Histone deacetylase inhibitors: mode of inhibition and histone deacetylase phosphorylation

By

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phosphorylation

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of

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LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nm
Co-IP	co-immunoprecipitation
CHD	chromo-helicase DNA-binding
CoREST	Co-repressor REST
cpm	counts per minute
NuRD/NRD	The nucleosome remodeling and Deacetylating
Dnmt1	DNA methyltransferase
DSP	Dithiobis (succinimidyl) propionate
FPLC	fast protein liquid chromatography
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	HDAC inhibitor
HDLP	histone-deacetylase-like protein
HMG	high mobility group
IP	immunoprecipitation
IPG	immobilized pH gradients
kDa	kilodaltons
MBD	methyl-CpG-binding domain
MTA	metastasis-associated
MDa	Megadalton

N-CoR	Nuclear Receptor CoRepressor
P	phosphor
Phsopho-HDAC	phosphorylated HDAC
RTT	ReTT syndrome
REST	RE1 silencing transcription factor
SAHA	suberoylanilide hydroxamic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMRT	Silencing Mediator for Retinoid and Thyroid hormone Receptors
SANT	SWI3/ADA2/N-CoR/TFIII-B
Ser	Serine
TSA	Trichostatin A
VPA	valproic acid
Western blot	WB

ABSTRACT

Histone deacetylases (HDACs) play a pivotal role in gene expression through their involvement in chromatin remodeling. The abnormal targeting or retention of HDACs to DNA regulatory regions is observed in many cancers, and hence HDAC inhibitors (HDACis) are being tested as promising anti-tumor agents. The results of previous kinetic studies, indicating that trichostatin A (TSA) and butyrate were non-competitive inhibitors, conflict with crystallographic and homology modeling data suggesting that TSA should act as a competitive inhibitor. Our results demonstrate that the three HDACis TSA, valproic acid (VPA) and butyrate act in a competitive fashion. Co-IP studies showed that the extensive level of association, in the human breast cancer cell line MCF-7, between HDAC1 and HDAC2 was not disturbed by inhibition of their activity with TSA. Moreover, inhibition of HDAC activity with TSA did not interfere with the interaction of HDAC1 and -2 with Sin3A, the core component of the Sin3 complex. Thus repressor complexes such as Sin3 appear to be stable in the presence of TSA. The association of HDAC1 and -2 with transcription factor Sp1 was also not affected by TSA.

Also Co-IP experiments have demonstrated that the interaction of HDAC1 and 2 with associated proteins is phosphorylation dependent. Although most of the HDAC2 is not phosphorylated in these cells, it is the phosphorylated form that is preferentially cross-linked to chromatin with formaldehyde or cisplatin.

Therefore, in this study we also determined the effect of HDACi (TSA) on HDAC2 phosphorylation by using 2D gel electrophoresis. Our results demonstrate that TSA does not have any effect on HDAC2 phosphorylation.

Moreover, Schreiber's study in 2001 has showed that in Jurkat cells, which contain low amounts of endogenous HDAC2, HDAC1 can get phosphorylated. In addition, mutagenesis studies have demonstrated that phosphorylation of HDAC1 and/or HDAC2 is required for the enzyme's association with RbAp48, which is a component of both the Sin3A and NuRD HDAC complexes. However, it is recently reported that HDAC1 C-terminal region was dispensable for binding to RbAp48 with the N-terminal section of HDAC1 being bound to RbAp48. Thus, we re-evaluated phosphorylation status of HDAC1 in Jurkat and HCT116p53 null cells which do not express HDAC2.Our results show that HDAC1which binds to RbAp48 is not phosphorylated. We propose that when HDAC2 is expressed, the HDAC2 phospho-form undergoes a conformational change exposing the RbAp48 binding site on HDAC2 to RbAp48.In contrast in the absence of HDAC2; RbAp48 binds to unmodified HDAC1 through its N terminal domain.

1.0 INTRODUCTION

1.1 Chromatin

The chromatin of the eukaryotic cell is a dynamic macromolecular complex structure situated in the nucleus and consists of DNA, histone proteins, and non-histone proteins (*1-3*). DNA and histones form the nucleosome which is a basic repeating unit of chromatin and a dynamic protein-DNA complex (*4, 5*). The nucleosome is composed of 146bp of two superhelical turns of DNA wrapped around the histone octamer containing two copies of each of four core histone proteins (H2A, H2B, H3, H4) (*3, 6*). The core histone octamer is composed of a H3-H4 tetramer and two H2A-H2B dimers. The tetrameric nucleosomic histone core involves three domains: the C-terminal domain, which is located inside the nucleosome core, the N-terminal domain with lysine residues extending out of the nucleosome, and a globular domain organized by the histone fold (*7*). Histone proteins are very basic and are highly conserved throughout evolution (*1, 8*). All histones are small basic proteins rich in amino-acids lysine and arginine (*2*). They are subject to posttranslational modifications. In general, histone modification is reversible (*3*). Nucleosomes are linked together by variable size linker DNA (*1, 2*). Although all four core histone tails can undergo modifications, H3 and H4 are most commonly targeted for various modifications such as methylation of lysine 4 and 27 of H3 and lysine 20 of H4 (*2*).

1.2 Modification of histones and regulation of transcription

Remodeling of chromatin proteins involving reversible post-translational modification of amino acids in the histone tails occurs by acetylation of lysines, methylation of arginines and lysines, phosphorylation of Ser ^{9} (Serine), and ubiquination and sumoylation of lysines (*5*). These covalent modifications lead to changes in chromatin structure that, in turn, lead to alteration in gene expression. The same modification can also occur on non-histone proteins which modulate their activity. For example, the tumor suppressor p53 is inactivated when deacetylation happens by human Sir2 (*1*). Histone modifications alone or in combination represent a code that can be recognized by non-histone proteins leading to formation of complexes involved in regulation of gene expression (*1*).

1.3 Histone acetylation and deacetylation

Histone acetylation of lysine residues is a dynamic, reversible process regulated by two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs transfer an acetyl group from acetyl-CoA to the ε-amino groups of lysine residues of histones. HDACs cleave the acetamide and remove the acetyl groups from the ε-amino groups of lysine residues, thus reversing the acetylation process (Fig 1) (*2*). Therefore, chromatin relaxes/opens and enables greater accessibility to proteins by acetylation or condenses/restricts access of proteins by deacetylation. It is important to remember that chromatin is a dynamic structure and DNA continually unwraps from and rebinds to the histone octamer (*9*). HATs together with HDACs control the dynamic acetylation

and deacetylation of core histones and nuclear regulatory proteins such as p53 (*10, 11*).

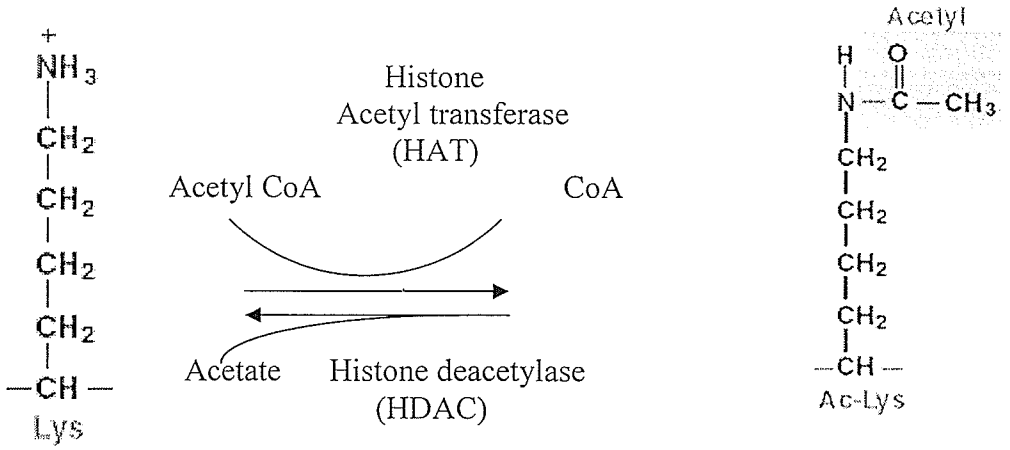


Fig. 1. Histone acetylation and histone deacetylation

1.3.1 Histone acetylation and transcriptional activation

During repression of gene transcription chromatin is tightly compacted to prevent accessibility of transcription factors. During activation of gene transcription the compact inaccessible chromatin becomes available to DNA binding proteins or transcription factors by modification of nucleosome histones (*1, 4*).

In comparison to phosphorylation and methylation, the histone acetylation is probably the best understood of these modifications: hyperacetylation leads to an increase in the expression of particular genes, and hypoacetylation has the opposite effect. Histone acetylation occurs at the ε amino groups of evolutionarily conserved lysine residues located at the N-terminal of histones (*1*). This results in neutralization of the positive charge of the lysine residues in the N-terminal tail of

the histones and relaxation or accessibility of a chromatin structure to different factors (*2*). Although all core histones are acetylated *in vivo*, modifications of histones H3 and H4 are much more extensively characterized than those of H2A and H2B (*12*). Important positions for acetylation are lysine^{9} and lysine^{14} on histone H3, and lysine^{5}, lysine^{8}, lysine^{14} and lysine^{16} on histone H4 (*4*). Acetylation occurs by several reported families of HATs such as PCAF/GCN5, P300/CBP, TAF250, SRC1 and MOZ. Among these, GCN5 was the first HAT discovered (*11*). Two families of PCAF/GCN5 and P300/CBP are the most characterized ones because they are potent HATs and are linked to signaling pathways and transcription factors (*11*). On the other hand, other explanations for the low activity of other HATs towards histones may be that the true target for these enzymes are non-histone proteins (*11*). In fact, the bromodomain, found in transcription factors and several HATs allows for the preferential recognition of acetylated histone tails (*9*). Substrates for acetylation now include DNA-binding proteins such as histones, transcription factors, HMG1 and non-nuclear proteins such as tubulin and proteins that shuttle from the nucleus to the cytoplasm such as importin-α family of nuclear import factors (*11*).

