and apoptosis (Fig. 6B). TSA-induced accumulation of histone H4 acetylation seemed to plateau at 0.3-1 μ M while MS-27-275-induced acetylation reached maximum at approximately 3 μ M. For TSA, the maximum apoptosis was reached at approximately 1 μ M, while 10 μ M MS-27-275 was required to reach maximal level.

HDAC inhibitors have been shown to activate artificial promoters containing Sp1 site (Sowa et al., 1997). We thus compared the effect of TSA and MS-27-275 on the SV40 early promoter. Both compounds significantly induced the promoter activity in a dose-dependent manner (Fig. 6C). TSA was more potent with $EC_{50} \sim 100$ nM, while the EC_{50} for MS-27-275 was approximately 300 nM.

Novel HDAC Inhibitors In an attempt to identify novel and selective HDAC inhibitors, a HDAC8 enzyme-based high-throughput screen (Hofmann et al., 2000) was developed and used to screen a chemical compound collection. In addition, a luciferase reporter assay using TK (thymidine kinase) promoter was also used to screen a similar chemical collection. Resulting compounds were analyzed for HDAC inhibitory activities as well as for activation of a SV40 early promoter (Hu et al., 2000). Three representative compounds were identified (Fig. 7A). SB-556629, also known as Scriptaid (Su et al., 2000), demonstrated inhibitory activity towards all three class I HDAC tested (IC_{50} : ~ 0.6 μ M for HDAC1 and 3, ~ 1 μ M for HDAC8). SB-429201 preferentially inhibited HDAC1 ($IC_{50} \sim 1.5$ μ M), and SB-379872-A only inhibited HDAC8 ($IC_{50} \sim 0.5$ μ M) (Fig. 7B). When these compounds were added to SW620 cells, dose-dependent increases in histone acetylation were observed for Scriptaid. A modest dose-dependent increase was also seen for SB-429201, while no effect in histone acetylation was observed for SB-379872-A (Fig.7C). A similar activation in SV-40 early promoter in response to these three compounds was also observed (Fig.

7D). These results suggest that the inhibition of HDAC1 could underlie the observed increase in H4 acetylation as well as the increase of Sp-1 mediated SV40 promoter activity in these cell types. Our data, however, does not exclude the possibility that other members of class I or class II are involved in either histone acetylation or SV40 promoter activation. Further experiments are required to examine the effect of these inhibitors on the activities of other members of HDAC family.

Discussion

HDACs belong to a large and expanding protein family that now encompasses at least 11 members. They are classified into class I and class II HDACs, based on 1) size and sequence characteristics and 2) distinct association with different transcription co-factors. It has been suggested that distinct HDACs play specific role in diverse cellular processes including regulation of gene expression, chromosome remodeling and cell proliferation. Since almost all of the class I and class II mammalian HDACs identified thus far are sensitive to sodium butyrate and TSA inhibition, there is intense interest in identifying isoform specific inhibitors for each HDACs and such tool inhibitors would facilitate the functional analysis of HDACs.

In this paper, we demonstrated that recombinant human HDAC1, 3 and 8 can be used to identify such isoform specific inhibitors. All three HDACs were expressed as soluble enzymes and thus were amenable to biochemical and enzymatic analysis. In vitro, purified HDAC1, 3 and 8 demonstrated deacetylase activity towards purified histone and this activity was completely inhibited by TSA. Our results demonstrated that MS-27-275, a compound originally isolated by its ability to suppress tumour growth (Saito et al., 1999), was in fact a HDAC1-selective inhibitor. MS-27-275 inhibited HDAC1 with IC_{50} of approximately 300 nM, while its IC_{50} for HDAC3 was 8 μ M, and it only marginally inhibited HDAC8. These results confirm the notion that HDACs display differential sensitivity toward different pharmacological inhibitors and that a highly selective HDAC inhibitor could be obtained via high-throughput

screening of diverse chemical banks and medicinal chemical approaches. This point is further strengthened by the identification of isoform-selective as well as non-selective HDAC inhibitors using high-throughput screening technology. A relatively non-selective inhibitor Scriptaid, a selective HDAC1 inhibitor SB-429201, and a potent HDAC8 inhibitor SB-379278-A were identified in this fashion. Interestingly we observed a dramatic increase of histone acetylation when SW620 cells were treated with Scriptaid and SB-429201, but not with SB-379278-A. Moreover, very little SV40 promoter activation was observed for HDAC8 inhibitor SB-379278-A, while all the other inhibitors elicited strong activation. It is noteworthy that Scriptaid and SB-429201 were identified from a reporter based screen, while SB-379278-A was found via a HDAC8 enzyme activity based screen. It is thus possible that a functional screen such as reporter assay could skew toward HDAC1 and thus identify selective set of inhibitors. On the other hand HDAC enzyme activity-based screening could alleviate such bias and may be useful to identify compounds with selectivity towards individual HDACs. Selectivity of HDAC inhibitors could help reduce the potential adverse effects of a pan-HDAC inhibitor on cardiovascular and other systems (Rossig et al., 2002; Zhang et al., 2002).

With the expanding family of HDACs, a logical question is which one(s) of these 11 HDACs might be primarily responsible for the diverse effects of HDACs, such as cell proliferation and/or differentiation and therefore, most relevant to cancer cell growth? Using the HDAC1-selective inhibitor MS-27-275, we observed a good correlation between HDAC1 inhibition, cellular histone

acetylation, SV40 promoter activation and apoptosis. Similar findings were also observed for a novel HDAC1 selective inhibitor SB-429201. In contrast, a potent HDAC8 inhibitor, SB-379278-A, did not affect cellular histone acetylation or SV40 promoter activity, suggesting either that proteins other than histones may be the physiologic substrate of HDAC8 or that HDAC8 is unimportant for these activities in SW620 cells. These data also suggest that of the class I HDACs evaluated in this study, HDAC1 is more essential for cancer cell growth and may be the therapeutic target for intervention. HDAC1 is the first histone deacetylase identified (Taunton et al., 1996) and there is considerable evidence linking this enzyme to cell growth and tumorigenic transformation. HDAC1 is also known to be induced by growth stimuli (Bartl et al., 1997); It is involved in deacetylation of acetylated p53, thereby modulating its activity (Luo et al., 2000). HDAC1 expression is increased in human gastric and prostate cancers (Choi et al., 2001; Patra et al., 2001). More recently, HDAC1 has been shown to be induced by hypoxia (Kim et al., 2001). This evidence, combined with our results with selective HDAC1 inhibitors suggests the important role of HDAC1 in tumorigenesis and its potential as therapeutic target.

It is worth noting that our study did not examine the effect of the novel HDAC inhibitors on class II HDACs and it is unknown whether these compounds also inhibit any of the class II HDACs. Class II HDACs have more tissue-restricted distribution and may participate in a biological functions related to a particular tissue or cell type rather than cell proliferation per se. For example, HDAC4 and 5 are highly expressed in cardiomyocytes and are involved in cardiac

hypertrophy (Zhang et al., 2002). Interestingly, a selective HDAC6 inhibitor, tubacin, has been recently identified that seems to specifically increase α -tubulin acetylation while it has no effect on histone acetylation, TSA-inducible gene expression and cell cycle progression (Haggarty et al., 2003). In contrast, FK901228, a HDAC inhibitor undergoing phase I clinical trial for cancer, showed strong inhibition for HDAC1 and HDAC2 but weak activity toward HDAC4 and 6 (Furumai et al., 2002). These data are consistent with the notion HDAC1 may be more important for tumor growth. Further studies are warranted to identify inhibitors that can distinguish class I and class II HDACs and examine their cellular effects.

HDACs are known to form complexes with other co-factors such as p300, MEF2 and even other HDACs. For instance, HDAC1 and 2 are present in Sin3, NuRD and coREST nuclear complexes while HDAC3 is found in NcoR complex (Grozinger and Schreiber, 2002). Recent data also showed that class I HDACs associate with chaperone proteins such as Hsp70 and the formation of such complexes enhances deacetylase activity (Johnson et al., 2002). It is currently unknown if HDAC activity is modulated in other complexes and if their sensitivity to various inhibitors are different as compared with purified HDACs. The availability of selective inhibitors and purified components of these complexes will allow us to begin to explore these questions.

Currently several HDAC inhibitors are in clinic trials (Kelly et al., 2003) for anti-tumour therapy, however, most of these have not been tested for selectivity versus individual purified HDACs. Many, if not all of these

compounds inhibit multiple HDACs. For example, hybrid polar compounds (HPCs) have been shown to suppress both HDAC1 and 3 activities at similar potencies (Richon et al., 1998). All of these inhibitors increased histone acetylation, thus suggesting that at least HDAC1 inhibition is involved. The method of expressing recombinant HDACs we described here could be used to systemically examine the effect of these inhibitors on HDAC enzymatic activity and correlate their effects on gene expression, cell proliferation and apoptosis. This would allow identification of compounds with better therapeutic potency and reduced side effects.

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Figure Legend:

Figure 1: Construction of baculoviral expression vector for hHDAC1 and hHDAC3. pFB is viral polyhedrin promoter. HDAC1 and HDAC3 were inserted as a in-frame fusion to GST.

Figure 2: Expression of recombinant GST-hHDAC1 and 3. Baculoviral expression construct for hHDAC1 and 3 were outlined in A. Purified GST-hHDAC1, GST-hHDAC3 and his-hHDAC8 were separated on 10% SDS-PAGE and stained with coomassie blue. Lane 1 is molecular weight marker, lane 2, 3 and 4 are GST-hHDAC1, GST-hHDAC3 and his-HDAC8, respectively. The position of GST as well as GST-hHDAC1 and 3 or his-hHDAC8 are indicated by arrows.

Figure 3: (A) Activity of recombinant GST-hHDAC1 and 3. Purified GST-hHDAC1 was used in routine HD assay (see Methods). Increasing amounts of GST-hHDAC1 and GST-hHDAC3 were used in a 100 ul assay (0.1 ug to 1.5 ug) in the presence or absence of TSA (1000 nM). 5,000 cpm of H³ labeled histone H4 peptide was used as substrate. (B) Deacetylation of purified histones. 20,000 cpm of metabolically ³H-labeled histone was incubated with indicated HDACs for 1 hour at 37° C. After incubation, the entire reaction mixture was denatured by adding SDS loading buffer and proteins were separated on a 4-12% gradient gel (Nu-PAGE). The gel was stained with Coomassie Blue, dried and exposed to X-ray film. Lane 1 was sample treated with 1 ug of GST-hHDAC1. Lanes 3 and 4 were samples treated with 1 and 0.5 ug of GST-hHDAC3, respectively. Lane 6

was sample treated with 1 ug of his-HDAC8. Lower panel is densitometry analysis. Mean+/-SEM of combined H1-H4 radioactivity (band intensity) are plotted. Values were normalized to samples without HDACs treatment (as 100). The experiments were repeated 4 times. In separate experiments, the TSA-sensitive release of ³H radioactivity from labeled histone were monitored by acetylacetate extraction, and released ³H were approximately 10-30% of input (20,000 cpm).

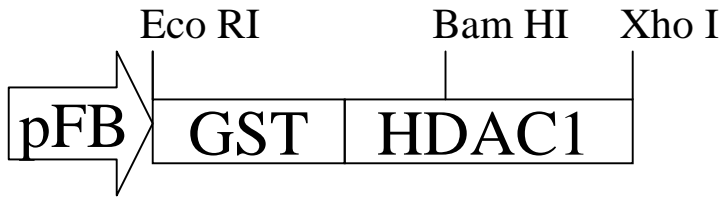
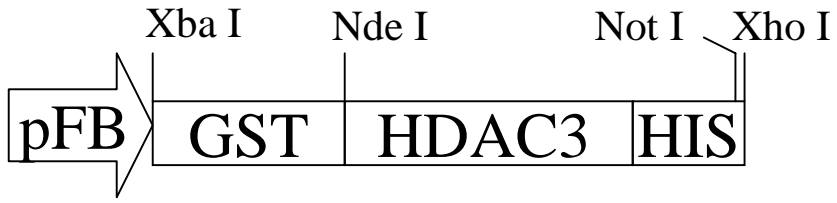
Figure 4: (A) Inhibition of HDAC1, 3 and 8 by TSA. (B) Inhibition of HDAC1, 3, and 8 by MS-27-275. Experiments were duplicated and similar data was obtained. (C). Reversible inhibition of HDAC1 by TSA and MS-27-275. Experiments were performed as described in Methods. Individual experiments were repeated 3 times with similar results.