Acetylation has many different effects such as preserving stability of a protein by competing with ubiquitylation. In this case HDACs can expose the lysine residue for ubiquitylation since both acetylation and ubiquitylation can happen on lysine, leading to regulation of protein function. An example is the transcription factor SMAD7. Acetylation can also have effects on protein-protein interactions, protein

localization and DNA binding and might regulate transcription factor activity (*13*).

The turnover of acetylation on the core histones occurs rapidly and the level of acetylation is balanced by the equilibrium of HATs and HDACs (*14, 15*).

1.3.2 Histone deacetylation and transcriptional repression

The mechanism of action of the HDAC enzymes involves removing the acetyl group from the histones in the nucleosome. Hypoacetylation results in a decrease in the space between the nucleosome and the DNA that is wrapped around it. Tighter wrapping of the DNA results in less accessibility for transcription factors, leading to transcriptional repression (*4*).

The fact that acetylation is a dynamic process that regulates chromatin structure and gene expression has stimulated the study of HDACs in relation to the aberrant gene expression observed in cancer. Although no direct alteration in the expression of HDACs has yet been demonstrated in human oncogenesis, it is now known that HDACs associate with a number of well characterized cellular oncogenesis and tumor-suppressor genes such as Mad and retinoblastoma protein (Rb), leading to an aberrant recruitment of HDAC activity, which in turn results in changes in gene expression (*4, 16*). HDAC enzyme often operates with other proteins. Many proteins with different functions such as recruiter, corepressor or chromatin remodeller are involved in forming a repressor complex. The most important signal involved in the initiation process of repression is situated in the

DNA itself. Methyl groups bound to the cytosine residues situated 5' to guanosines in DNA, are directly responsible for recruitment of the HDAC complex. This happens via proteins such as methylated-CpG binding proteins and methyl-CpG-binding-domain-containing proteins, or via the enzymes that methylate CpG, the DNA methyltransferases. Although it seems that HDAC could be solely responsible for the repression of gene transcription via recruitment to methylated CpG, this is not the case. When HDAC activity is inhibited, the transcription of the gene under study is not always (completely) restored (*4*).

In addition to deacetylation of histones, other proteins can also be deacetylated by HDACs, including p53, E2F, α-tubulin and MyoD, illustrating the complex function of HDACs in many processes in the cell (Table 1) (*4*) .

HDACs are believed to be a good target for therapeutic interventions since they result in reversing aberrant epigenetic states that cause cancer. Aberrant recruitment of HDACs to promoters by direct contact to oncogenic DNA-binding fusion proteins has been provided as a first reason for target potential of HDACs in cancer treatment. For example in Acute lymphocytic leukemia (ALL), recruitment of HDAC2 by p21 WAF1/Cip1 which prevents turning on expression of the genes on promoter. However HDACis in cancer treatment reverse this effect (*17*). In addition, in some other studies over-expression of individual HDACs in tumor onset and progression has been reported as a reason for them to be therapeutic targets. For example, over-expressed HDAC1 is found in prostate, gastric, colon and breast cancers, while HDAC2 is increased in colorectal, cervical and gastric cancers and HDAC3 is expressed more than other HDACs in

colon tumors. Therefore, in recent years HDACis have increasingly been developed (*17*).

Table 1. Non-histone proteins that can get deacetylated by HDAC.

Proteins	Function
p53	Tumor suppressor
NF-κB	Regulates antiapoptotic responses
Sp3	Transcriptional activator or repressor
YY1	Multifunction transcription factor
α-tubulin	Microtubule component
HMGI	Essential architectural component for enhancesome assembly
HMG-17	Unfolds higher order chromatin structure
E2F1	Cell cycle activator — required for progression
MyoD	Stimulates cdk inhibitor p21
c-Myb	Proto-oncogene — regulates proliferation and differentiation
Importin-α	Nuclear import factor
TFIIE	General transcription factor
TFIIF	General transcription factor
Ku70	Suppresses apoptosis
C-Jun	Transcription factor
GATA-1	Differentiation of blood cells
TCF $\downarrow$	Transcriptional regulator
HSP90	Chaperon proteins
Reh1	Nuclear import factors

1.4 Classification of HDACs in yeast and in mammals

There are three classes of mammalian HDAC enzymes (Table 2). For chromosomal localization of classes I and II of human HDACs see (Table 3). Class I includes HDACs 1, 2, 3, and 8; these are related to yeast RPD3 deacetylase, have molecular masses of 22–55 kDa, and share homology in their catalytic sites. Class II deacetylases include HDACs 4, 5, 6, 7, 9, and 10, are larger molecules with molecular masses between 120 and 135 kDa, and are related to yeast HDAC1 deacetylase. They have a more complex domain organization; for example HDAC6 contains two catalytic domains, HDAC9 gene encodes multiple protein isoforms, some of which display distinct cellular localization patterns and HDAC 10 is a novel class II HDAC that has two sequence variants based on two C-terminal retinoblastoma protein-binding domains and a nuclear receptor-binding motif (*13*). HDAC 10 interacts with HDAC 3 but not with HDAC 4 or 6. HDAC 11 contains conserved residues in the catalytic core region shared by both class I and II enzymes. Class I and II HDACs each map to different chromosomal sites. A third class of HDACs which consists of seven subtypes in humans has been identified, members of which are NAD dependent and show homology to the yeast silencing protein Sir2. This group is not inhibited by HDACis (*3*). Class IV consists of a single HDAC, HDAC11. All classes of HDACs except HDAC IV are found in eukaryotic organisms. Class IV are not expressed in fungi (*13*).

Table 2. Classification and properties of histone deacetylases. S, Sentitive to inhibition by SAHA or TSA; ND, Not Determined.

Human HDAC classes	**Human HDACs**	**Inhibitor sensitivity**	**Properties**
Class I	HDAC 1,2,3,8	S	-Homologous to yeast RPD3 protein -Detected almost exclusively in the nucleus -Ubiquitous expression in various human cell lines and tissues
Class II	HDAC 4,5,6,7,9, 10	S	-Homologous to yeast HDA1 protein -Less restricted location, shuttling between nucleus and cytoplasm in response to certain cellular signals -May be involved in cellular differentiation and development
Class III	SIRT 1,2,3,4,5, 6,7	ND	-Homologous to yeast SIR2 protein -Require NAD+ for gene regulatory activity
Class IV	HDAC11	ND	-Recently discovered; -Shares features of Class I and Class II HDACs

Table 3. Chromosomal localization of HDACs. Data were obtained from NCBI SAGE database, freely available on the internet.

HDAC Class	Enzyme	Human chromosome location
I (RPD3-like)	HDAC1	1q34
	HDAC2	6q21
	HDAC3	5q31
	HDAC8	Xq13
	HDAC11	3p25.2
II (HDA1-like)	HDAC4	2q37.2
	HDAC5	17q21
	HDAC7	12q13.1
	HDAC9	7q21-p15
	HDAC6	Xp11.22-23
	HDAC10	22q13.31

HDACs are divided into three structural classes. Class I and II stand for "classical" HDACs operating by a Zn^{+2} dependent mechanism. The removal of the acetyl group via a charge-relay system is dependent on the zinc ion that weakens the acetamide bond and facilitates the hydrolytic attack of a water molecule (*1*). Deacetylation reaction in class I and II HDACs is proposed to be very similar to metallo- and Ser- proteases. The deacetylation requires zinc ion, water molecule, carboxyl oxygen and coordination among them to produce acetate and lysine products. In this proposed reaction, first carboxyl oxygen of the N-acetyl amide bonds coordinate with the zinc ion and put it closely to a bound water molecule which will attack the amide bond. The water could be activated by interaction with negatively charged histidine-aspartate pair and coordination with

zinc ion. After that water attacks on the carbonyl carbon which produces oxy-anion intermediate product. This intermediate product will be stabilized by zinc ion and hydrogen bonding to a nearby tyrosine. The final step is production of acetate and lysine products by breakdown of intermediate product which leads to disruption of carbon-nitrogen bond and provide a proton by histidine for the nitrogen. It is unlikely that zinc ion can activate the water and stabilize the oxy-onion intermediate since the geometry of electrostatic interactions in this system does not allow both (*18*). Class III in contrast to classes I and II is NAD dependent. The acetyl group is removed from the histone tail and simultaneously a mixture of 2'- and 3'-O-acetyl-ADP-ribose and nicotinamide is formed (*1*).

1.5 Crystal structure of HDACs

In 1999, for the first time the crystal structure of a bacterial HDAC homologue called histone-deacetylase-like protein (HDLP) was reported (*19*). The catalytic domain of HDLP was similar to both class I and II HDACs which contain 390 amino acids, consisting of a set of conserved amino acids. Therefore, presumably they both have similar mechanisms of deacetylation. HDLP has a single domain structure related to open α/β class of folds. HDLP pocket consists of 8 central standard parallel β sheet and 12 α helices. Four α helices out of 12 stand on either side of central sheets. The other 8 helices plus large loops in the β sheets extend the central structure and form a deep, narrow pocket with an adjacent internal cavity. The pocket represents the active site of the enzyme and binds to TSA (*18*). In HDACs the active site consists of a gently curved tubular pocket with a wider

bottom (*19*). For HDLP activity, Zn^{+2} ions are located near the bottom of the pocket and are coordinated by several histidine and aspartate residues. There is a similar charge-relay system in HDACs, consisting of two adjacent histidine residues, two aspartic residues (located approximately 30 amino acids from the histidines and separated by approximately 6 amino acids), and one tyrosine residue (located approximately 123 amino acids downstream from the aspartic residues) (*20, 21*). An essential component of the charge-relay system in HDACs is also a Zn^{+2} ion. For HDAC activity, again this atom is bound to the zinc binding site on the bottom of the pocket. However, other cofactors are required for HDAC activity (Table 4).

At that time co-crystalization of trichostatin A (TSA) with HDLP provided structural property information as to the postion of these HDACis on the enzyme. TSA binds to the pocket which represents the active site of the enzyme and consists of a cap group, an aliphatic chain and a terminal hydroxamic acid functional group. The terminal hydroxamic acid coordinates with the zinc ions and makes hydrogen bonds to several amino acids in the active site. The aliphatic chain of acetyl-lysine amino acids fits in the channel which is surrounded by hydrophobic residues and leads to the active site. This chain makes van der Waals contacts with the residues.

Lining the channel and its optimal length is 5-6 carbon residues. The cap group resides on the rim of the pocket by interacting with the amino acids (tyrosine 91 and proline 22) near acetylated lysine residue in the histones. When TSA fits into

the channel the only residue whose conformation changes are tyrosine 91 located on the rim of the pocket so that the cap group can fit tighter.

HDACis function by displacing the zinc ion and thereby rendering the charge-relay system dysfunctional. TSA, with its hydroxamic acid group and its five-carbon atom linker to the phenyl group, has the optimal conformation into the active site (*20*). TSA is the most potent reversible HDACi currently known, with an IC50 in low nanomolar range (*22*). Class I and II HDACs are thought to be approximately equally sensitive to inhibition by TSA (*3, 18, 23, 24*). The catalytic domains of the nine human HDACs are very well conserved, however there are some differences allowing for production of specific inhibitors. HDLP structure in the residues which interact directly with TSA is completely conserved in almost all human HDACs except in the region of tyrosine 91 of HDLP which is positioned on the rim of the channel and interacts directly with the cap group of TSA. Thus, with this consideration it is possible to develop specific inhibitors by altering the structure of this cap group for class I and II HDACs. In terms of the regions surrounding the active site, there is less conservation in between class I and II HDACs. The structural differences in the active sites of the HDACs are very valuable for inhibiting the specific HDAC that is associated with a particular disease by altering the cap group of inhibitors (*18*). For example, alteration of the cap group of one HDACi called apicidin leads to development of more potent apicidin derivatives for inhibition of malarial HDACs than human HDAC1 (*25*). Furthermore, HDAC6 has the greatest difference from the other HDACs in the

rim region near tyrosine 91 in HDLP (*26*) and is not inhibited by any cyclic tetrapeptide inhibitors (*18*) .

1.6 HDAC complexes

Basically there are three major complexes containing HDAC1 and HDAC2: Sin3, the nucleosome remodeling and deacetylating (NuRD) and CoREST which dynamically regulate transcription (Fig. 2). Among them Sin3 and NuRD contain a core complex, consisting of HDAC1/2 and the histone binding proteins RbAp46/48, which associate with proteins *in vivo* and *in vitro*. The CoREST complex, however, contains no RbAp46/48 in its core complex. Sin3 complex consists of a core complex, the complex stabilizers: SAP18 and SAP30 and a scaffold protein called mSin3 for the assembly of the complex and interaction with other DNA binding transcription factors called Mad, Ikaros, REST and nuclear hormone receptors (*27*). The NuRD complex also contains the core complex, MTA2 (related to MTA1 and overexpress in metastatic tumor cells), CHD3 (Mi-2α), and CHD4 (Mi-2β), methyl-CpG-binding domain 3 (MBD3) and metastasis-associated (MTA1,2), having DNA helicase/ATPase domains which also are found in the SWI/SNF family of chromatin remodeling proteins. Therefore it affects transcription with combination of its deacetylation and ATP-dependent nucleosome remodeling functions (*27*). In contrast to the above complexes, CoREST complex consists of a core complex and a protein homologous to MTA1 and MTA2 called CoREST and p110. CoREST similar to MTA1, MTA2, SWI3, NCoR and other proteins involved in transcriptional

regulation contains a SANT domain (*28*). This SANT domain resembles the DNA binding domains of Myb-related DNA binding proteins and mediates protein-protein interaction. For example, CoREST contains two 50 amino acid SANT domains whose N-terminal recruits HDAC1 to promoters (*29*). P110 is homologous to polyoxidases in the CoREST complex which contains a FAD binding domain and its enzymatic activity has not been clarified yet (*29, 30*). One study suggested that FAD does not bind to P110 (*30*). In these complexes three proteins called MTA2 in NuRD, CoREST in CoREST and Sds3p in Sin3/Rpd3 activate class I HDACs (*23, 29, 31*).

Other HDACs also are in complexes either with HDACs or with corepressor proteins and transcription factors (*27, 29-34*). Moreover, other studies have shown that HDAC interacts with other proteins such as nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid receptor (SMRT) through their deacetylase activating domain (DAD) or SANT domain. SANT domain is a putative DNA binding domain and is necessary for activating HDAC enzymatic activity (*31*). For instance, HDAC 3, 4, 5 and 7 are directly associated with the corepressors NCoR and SMRT (*35-39*).

Also, N-terminal extensions of HDAC 4, 5 and 7 interact directly with MADs box domain of myocyte enhancing factor (MEF) family of transcription factors (*40-42*). Specifically, HDAC3 is highly homologous to and associates with HDAC 4 and 5 and probably HDAC7. Also, HDAC4 but not HDAC5 associates with RbAp48 (*43*).

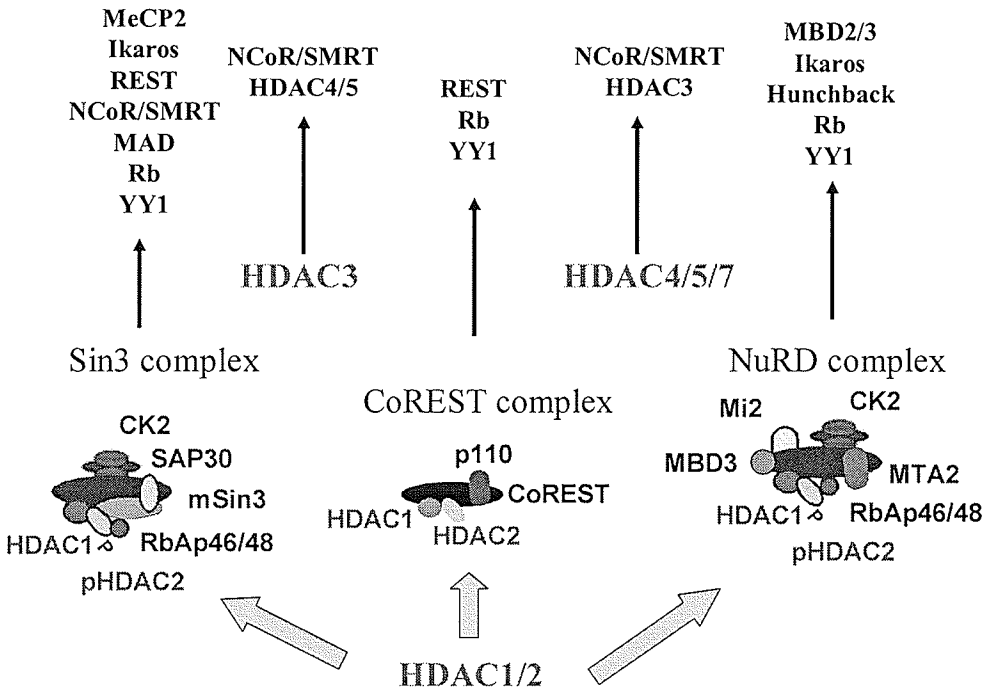


Fig. 2. HDAC complexes. HDACs are components of multiprotein complexes that interact with other proteins such as transcription factors.