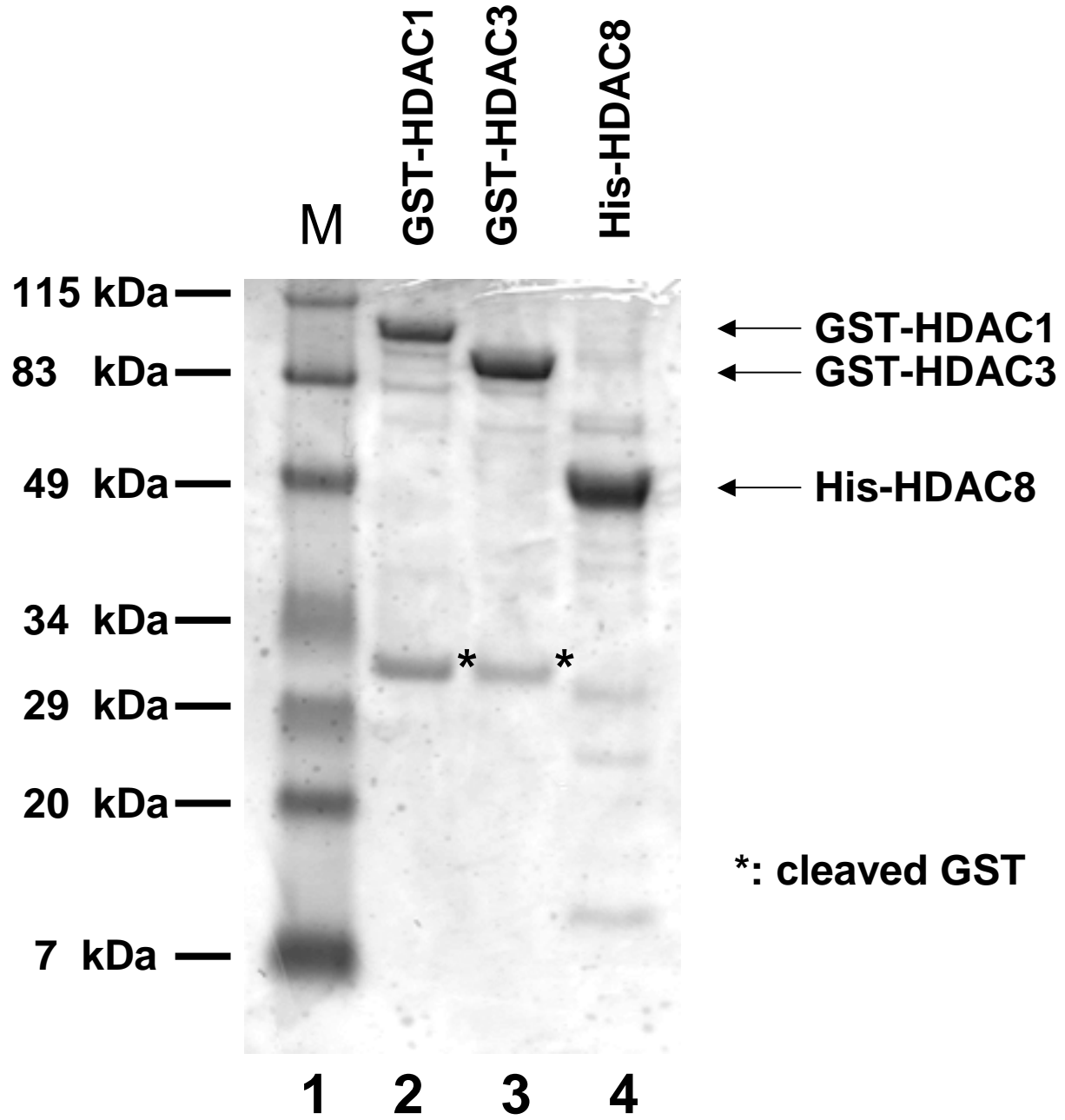
Figure 5: (A). Expression of HDAC1, 3 and 8 mRNA in control and TSA(300 nM)-treated SW620 cells. Northern blots are shown. Actin was used to normalize RNA loading. (B). Expression of HDAC1, 3 and 8 protein in control and TSA-treated SW620 cells. Western blots were performed with whole cell lysates. 100 ug of total protein was separated on a 4-12% Nu-PAGE (SDS) gel and blotted using anti-HDAC1, 3 and 8 antibodies. Densitometry analysis was performed using ImageJ software for HDAC3 mRNA and protein. TSA induced approximately 1.3 and 1.5 elevation for HDAC3 mRNA and protein, respectively.

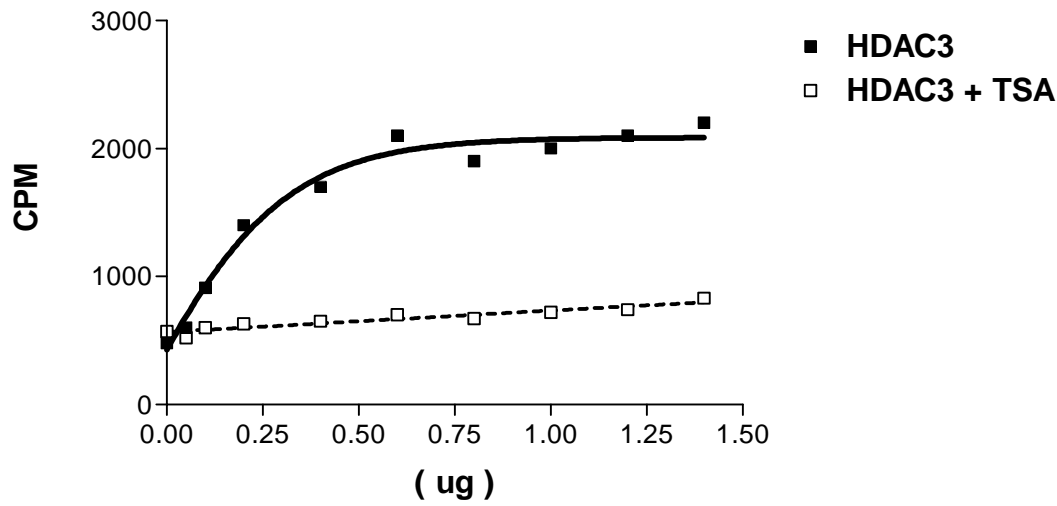
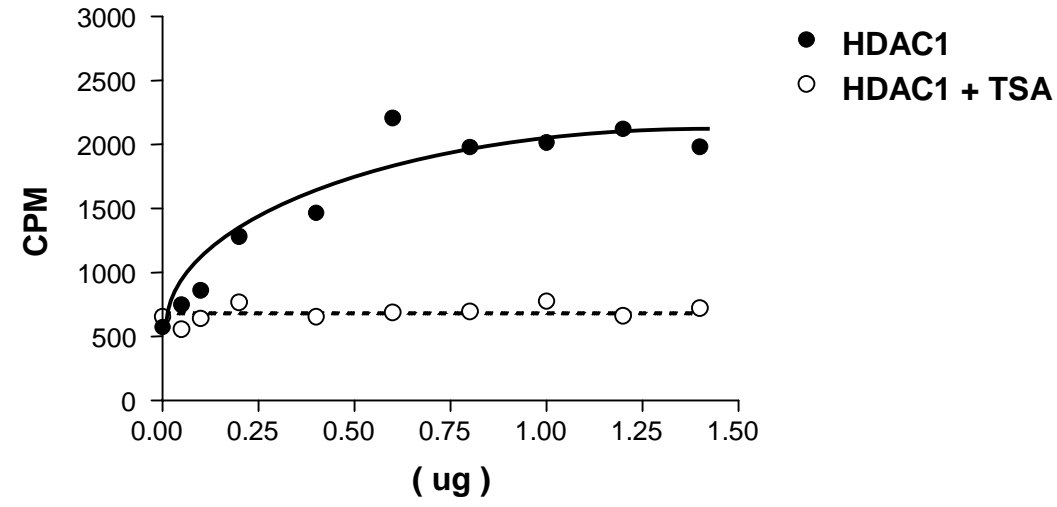
Figure 6: (A). Histone acetylation induced by TSA and MS-27-275. Increasing concentrations of TSA (0, 0.1 uM, 0.3 uM, 1 uM and 3 uM) and MS-27-275 (0, 0.3 uM, 1 uM, 3 uM and 10 uM) were added to SW620 cell for 24 hours. Cell

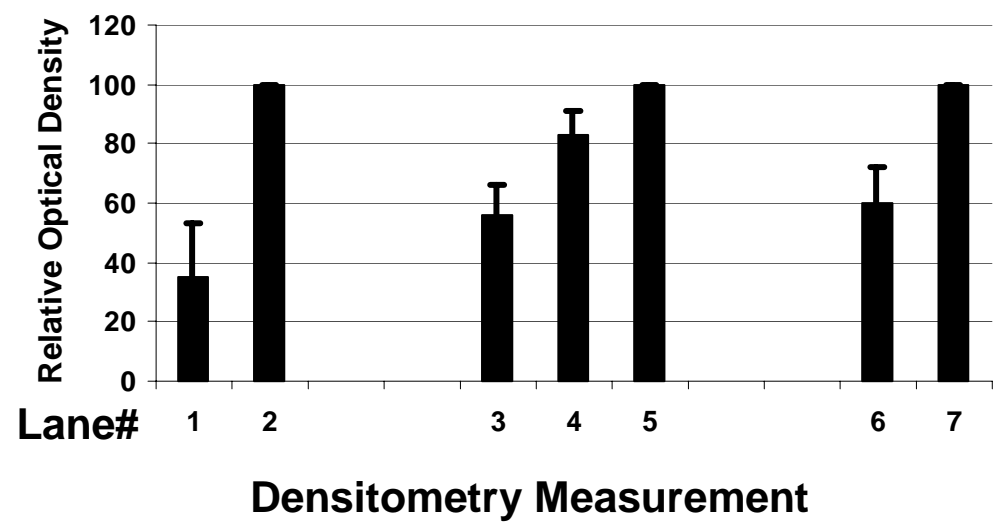
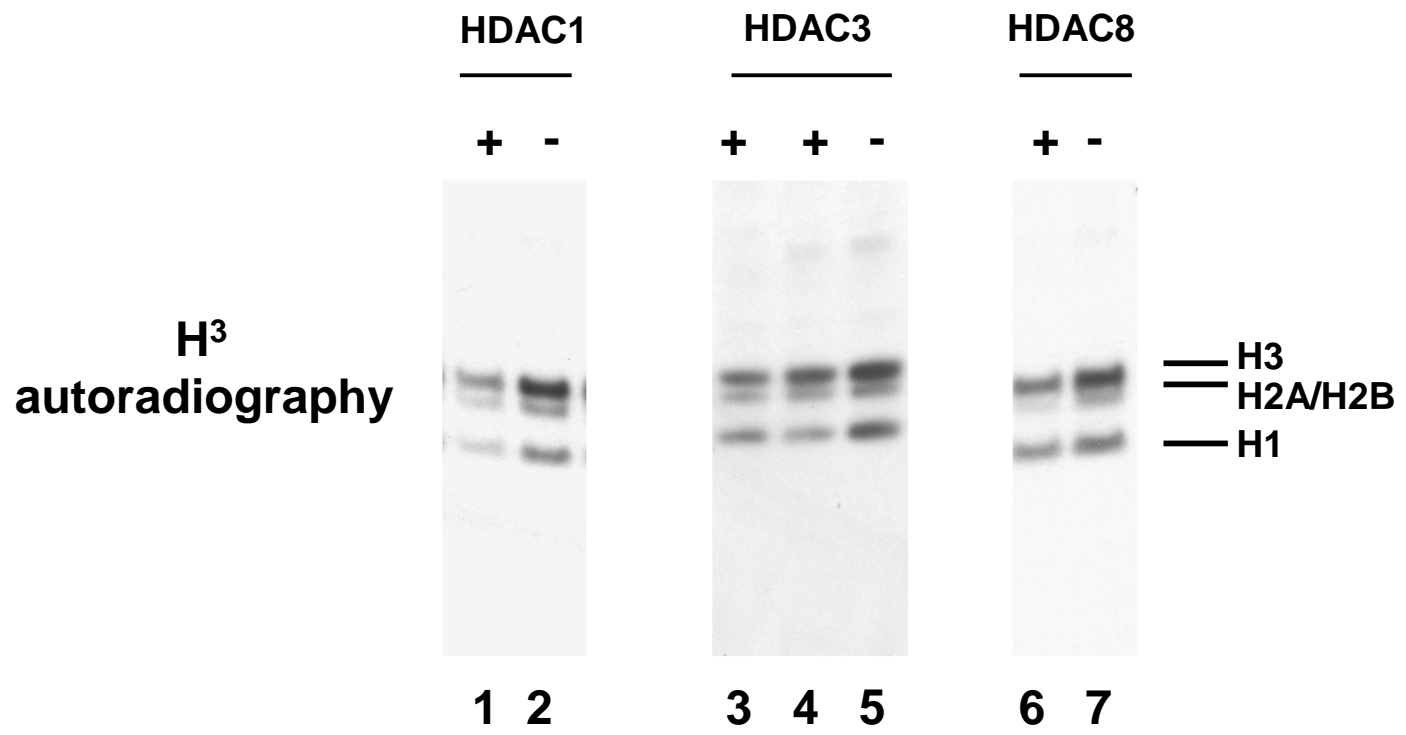
lysates were analyzed for histone H4 acetylation using anti-acetylated H4 antibody (Upstate Biotechnology, Lake Placid, NY). **(B)**. SW620 apoptosis induced by increasing concentration of TSA and MS-27-275. **(C)**. Activation of SV40 early promoter by TSA and MS-27-275.