1.7 Transcriptional repression by class I HDAC complexes

1.7.1 mSin3 repressor complexes

Mad family proteins have a basic helix-loop-helix-leucine zipper (bHLH-Zip) structure and are involved in cell proliferation and differentiation control (*44, 45*). The four identified Mad family members, Mad1, Mxi1, Mad3, and Mad4 form heterodimers with another bHLH-Zip protein, Max, to repress transcription (*46, 47*). Transcriptional repression by Mad:Max heterodimers is mediated by ternary complex formation with either corepressor mSin3A or mSin3B which are capable

of binding DNA (*15*). Yeast Sin3 and its mammalian orthologs Sin3A and Sin3B possess four conserved paired amphipathic helices (PAH) and a histone deacetylase interaction domain (HID), and are found associated with HDACs in large multimeric complexes (*48*). Therefore, mSin3A is a large protein (150kDa) with multiple protein-protein interaction domains on which other corepressor associated proteins assemble a complex (*15, 49-52*). It is reported that HDAC1, HDAC2, RbAp46, RbAp48, SAP30 (*49, 53*), RBP1(*54*), and p33ING1b (*55, 56*) associate with mSin3A with relatively high stoichiometry and as such, may represent components of a "core" mSin3A corepressor complex. Other components such as SAP18 appear to associate with mSin3A in a cell-type-specific or perhaps regulated manner (*53, 55*). SAP180, SAP130 and SAP45 have been shown to be in mSin3 complexes (*52*). The HDAC/RbAp subcomplex is required for much of the transcriptional repression capacity of the mSin3A corepressor (*32, 33*), whereas the other mSin3A associated proteins appear to function in the assembly of the complex and the targeting of mSin3A to other proteins or multi-protein complexes.

mSin3A and mSin3B are close paralogs and were first identified as corepressors required for the transcriptional and growth suppression functions of the Mad1 and Mxi1 proteins (*14, 15*).

1.7.2 NuRD repressor complexes

In 1998, several different groups simultaneously described the protein composition of a macromolecular histone deacetylase complex variously termed

the Mi-2 complex (*57*), NuRD (*58*), NURD (*59*) or NRD (*60*). The Mi-2/NuRD complex is a multi-subunit protein complex containing both histone deacetylase and nucleosome-dependent ATPase subunits (*61*). Current models predict that this complex functions primarily in transcriptional repression.

Interestingly there were minor differences between what different laboratories reported about the composition of NuRD complex using multiple cell types. All groups agree that the sole and largest component of this complex is 240kDa and consists of Mi-2β (CHD4) and Mi-2α (CHD3) which is less abundant than Mi-2β (*52-55*).

CHD4 has two plant homeodomain (PHD) zinc fingers for protein-protein interaction and transcriptional regulation, two chromodomains for establishing transcriptionally silenced chromatin regions by targeting to heterochromatin and a SWI/SNF-like ATPase domain which is the enzymatically active component of chromatin-remodeling complexes and is involved in DNA repair and recombination (*34*). Mi-2 is an autoantigen in the human connective tissue disease dermatomyositis (*62, 63*) and a member of the SNF2 superfamily of chromatin remodeling ATPases (*64*). Schultz et al. established that unique Mi-2/NuRD complex components might serve as molecular markers for functional specialization (*65*). For example, biochemical and functional exploration by his group led to the conclusion that KAP1 which is a corepressor utilizes the far carboxyl terminus of Mi-2α-containing complex with a subunit composition similar to the NuRD complex as a mediator of transcriptional repression so that

Mi-2/NuRD complex components might serve as molecular markers for functional specialization (*65*).

In addition to HDACs 1 and 2, the NuRD complex contains RbAp46/48 and proteins with apparent molecular masses of 32 and 70kDa. p32 is a novel protein, whereas p70 comprises multiple, closely migrating protein species, including MTA1 and MTA2 (*34*). In contrast to Mi-2 isoforms, MBD3 is the smallest subunit of the Mi-2/NuRD complex in human and Xenopus laevis (*23, 66*). The difference between human and Xenopus MBD3 protein is that in human the MBD3 can not bind to methylated DNA with selectivity (*66*). However, Feng and Zhang demonstrated that although the majority of the NuRD complexes do not contain MBD2 (methyl CpG binding protein), they can be recruited to methylated loci through interaction with MBD2 which has been shown to have strong affinity for interaction with methylated DNA (*23, 67, 68*). Co-IP studies have showed that HDAC1 but not HDAC2 interact with MBD2, confirming Zhang's study, and have also shown that the interaction between MBD2 and human Mi-2/NuRD complex is only evident in a subset of the complexes (*30*).

Four different groups reported on the identity of another major Mi-2/NuRD polypeptide of approximately 70–80kDa. In the NuRD human complex, this protein was called metastasis-associated (MTA) protein family (*23*). There are three genes in the MTA protein family that code for five proteins in human, MTA1, MTA1s (splice variant of MTA1), MTA2, MTA3 and MTA3L (*32*). MTA1, a founder of this family was discovered a decade ago in a differential display screen comparing mRNA from rat breast cancer cell lines with different

growth properties. Injection of mRNA into nude mice resulted in higher level expression of mRNA in cell lines and higher potential for invasion and metastasis. Thus, the protein was named *metastasis associated* protein 1 (*69, 70*). While MTA1 has been demonstrated to associate with components of the Mi-2/ NuRD complex (*59, 71*), the splice variant MTA1s, which lacks a nuclear localization sequence, is found predominantly in the cytoplasm (*72*), and is unlikely to be a component of Mi-2/NuRD. The MBD3 has two isoforms based on alternative splicing in human. The shorter one is more abundant than the longer protein known as MTA3, however both are associated with the Mi2-NuRD complex (*73*). Two independent studies support the notion that MTA family members demarcate functionally specialized Mi-2/NuRD complexes which means that for instance, MTA1 is unable to substitute for MTA3 and the function of MTA1 is specific for this member of MTA (*71, 73*). Therefore, there is a subunit heterogeneity in the vertebrate Mi-2/NuRD complexes (*74*). Furthermore, other data showed that MBD2/NuRD and MBD3/NuRD are distinct protein complexes with different biochemical and functional properties (*75*). In conclusion, the NuRD complex functions in transcriptional repression; for example, transcriptional repressor YY1 can recruit NuRD complex to DNA by binding to HDACs (*76*); however, additional roles for the NuRD complex in other chromatin-dependent processes, such as remodeling, recombination, replication and DNA repair cannot be ruled out (*34*).

1.7.3 N-CoR/SMRT mediated repression by mSin3-HDAC

Gene expression and transcription pursues after binding of retinoic acid or thyroid hormone ligands to their receptors, retinoic acid and thyroid hormone receptors (transcription factors). These receptors regulate transcription negatively and positively. There are two types of repressor proteins: N-CoR (Nuclear Receptor CoRepressor) or its related protein SMRT (Silencing Mediator for Retinoid and Thyroid hormone Receptors). These repressors, other hormone receptors and DNA binding proteins can bind to transcription factors such as retinoic acid and thyroid hormone receptors in the absence of their respective ligand and prevent transcription. Co-IP experiments and microinjection studies showed that N-CoR (270 kDa) binds both mSin3A and mSin3B; SMRT binds only mSin3A (*32, 77-79*). However, no direct interaction could be detected between N-CoR and mRPD3 or mSin3A/B and mRPD3. In addition, mSin3 and SAP30 are essential for N-CoR mediated repression (*49, 77*).

mSin3 is in a complex containing enzymatically active HDACl or HDAC2. N-CoR or SMRT are found in several studies in mSin3- and HDAC1-containing complexes and act as corepressors by binding to HDAC1 through mSin3 and mediate repression through mSin3/HDACl (*37, 77*). However, N-CoR/SMRT associate with HDAC4 in a complex that does not contain mSin3A or HDAC1 (*37*). *In vivo* and *in vitro* studies by Kao showed that SMRT can also directly interact with HDAC5 and HDAC7 and mSin3A (*38*). Therefore, nuclear hormone receptor corepressors such as N-CoR/SMRT complexes, which at times can

associate with different HDACs simultaneously and at other times, exist in distinct complexes (*37, 38*).

1.7.4 MeCP2 mediated repression by mSin3-HDAC

Gene expression is under control of DNA methylation and histone deacetylation as established by biochemical experiments (*80-82*). The transcriptional repressor methyl-CpG binding protein 2 (MeCP2) is the best characterized family member of methyl binding proteins that can bind to heterochromatin specially 5'cytosine residues in CpG dinucleotide repeats within the promoter regions and stably repress transcription (*67, 80, 83, 84*). Consequently, in the genomes of higher organisms methylation correlates to gene silencing. MeCP2 contains a methyl-binding domain (MBD) which helps it associate with heterochromatin by recognizing a symmetrically methylated CpG residue in naked DNA or chromatin (*80, 85*). It also contains a transcriptional-repression domain which recruits the corepressors mSin3 and HDACs (*81, 82*). Therefore, transcription regulation can be achieved by preventing the transcription machinery and/or factor binding to the 5'-methylated cytosine residues within the recognition sequence or by recruitment of HDAC corepressor complexes via proteins that specifically interact with methylated or sometimes hypermethylated DNA sequences (*80, 85*). Moreover, repression by MeCP2 is partially overcome by incubation of cells with the HDACi TSA, suggesting HDAC-independent repressors are involved (*80-82*). Evidence of links between DNA methylation and histone hypoacetylation is

accumulating and is related to silencing of gene expression and results in self-reinforcing epigenetic states. Depression by TSA was comparable to depression and demethylation by the inhibitor of DNA methylation, 5-azacytidine. Also, several possible models are discussed for the observed selective demethylation induced by TSA in *Neurospora crassa* (*86*). It was also reported that for organizing nuclear architecture MeCP2 may have a role since it has been shown that chicken matrix attachment region binding protein (ARBP) was found to be homologous to the rat protein MeCP2 (*87*). Rett Syndrome (RTT) is usually caused (95% or more) by a mutation in the gene encoding *methyl-CpG-binding protein-2*, MeCP2 which is found near the end of the long arm of the X chromosome at Xq28. RTT is a severe neurological disorder diagnosed almost exclusively in girls. Children with RTT appear to develop normally until 6 to 18 months of age, when they enter a period of regression, losing speech and motor skills. Most develop repetitive hand movements, irregular breathing patterns, seizures and extreme motor control problems. Currently, there is no cure for this disorder.