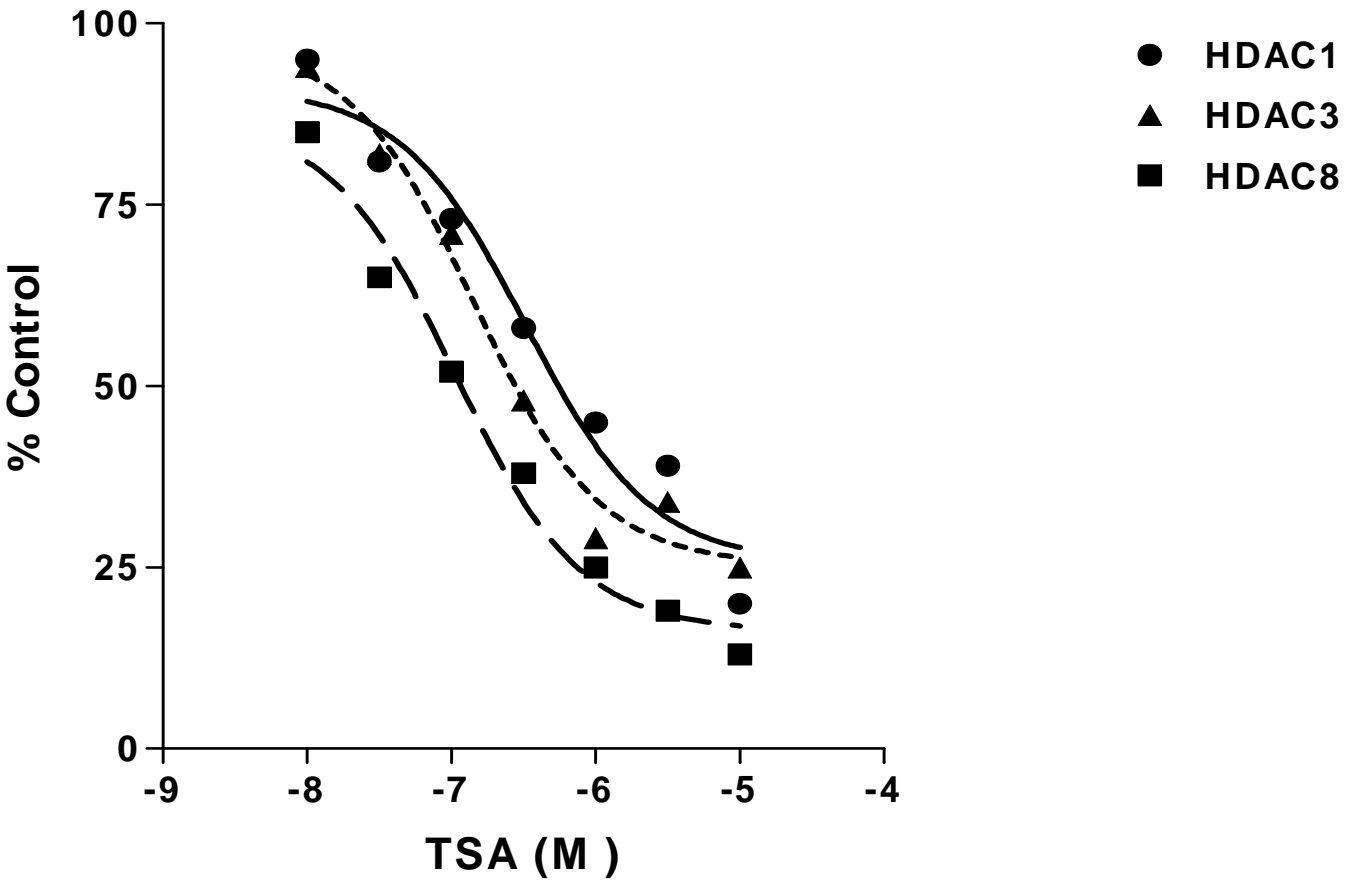
Figure 7: **(A)** Structures of novel HDAC inhibitors; **(B)** Effects of selective HDAC inhibitors on deacetylase activity of purified HDAC1, 3 and 8. **(C)** Effect of novel HDAC inhibitors on histone H4 acetylation in SW620 cells. control (0) and TSA (T, 300 nM) were used as positive controls. Increasing concentration of SB-556629 (Scriptaid), SB-429201 and SB-379278A (0.01uM, 0.03uM, 0.1uM, 0.3uM, 1uM, 3uM, 10uM, 30uM) were used. **(D)** Effect of novel HDAC inhibitors on SV40 early promoter activity. Individual experiments were repeated at least three times and a representative data set is shown.

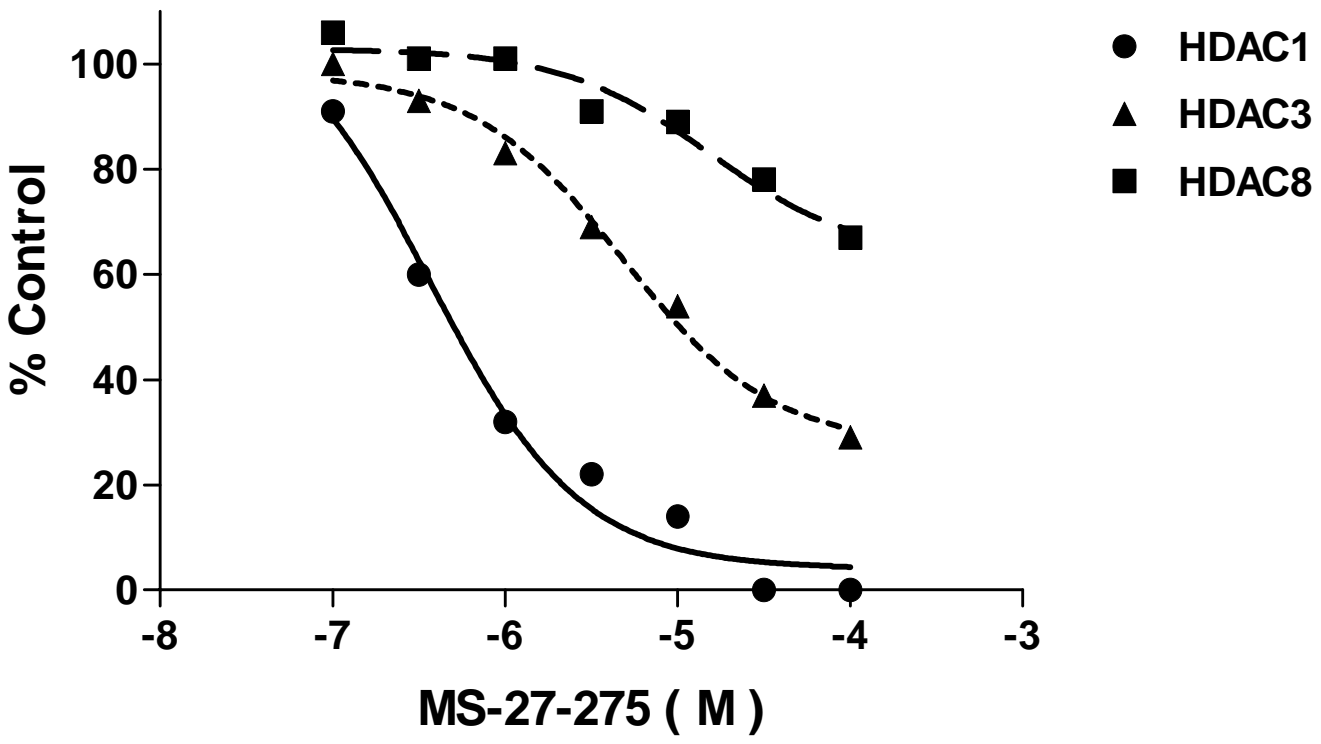


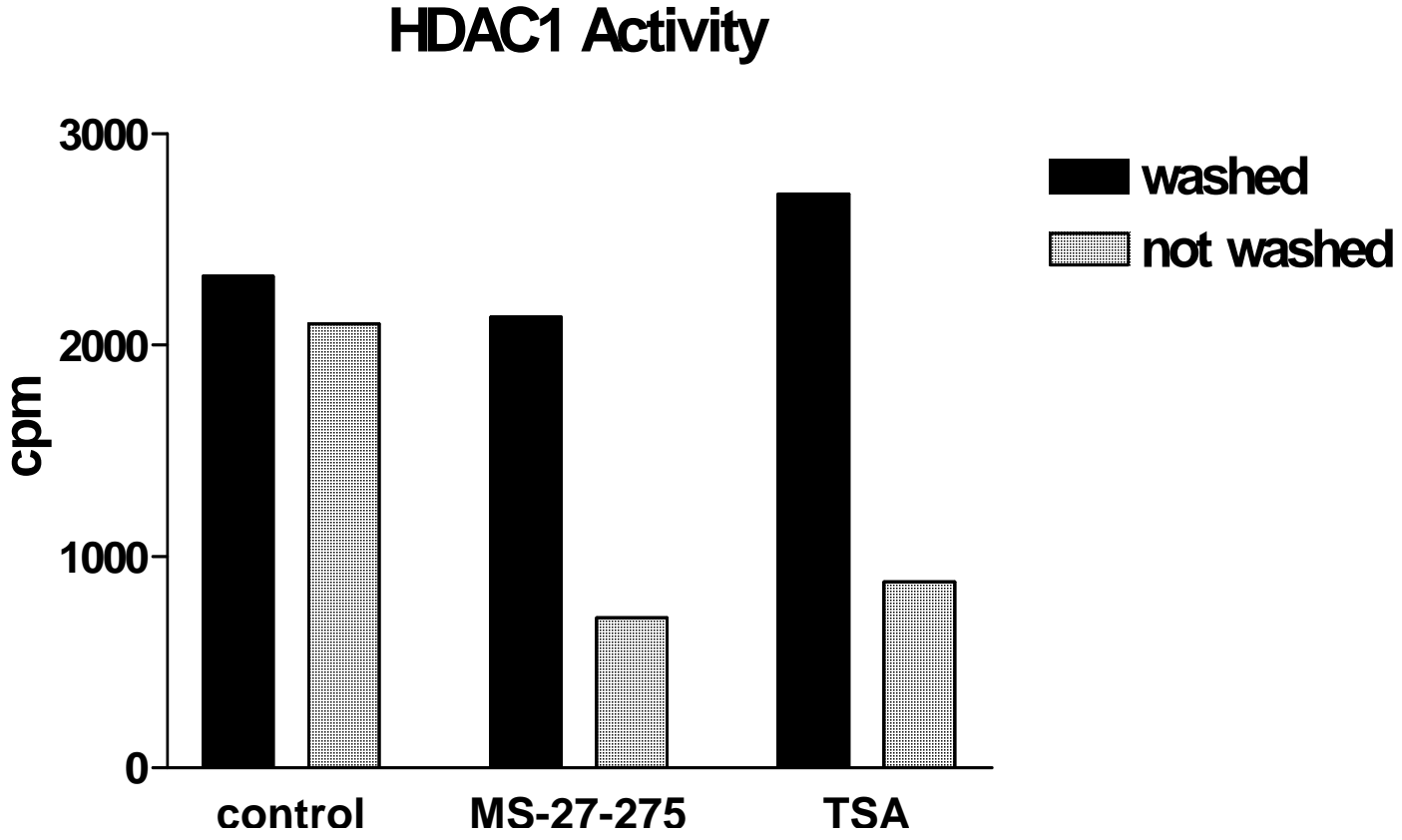




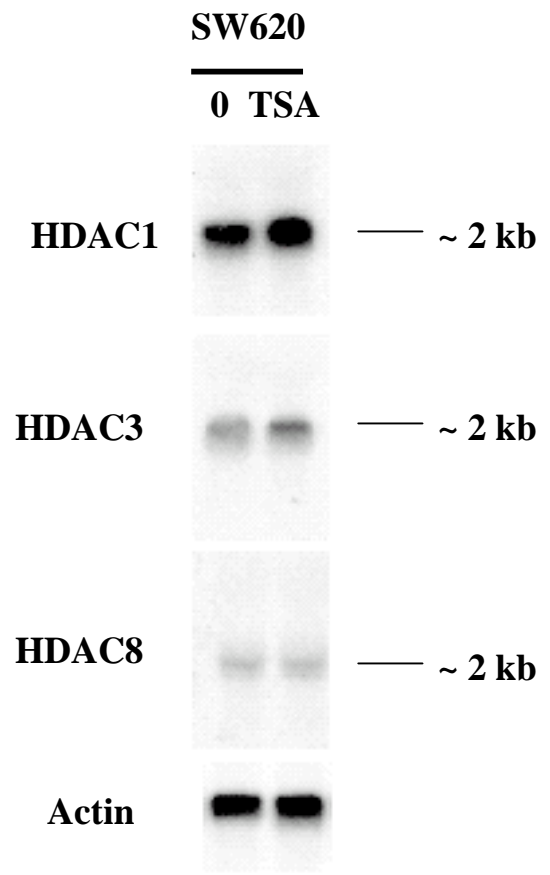