1.7.5 p53 mediated repression by mSin3A-HDAC

There is growing evidence that the p53 tumor suppressor protein is both a transcriptional activator and repressor. When activated, p53 stimulates expression of genes involved in cell cycle control and down-regulates some of those involved in cell growth. In some cases, p53 initiates apoptosis in response to severe cellular

damage. p53 recruits HDAC to the promoters of p53-repressed genes in order to repress transcription. The p53-HDAC interaction is not direct and is mediated by the co-repressor protein Sin3. The polyproline-rich region of p53, from amino acids 61–75 domain of p53 interacts with Sin3. Point mutants within this domain of p53 that fail to interact with Sin3 *in vivo* make it incapable of repressing the expression of p53-repressed genes, such as survivin, Map4 and stathmin genes but capable of activating p53-induced genes. Notably, while Sin3 alone does not inhibit tumor cell growth, Sin3 combined with p53 leads to complete inhibition of tumor cell growth. TSA can inhibit p53-mediated transcriptional repression and apoptosis induction by p53. These data point out the importance of p53-mediated transcriptional repression for apoptosis induction by p53 (*88*).

1.8 Other HDAC-interacting proteins

Histone deacetylases may exist in complexes not containing mSin3 or ATP-dependent remodeling activities. HDACs may interact directly with regulatory proteins to exert their effects on a subset of genes. Some examples are described below:

1.8.1 Ikaros family proteins

Ikaros is a zinc finger DNA binding protein and the founding member of a family of zinc finger nuclear factors that includes Aiolos and Helios (*89*). Ikaros is a

component of mSin3 and ATP-dependent chromatin remodeling (Mi2/NuRD) complexes (*89, 90*). In mSin3A it binds in between PAH3 and PAH4. It can also bind mSin3B and a mSin3B isoform at a region spanning PAH1 and PAH2 (*89*). It can also interact with SAP18, HDAC1, and HDAC2, but not with HDAC3 or SMRT (*89*). In NuRD complex it interacts with a large 2MDa immunopurified complex containing Mi-2, HDAC1, HDAC2, and RbAp48 (*90*). There is evidence that a small proportion of Ikaros and Aiolos interacts with Brg-1, hSWI-3, and BAF430, components of the homologous SWIISNF complex in higher eukaryotes (*90*). It has been proposed that Ikaros family members modulate gene expression during lymphocyte development by recruiting distinct histone deacetylase complexes to specific promoters (*89*).

1.8.2 Rb, BRCA1 and Dnmt1

The Rb protein is a product of the retinoblastoma susceptibility gene, and functions partly through transcriptional repression of E2F-regulated genes. The Rb family of proteins binds and sequesters the transcription factor E2F.

Repression by Rb is mediated, at least in part, by a histone deacetylase complex, whose enzymatic activity relies on HDAC1, HDAC2 or HDAC3. Rb-associated histone deacetylase complex contains RbAp48 protein, which interacts with HDAC1 and HDAC2 (*91*). Therefore, RbAp48 is necessary for transcriptional repression of E2F activity. It was also found by GST pull-down assay that

hypophosphorylated Rb can act as a powerful repressor by directly binding HDAC1 or HDAC2 (*92*).

HDAC3 as well as HDAC1 helps recruitment of RbAp48 to Rb; however HDAC3 does not increase Rb–E2F1 interaction. Surprisingly, HDAC3 as well as HDAC1 and HDAC2 can physically interact with RbAp48 both *in vitro* and in living cells. In conclusion, Rb mediates the recruitment of a repressive complex containing HDAC1, HDAC2 or HDAC3 and RbAp48 to E2F-regulating promoters (*91*). Inhibition of histone deacetylase activity by trichostatin A (TSA) inhibits Rb-mediated repression of a chromosomally integrated E2F-regulated promoter (*92*).

BRCA1 is a tumor suppressor gene whose mutation is associated with an increased susceptibility to breast and ovarian cancer (*93*). BRCA1 interacts with Rb-binding proteins, RbAp46 and RbAp48 as well as Rb *in vitro* and *in vivo*. In addition, BRCA1 domain associates with the histone deacetylases HDAC1 and HDAC2 suggesting BRCA1 involvement in multiple processes such as transcription, DNA repair, and recombination (*93*). Expression of BRCA1 can also lead to suppressed cell growth, dephosphorylation of Rb and decreased cyclin dependent kinase activity. Moreover, BRCA1 can induce apoptosis via binding to p53 (*93*).

The DNA methyltransferases (Dnmt1) are responsible for addition of methyl groups to cytosine and have a role in gene silencing (*94, 95*). As mentioned earlier, DNA methylation represses genes partly by recruitment of the methyl-CpG-binding protein MeCP2, which in turn recruits histone deacetylase activity

(*81, 82*). In the case of Dnmtl, HDAC1 is directly bound to it *in vitro* and *in vivo*. Therefore, HDAC1 is directly recruited to specific gene sequences through Dnmt1 and represses transcription. In addition, it is suggested that the process of DNA methylation, mediated by Dnmt1, may depend on or generate an altered chromatin state via histone deacetylase activity (*96*). Dnmtl acts with the other methyl-DNA binding proteins to maintain unacetylated histones within target chromatin regions containing methylated DNA.

1.9 Transcription factors interacting with HDAC

1.9.1 Sp1

Sp1 and Sp3 are mammalian transcription factors that function as activators or repressors. Sp1 and Sp3 form different complexes in estrogen-dependent human breast cancer cells. They both associate with histone deacetylases (HDACs) 1 and 2. Interestingly, they share a common domain in forming multimers. Our group has shown that in breast cancer cells most HDAC2 is not phosphorylated but Sp1 and Sp3 are more likely bound to phosphorylated HDAC2 that has a reduced mobility in SDS-polyacrylamide gels (*97*). HDAC2 is preferentially cross-linked to chromatin in its phosphorylated form. Protein kinase CK2 is associated with and phosphorylates HDAC2. CK2 phosphorylation of HDAC2 recruited by Sp1 or Sp3 could regulate HDAC activity and alter the balance of histone deacetylase and histone acetyltransferase activities and dynamic chromatin remodeling of estrogen regulated genes (*97*).

1.9.2 YY1

The zinc finger transcriptional factor yin yang 1 (yy1) is able to positively and negatively regulate transcription. When it is in interaction with HDACs, it will cooperate for repressing transcription.

Copurification assays have shown YY1 is a transcription factor whose zinc-finger domain helps other transcription factors such as LSF bind to a sequence located at the region from -10 to +27 (RCS site) of the HIV-1 long terminal repeat (LTR). LSF, YY1 interaction recruits HDAC1 to cooperate in the repression of HIV-1 LTR and inhibit transcription. Therefore, YY1 is capable of recruitment of HDAC1 and its zinc finger can regulate transcription by regulating occupancy of HDAC1 at a positioned nucleosome (nuc 1) near the transcription start site of integrated LTR. YY1 expression down-regulates LTR expression by increasing HDAC1 occupancy and decreasing acetylation at nuc 1. HDAC1 recruitment and histone hypoacetylation were also seen by the overexpression of YY1. YY1 mutants are incompetent for HDAC1 interaction and fail to recruit HDAC1 to LTR or decrease nuc 1 acetylation. Also, dominant-negative mutant of LSF (dnLSF), which inhibits LSF occupancy and LTR repression showed decreased HDAC1 occupancy at nuc 1 (*98*). On the other hand, TSA results in the displacement of HDAC1 from nuc 1, in association with increased acetylation of histone. Table 4 shows a summary of some HDACs' chractristics and their interacting proteins.

Table 4. Characteristics of HDACs.

	Complex	Cofactors (Modulate HDAC activity)	Interacting proteins	Expression
Class I				
HDAC1 HDAC2	NuRD, Sin3, CoREST	MTA2, p70, p32 and CoREST	Ikaros, MBD3, YY1, Sp1, p53, BRCA1, Rb, MeCP2, HDAC10, MBD2, YY1, BRCA1, Rb, MeCP2, HDAC10, NF-kB	Nucleus
HDAC3		N-CoR,SMRT	HDAC4, HDAC5, HDAC10, HDAC9 HDAC7, YY1, Rb	Nucleus/ cytoplasm
Class II				
HDAC4		N-CoR, SMRT, HDAC3	MEF2	All:
HDAC5		N-CoR, SMRT, HDAC3	MEF2	Nucleus/ cytoplasm
HDAC6				
HDAC7		N-CoR, SMRT, HDAC3	MEF2	
HDAC9		N-CoR, SMRT, HDAC3	MEF2	
HDAC10				
Class III			p53	Nucleus

1.10 Phosphorylation versus Acetylation

Protein phosphorylation is a post-translational modification that mediates intracellular signal transduction and is regulated by kinases and phosphatases (*99*). The first similarity between acetylation and phosphorylation is the diversity of their substrates. In the case of phosphorylation, substrate can be nuclear or

cytoplasmic. Also, acetylation although not as prevalent as phosphorylation can occur on DNA binding proteins, acetylases, nuclear import factors and tubulin.