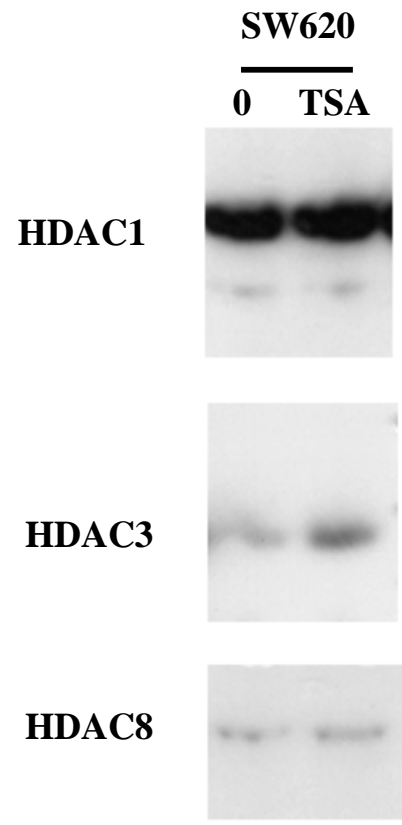


A:



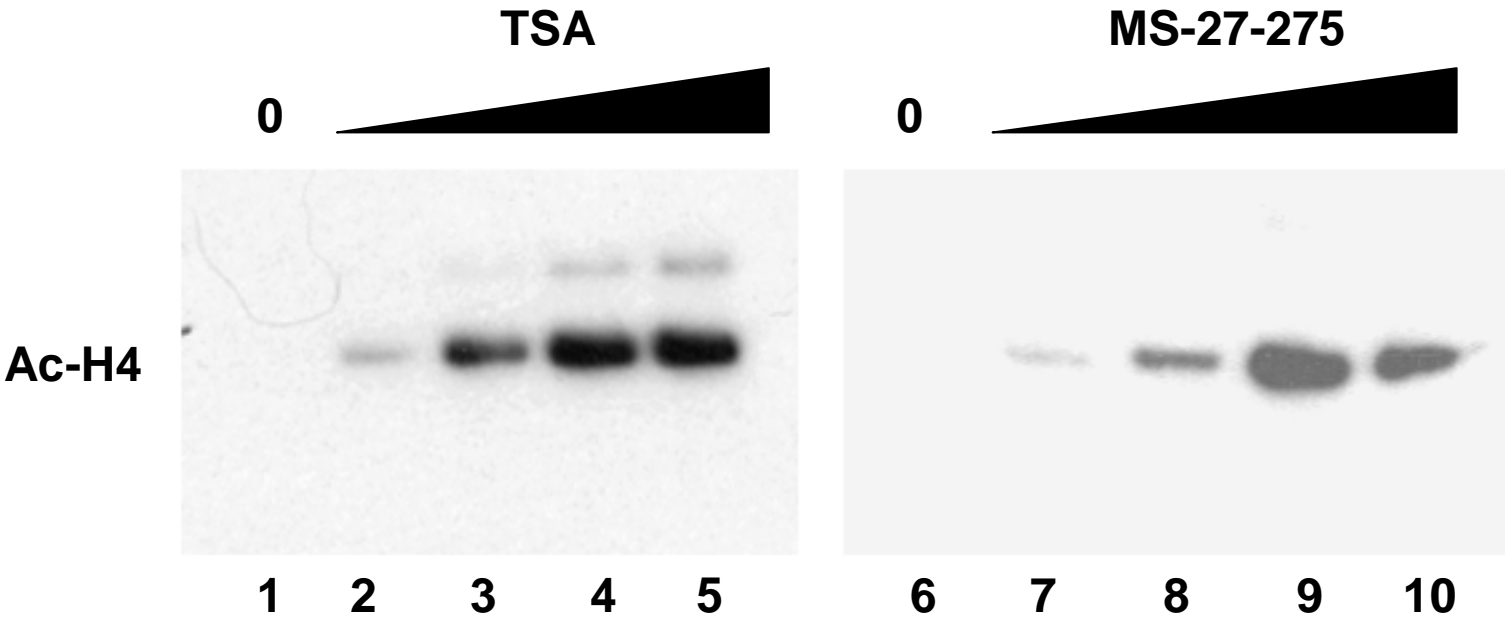
Northern Blots

B:

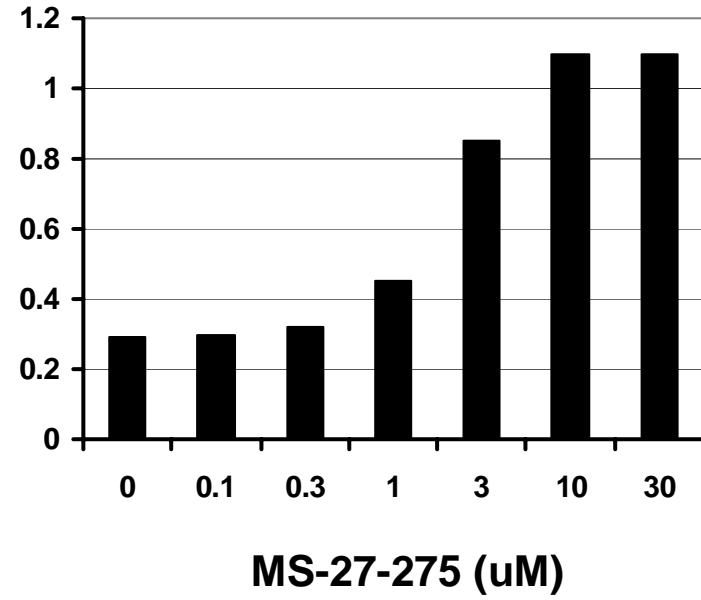
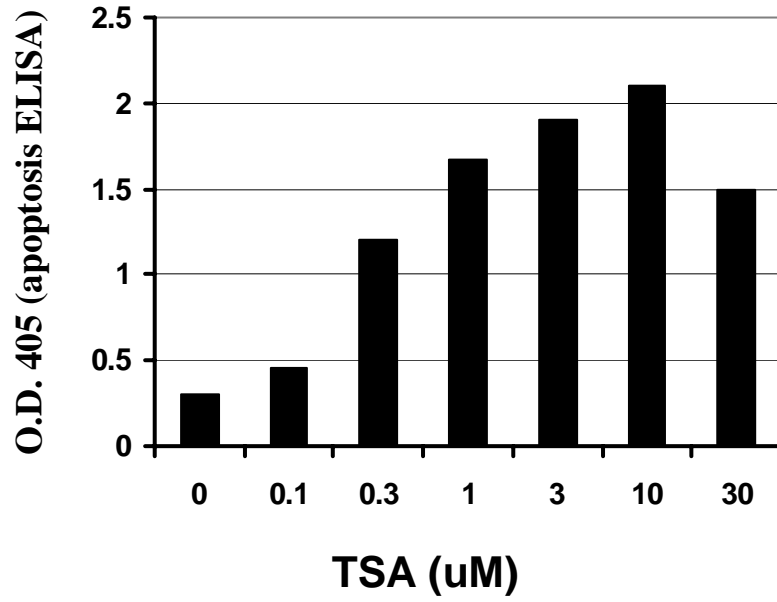


Western Blots

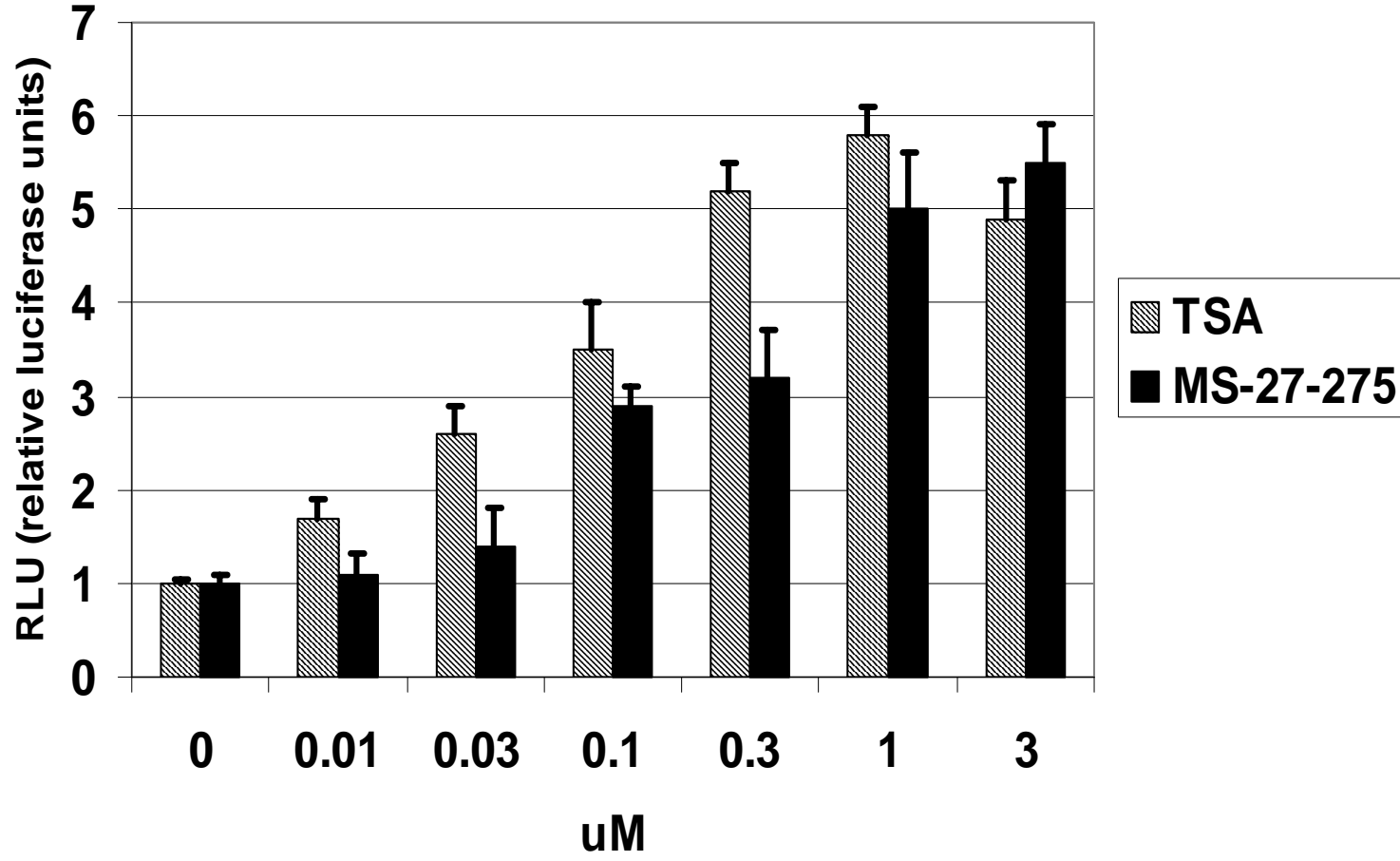
A: Histone acetylation

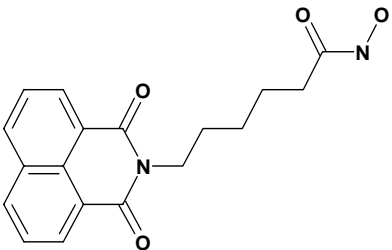


B: Apoptosis:

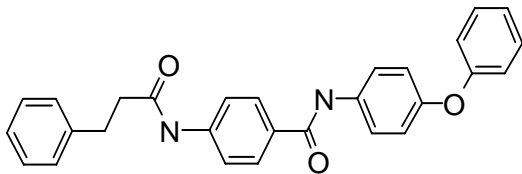


C: Reporter activity

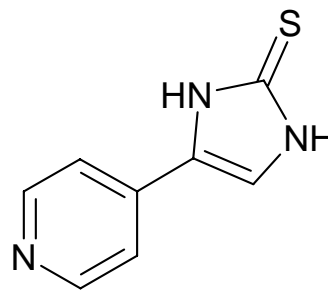




SB-556629
(Scriptaid)



SB-429201



SB-379278A