The second similarity is that both can regulate different functions. Thus both acetylation and phosphorylation are analogous in the fact that both modify different proteins and regulate them in a variety of different ways (*11*).

There are some differences between them as well. First, kinases in contrast to acetylases do not associate with their substrates. Second, there are fewer kinases than acetylases. Third, there is more homology within the catalytic domain between kinases than acetylases (*11*).

1.10.1 HDAC1 and HDAC2 and their phosphorylation

The first HAT which had a high degree of amino acid sequence similarity to the yeast transcriptional adapter GCN5 was discovered in Tetrahymena (*100*). HDACs are in multiprotein complexes including transcription activators and co-activators that target HDAC1/2 directly to specific promoters (*101-103*). HDACs are characterized in different classes as discussed earlier and each may have a particular histone substrate and each may deacetylate different histone amino acids. In addition, some may deacetylate non-histone proteins as well as histones (*104*). The first human HDAC, HDAC1 was purified by Schreiber and colleagues (*105*). Sequence analysis of HDAC1 revealed that it is related to the yeast protein RPD3. Human HDAC1 sequence analysis was identified by mass spectrometry. Two phosphorylated Sers were identified at Ser^{421} and Ser^{423} by mutagenesis experiments (*27*). Moreover, it was reported *in vitro* that phosphorylation is done

by protein kinase casein kinase 2 (CK2) at carboxyl-terminal region of HDAC1 Ser^{421} and Ser^{423}, leading to increased enzymatic activity and HDAC1 complex formation. The charge and size of phospho-Sers are crucial for HDAC1 function, as glutamic acid and aspartic acid only partially substituted for phosphorylated Ser in HDAC1 (*27*). On the other hand, in Cohen et al.'s study, it was found that phosphorylation did not influence HDAC1 enzymatic activity using a human histone H4 N-terminal peptide as the substrate. It was confirmed that phosphorylation occurred in the C-terminal domain using HDAC1 deletion mutants (*106*). Another study has shown that the N-terminal domain of HDAC1 is also necessary for *in vitro* binding to HDAC2, HDAC3 and association with RbAp48 and for catalytic activity of the enzyme. The report claimed that the C-terminal domain of HDAC1 was only important for nuclear localization of mouse HDAC1 (*10*).

A second human histone deacetylase protein, HDAC2, also with high homology to yeast RPD3, was identified by Seto et al. (*76*). Human HDAC1 and HDAC2 are highly homologous protein with 75% identity in DNA sequence and 85% identity in protein sequence. Detailed analyses of the HDAC1/2 complexes revealed an unprecedented connection between deacetylation, DNA methylation, and chromatin remodeling (*104*).

The function of class II HDACs is regulated by phosphorylation (*107*).We have showed that HDAC2 co-exists in the same protein complexes as HDAC1 leading to gene regulation (*108*). It has also been reported by mutational analysis that HDAC2 is phosphorylated at the C-terminal portion of the protein exclusively at

Ser residues of Ser^{394} and Ser^{411}, Ser^{422}, and Ser^{424} by protein kinase CK2 *in vivo*. In addition to gene regulation, HDAC2 phosphorylation is critical is for protein complex formation. It means that hypophosphorylated HDAC2 disrupts interaction with mSin3 and Mi2, but only fairly affects formation of HDAC1/2 complex, suggesting that the HDAC2 interacting domain is probably similar for mSin3 and Mi2 but different from HDAC1. This phosphorylation is important for enzymatic activity but not for transcriptional repression (*104*).

1.11 Regulation of HDAC activity

1.11.1 Regulation by protein complex formation

HDACs have enzymatic and functional activities. They exert their enzymatic activities by deacetylating histones or non-histone protein substrates and they regulate transcription with their enzymatic activities (*31*). Also, HDACs in general have no DNA binding activity and any proteins that target HDACs to DNA or histones can affect their functions *(33).*

1.11.2 Regulation by post-translational modifications (phosphorylation)

Phosphorylation is an important post-translational modification for regulating many enzymatic activities. All mammalian HDACs have potential phosphorylation sites. In 2001, two phosphorylated sites on HDAC1 Ser ^{421} and ^{423} were identified by mass spectrometry (*27*). In 2002, HDAC2 phosphorylation sites were reported at the C terminal portion of the protein at Ser ^{394}, Ser ^{411}, Ser ^{422} and Ser ^{424} (*104*). Furthermore, the protein kinases, casein kinase 2 (CK2),

protein kinase A (PKA) and protein kinase G (PKG) were shown to phosphorylate HDAC1; however, HDAC2 could only be phosphorylated by CK2 *in vitro* (*31*). Mutagenesis studies have revealed that these phosphorylations are critical for HDAC1 and HDAC2 enzymatic activity and complex formation with RbAp48, MTA-2, mSin3A and CoREST (*27, 104*). Phosphorylation of HDAC1 or 2 by altering their conformation into a more favorable enzymatically active form might enhance enzymatic activity; alternatively, phosphorylation might increase HDAC1, 2 interactions with proteins such as MTA2 and SDS3 leading to more enzymatic activity. However, in 2001 Cohen by using HDAC assays reported that HDAC1 phosphorylation did not affect HDAC activity towards an acetylated N-terminal peptide of human histone H4. HDAC assays were performed using immunoprecipitated HDAC1-FLAG protein from transfected COS-7 cells (*106*) . Yet another study observed regulation of HDACs by phosphorylation (*109*). In this study, the authors showed HDAC1 and HDAC2 are in three different phosphorylated forms: unphosphorylated, phosphorylated and hypophosphorylated. They treated cells with the phosphatase inhibitor okadaic acid (OA). This led to hyperphosphorylation of HDAC1 and 2 that correlated with a small but significant increase in the activity of both enzymes. This is consistent with Pflum et al.'s data suggesting that phosphorylation leads to higher enzymatic activity (*27*). However, Ahn group study showed that HDAC phosphorylation resulted in disruption of protein complex between HDAC1 and HDAC2 as well as HDAC1 and mSin3A or YY1 but it did not affect the interaction between HDAC1/2 and RbAp46/48 (*109*). In another study on breast cancer cells

phosphorylation of HDAC2 unlike HDAC1 was not necessary for transcriptional repression (*97*). In addition, in this study CK2- phosphorylated HDAC2 was preferentially associated with Sp1 and Sp3 and chromatine in human breast cancer cells and it is recruited by Sp1 or Sp3 on the chromatin to regulate HDAC activity (*97*).

In addition to HDAC1 and HDAC2, other HDAC1 family members such as HDAC3 and HDAC8 maybe phosphorylated by CK2 and PKA, respectively (*104*). One study in 2004 showed that phosphorylation of HDAC8 resulted in reduced enzymatic activity reflected by hyperacetylation of histones H3 and H4. The phosphorylation site was at non-conserved residues (Ser ^{39}) located at the N-terminal (*110*).

Other studies have analyzed phosphorylation of class II HDACs and role in regulation of HDAC function. It has been reported for instance that phosphorylation does not appear to affect HDAC enzymatic activity; instead it modulates subcellular localization (*31*). Involvement of phosphatases in regulating HDAC activity is equally important as HDAC kinases (*109*). PP1 has been reported in several studies as a major HDAC phosphatase that regulates phosphorylation and therefore activity in several human HDACs (*31, 111*).

1.11.3 Regulation by subcellular localization

Class I HDACs include HDACs 1, 2, 3 and 8. HDAC1, 2 and 8 are nuclear proteins and are located in the nucleus. Therefore, they need nuclear localization signals to bring HDACs in to the nucleus for regulating their activity. However,

HDAC3 can be found in the cytoplasm as well, and the nuclear/cytoplasmic ratio depends on the type of cell studied and the stimuli. As such HDAC3 activity can be regulated by nuclear localization signals (*21*). Class II HDACs include HDACs 4, 5, 7 and 9 which shuttle between the nucleus and cytoplasm (*31, 41, 107, 112*). These HDACs bind to 14-3-3 proteins by phosphorylation of their conserved N-terminal Ser residues and move to cytoplasm. Therefore, cytoplasmic localization negatively regulates class II HDACs by excluding them from the nucleus (*31*). In addition, it has been reported that sequences located at the C-terminal of HDAC4 act as nuclear export signals which help cytoplasmic retention of HDAC4 (*113, 114*). Moreover, there is a conserved nuclear import signal at the N-terminal of HDACs 4, 5, 7 and 9 which can be covered by protein 14-3-3, thereby inhibiting nuclear targeting (*114*). To enhance its nuclear localization, HDAC4 is associated with components of the Ras-MAPK signal transduction pathway (*31, 115*). Among class II HDACs, HDAC6 although predominately localized in the cytoplasm, is capable of shuttling to the nucleus (*116*). HDAC6 does not bind to protein 14-3-3 and the primary substrate of HDAC6 and SIRT2 is cytoplasmic α-tubulin and not histones (*117*). Consequently, cytoplasmic localization of HDAC6 and SIRT2 is important in their regulation (*31*).

1.11.4 Regulation by changes in gene expression

Changes in HDAC gene expression can regulate HDAC protein levels. Seiser and his colleagues analyzed the regulation of the mouse HDAC1 gene by deacetylases and acetyltransferases. The murine HDAC1 promoter lacks a TATA box

consensus sequence but contains several putative SP1 binding sites and a CCAAT box, which is recognized by the transcription factor NF-Y. HDAC1 promoter-reporter studies revealed that the distal Sp1 site and the CCAAT box are crucial for HDAC1 promoter activity and act synergistically to form HDAC1 promoter activity. Furthermore, these sites are essential for activation of the HDAC1 promoter by the deacetylase inhibitor trichostatin A (TSA). Chromatin immunoprecipitation assays showed that HDAC1 is recruited to the promoter by Sp1 and NF-Y, thereby regulating its own expression (*118*). Also, HDAC1 not only regulates its own expression but it also regulates HDAC2 and HDAC3 protein levels (*119*).

1.11.5 Regulation by metabolic cofactors

Class III HDACs are regulated in response to the metabolic status of the cells. NAD+ is a substrate for catalytic activity of Sir2 like enzymes (class III HDACs) in deacetylation of lysine residues. In the reaction class III HDACs remove acetyl groups which will transfer to NAD+ and cleave it to nicotinamide, resulting in acetyl-ADP-ribose. Therefore, Sir2 like enzymes get activated by increasing the NAD+ or decreasing the level of nicotinamide, an inhibitory product of Sir2 (*31*). Thus there is a link between redox cofactors such as NAD and histone deacetylase activity and these cofactors may play a critical role in transcriptional regulation.

1.11.6 Regulation by proteolytic processing

Protein degradation can be another factor for HDAC activity regulation. For example, HDAC1/mSin3A corepressor complex or HDAC3-associated protein N-CoR can be targeted for proteosome-mediated degradation as an indirect mechanism for controlling HDAC1 or HDAC3 activity (*24, 120*).

1.12 Tissue Distribution of HDACs

Surprisingly, no major differences were observed between the expression patterns in normal and malignant tissues. However, significant variation in HDAC expression was observed within tissue types (*4*) (see Table 5).The level of HDACs throughout embryonic development are variable, possibly indicating that different HDACs are involved in various stages of embryogenesis (*3*). In summary, class I HDACs are widely expressed whereas class II and IV are more tissue specific (*13*).

Table 5. General detectable HDAC expression by tissue type based on SAGE tag analysis. ND: Not Determined; ----: Not expressed.

Expression		Tissue (Normal)						
		Brain	Breast	Colon	Ovary	Pancreas	Prostate	Heart
ClassI	HDAC1	√	---	√	√	√	√	---
	HDAC2	√	---	√	√	√	√	√
	HDAC3	√	√	---	√	√	√	---
	HDAC8	---	---	---	---	√	---	---
ClassII	HDAC4	---	---	---	---	---	---	---
	HDAC5	√	√	√	---	---	√	√
	HDAC6	√	√	√	√	√	√	√
	HDAC7	√	√	---	√	√	√	√
	HDAC9	√	---	---	√	√	√	√
	HDAC10	√	√	√	√	---	√	---
		Tissue (Tumor)						
ClassI	HDAC1	√	√	√	√	√	√	ND
	HDAC2	√	√	√	√	√	√	ND
	HDAC3	√	√	√	√	√	√	ND
	HDAC8	√	---	√	√	√	√	ND
ClassII	HDAC4	√	---	√	√	√	√	ND
	HDAC5	√	---	√	√	√	√	ND
	HDAC6	√	√	√	√	√	√	ND
	HDAC7	√	√	√	√	√	√	ND
	HDAC9	√	---	√	√	√	---	ND
	HDAC10	√	√	√	√	√	√	ND

1.13 HDAC inhibitors (HDACis)

HDACis have been recognized to have potential anti-cancer effects and tumor specific activities. Some of these agents have been demonstrated to have potential early therapeutic effects in the early phase of clinical trials for hematological malignancies such as cutaneous T-cell lymphoma or diffuse B-cell lymphoma

(*17*). HDACis exert their anti-cancer effect by killing tumor cells via apoptosis, autophagy or necrosis. However, it has been shown that they induce extensive morphological changes characteristic of apoptosis more than other types of cell death (*17*).

HDACis induce cell cycle arrest in G1 or G2 phase and apoptosis and differentiation in transformed cells from hematological and epithelial tumor. HDACis could therefore inhibit tumor growth both directly by causing growth arrest, terminal differentiation and/or apoptosis of cancer cells, and indirectly, by inhibiting neovascularization of tumors. Both TSA and depsipeptide are reported to block angiogenesis *in vivo*. TSA inhibits hypoxia-induced angiogenesis in the Lewis lung carcinoma model and depsipeptide inhibits hypoxia-stimulated angiogenesis. Hypoxia induces HDAC1 and angiogenesis, so inhibition of HDACs might have a role in blocking new tumor blood vessel formation (*17*).

The effective concentration of HDACis depends on the concentration of required acetylated histones in *in vitro* study(*20*). HDACis cause an accumulation of acetylated histones in tumor and normal tissues (spleen, liver and peripheral mononuclear cells).Therefore the level of accumulation of acetylated histones is a useful marker of HDAC biological activity, and has been used to monitor dosing in clinical trials of cancer patients.For example a 13-year-old girl with acute promyelocytic leukemia (APL) who no longer responded to treatment with retinoic acid alone was treated with retinoic acid plus phenylbutyrate and had a complete clinical remission that was sustained for 7 months, during five treatment courses, before relapsing and becoming resistant to this treatment. The acetylation

of histones in her mononuclear blood cells was elevated during the period of administration of the phenylbutyrate. Unfortunately, HDACis in addition to their main effect on tumor cells might have side effects including neoplastic growth and survival by regulating host immune system response and tumor vasculature (*17*).

1.13.1 Classification of HDAC inhibitors

Several drugs that inhibit histone deacetylation are in clinical trials. These include butyrate, VPA, SAHA, pyroxamide, depsipeptide, MS-275 and CI-994. These are in phase I and II evaluation as monotherapy, as well as in combination with other different therapies and cytotoxins. In phase I trials, agents have been administered to patients with various types of solid tumor at advanced stages of the disease and to patients with hematological malignancies, including lymphomas and leukemias. In this phase, toxicity of a new therapeutic agent and its maximal safe dose is evaluated. A phase II clinical trial might focus on one or a few types of cancer, and to determine if a safe dose of a new therapeutic agent is effective in treating disease (*8*).

There is a wide range of HDACis which can be purified from natural or synthetically developed sources and today at least 11 are used in the clinic. They are divided into several different classes according to chemical structure:

1- Aliphatic acid such as VPA and butyrate

2- Hydroxamic acids such as trichostatin A (TSA), SAHA, oxamflatin, pyroxamide, m-carboxycinnamic acid bishydroxamide (CBHA)

3- Cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy-decanoyl moiety such as trapoxin A

4- Cyclic tetrapeptides not containing the 2-amino-8-oxo-9,10-epoxy-decanoyl moiety such as

FR901228, desipeptide (FK-228) and apicidin

5- A combination of the hydroxamate class and the cyclic tetrapeptides leads to hybrids such as CHAP compounds and trapoxin-hydroxamic acid (TPX-HA) analogue

6- Benzamides such as MS-275 and N-acetyl dinaline CI-994 (see table 6)

These drugs limit proliferation of transformed cells in culture and tumor growth in animal models (*3, 22*). These drugs inhibit HDAC activity with various degrees of efficiency. For example, MS-275 is more active against HDAC1 than HDAC3 and HDAC8 (*17*).

There are two modes of inhibition, reversible and irreversible. Reversible inhibitory mechanisms usually have high activity when the concentration of the inhibitor is low and low activity when the concentration of the inhibitor is high. Irreversible inhibition usually results from a covalent and permanent modification of a functional group on the enzyme, rendering the molecule inactive. There are four generally recognized classes of reversible inhibition: competitive, non-competitive, uncompetitive (Fig. 3) and mixed inhibition.To determine what type an inhibitor is:

1. Find Km and V_{max} for uninhibited from 1/Vo vs 1/[S] plot (Lineweaver-Burk graph).

2. On the same graph find Km' and V_{max}' for inhibited reaction.

a. If Vmax = Vmax' then the inhibitor is competitive (Vmax and Vmax' should not be more than 10% different).

b. If Vmax does not equal Vmax', and if Km = Km', then the inhibitor is non-competitive.

After inhibitor type was determined, equations were used to calculate Ki. Ki is the binding constant of inhibitor to the enzyme. Ki has the same units as the [I]. If [I]=mM, then Ki=mM. Equations used for calculating Ki values:

Competitive Inhibitor

$$K'm = Km\ (1+\frac{[Ic]}{Ki})$$

In competitive inhibition, the inhibitor competes with the substrate at the active site on the enzyme. Competitive inhibitors often share structural resemblances with the substrate. Non-competitive inhibition results from the interaction of the inhibitor and the enzyme at a site other than the substrate binding site. The inhibitor may bind to a functional group on the enzyme in such a manner that the active site is contorted so as not to bind the substrate properly. Uncompetitive inhibition results from the combination of the inhibitor with the enzyme-substrate complex directly, without the formation of an enzyme-inhibitor complex. Mixed

inhibition may be considered a consequence of several kinds of inhibition. Competitive and non-competitive inhibitors bind reversibly. Inhibitor constants, Ki, are quantitative measures of the inhibitory strength of reagents. Ki was determined by first performing a standard Km analysis. Next was repeating the entire procedure with the inhibitor in the assay buffer.

IC_{50} is a measure of concentration used in pharmacological research. IC_{50}, or the half maximal inhibitory concentration, represents the concentration of an inhibitor that is required for 50% inhibition of its target (i.e. an enzyme, cell, cell receptor or a microorganism).

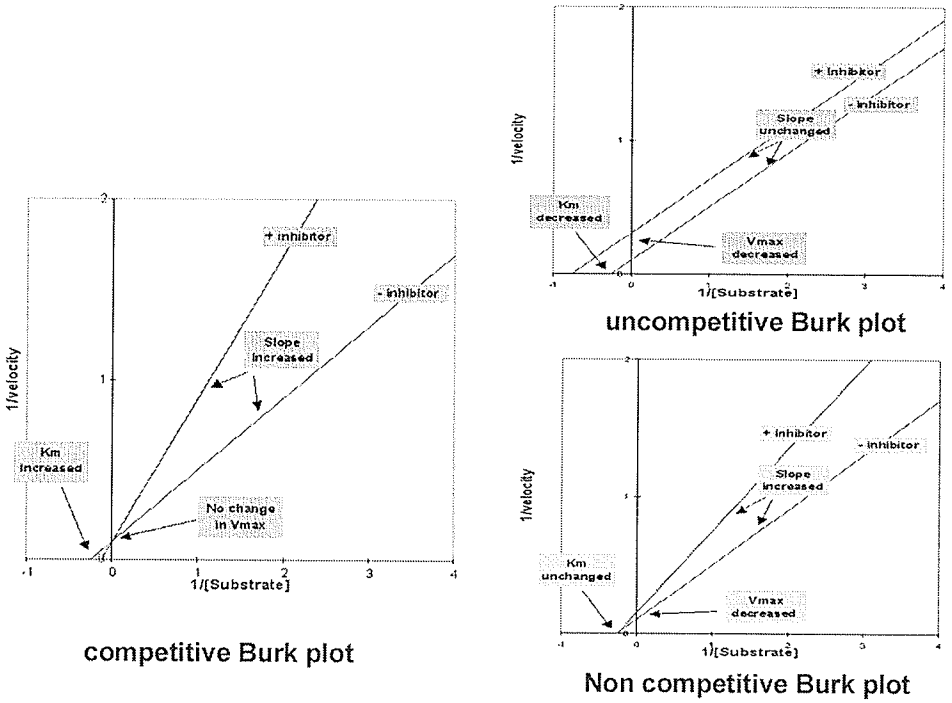


Fig. 3. Standard model of enzyme kinetic mode of action. Top right, uncompetitive mode of action. Bottom right, non-competitive mode of action. Left, competitive mode of action.

Table 6. HDAC Inhibitors.S, Synthetic; N, natural; NF, Not Found.

Group	**Drugs (HDAC inhibitors)**	**synthetic or natural sources**	**In vitro IC50 range**
Hydroxamic acids	Trichostatin A (TSA)	N	nM
	Suberoylanilide hydroxamic acid (SAHA)	S	μM
	M-carboxycinnamic acid bishydroxamide (CBHA)	NF	μM
	Azelaic bis-hydroxamic acid (ABHA)	NF	NF
	NVP-LAQ824	NF	NF
	LBH589	NF	NF
	Oxamflatin	NF	nM
	PXD101	NF	NF
	Scriptaid	NF	μM
	Pyroxamide		μM
Short-chain fatty acids	valproic acid (VPA)	S	mM
	Phenyl butyrate (PB)	S	mM
	Phenyl acetate (PA)	NF	mM
	Sodium butyrate (SB)	N	mM
	AN-9 (Pivanexs)	S	NF
Cyclic tetrapeptides	Apicidine	N	μM-nM
	Trapoxin	NF	μM
	HC-toxin	NF	nM
	Chlamydocin	NF	nM
	Depsipeptide (FK228; FR901228)	NF	nM
	Cepudesin	NF	μM
benzamide derivatives	MS-275	S	μM
	CI-994 (N-acetyldinaline) (Tacedinaline	S	μM

1.13.2 Cellular mechanism of HDAC inhibitors

It is proposed that activation of gene transcription is associated with histone acetylation (*22, 121*). The HDACis butyrate, TSA, depsipeptide, oxamflatin, MS-275 and SAHA induce expression of the genes regulating cell cycle proliferation including the *CDKN1A* gene, which encodes the cyclin-dependent kinase inhibitor WAF1 (also known as p21) or *CDKN2A* (which encodes INK4A, also known as p16), and the genes for cyclin E and thioredoxin binding protein 2 (TBP2) and the putative tumor suppressor gelsolin (*122-127*). It is postulated that there are specific sites in the promoter region of these genes (for example Sp1 sites) that bind HDAC1 recruiting transcription factor complex (TFC) on the promoter of these genes and repress gene transcription. In return activation of these silenced genes by HDACIs such as SAHA selectively increase acetylation of histones and relax the chromatin that is tightly wrapped around a deacetylated histone core. Therefore acetylation of histones result in increased transcription of these genes which eventually lead to downstream effect such as cell-growth arrest, differentiation and apoptotic cell death and as a consequence inhibition of tumor growth. For example, increased transcription of WAF1 by TSA inhibits cell-cycle progression, acting to block cyclin-dependent kinase activity and, as a consequence, causing cell-cycle arrest in G1 (*8*).

1.13.3 TSA

Trichostatin A is a hydroxamic acid-based compound and was initially isolated as a fungistatic antibiotic from *Streptomyces hygroscopicus* (*2, 128*). It is a potent

inhibitor of HDACs active at nanomolar concentrations (*22*). Another compound in this group is oxamflatin which inhibits HDAC activity at micromolar concentrations. Cyclic hydroxamic-acid containing peptide (CHAP) compound built from TSA and some cyclic tetrapeptides is hydroxamic-acid based and inhibits HDACs at nanomolar concentrations (*3, 22*). SAHA, which contains relatively less structural complexity, was found to be a nanomolar inhibitor of partially purified HDAC, as was pyroxamide (*3, 20, 129*). Hydroxamic-acid based compounds contain a polar site (the hydroxamic group), a six-carbon hydrophobic methylene spacer, a second polar site and a terminal hydrophobic group (Fig. 4). Two members of this group SAHA and pyroxamide are being used in clinical trials (*22*).

Members of the benzamide class, which includes MS-275 and CI-994, possess micromolar levels of activity (*127, 130*). Another class of HDACis includes the electrophilic ketones. These agents include various trifluoromethyl ketones and ketoamides, which are active at micromolar concentrations as inhibitors of HDAC activity (*131*).

The aliphatic acids, the least potent class of HDACis, possess millimolar levels of activity and include valproic acid (VPA) and phenylbutyrate (PB) (*132, 133*).

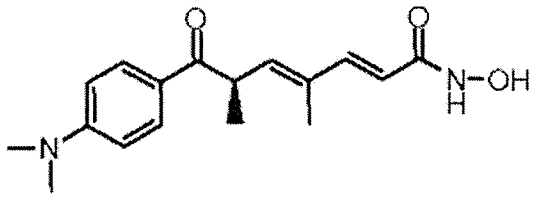


Fig. 4. Chemical structure of TSA.

1.13.4 Valproic acid (VPA)

VPA is a well-known anticonvulsive agent that emerged in 1997 as a short-chain fatty acid and broad spectrum antiepileptic drug (Fig. 5)(*134, 135*). It has been used in humans for treatment of bipolar disorders, neuropathic pain and migraine prophylaxis (*134*). The effectiveness of VPA as an anticonvulsant was discovered by chance when other compounds were dissolved in VPA for administration to animals used in experimental models of epilepsy (*136*). The mechanisms of action for VPA are currently unknown, although its antiepileptic effects are related to increased γ-aminobutyric acid (GABA) function and sodium/calcium interaction with VPA (*134*). Since then, VPA has been used to control a variety of seizures, including generalized and partial seizures (*136*). Recent studies have indicated that VPA may also have anticancer activity against a variety of tumor types (*137-141*) and it can induce growth arrest in several breast cancer cell lines.

Approximately 0.3-0.7mM VPA was used as a therapeutic concentration to inhibit HDAC enzymes in HeLa and neuro2A neuroblastoma cells (*133, 142*). VPA is currently in phase I and II clinical trials (*4, 8, 143*). Despite VPA being well tolerated and having a low incidence of serious side effects, one concern with VPA therapy is weight gain. VPA treatment in humans increases the serum level of two hormones, leptin and insulin, which are produced by the adipose tissue and pancreatic cells, respectively. After VPA treatment for 1 year, 37% of female patients with epilepsy who developed obesity had a 1.8-fold increase in fasting serum insulin and 3.4-fold increase in serum leptin levels (*144*). VPA also influences cell cycle regulation, differentiation, apoptosis, and angiogenesis (*135*).

VPA, like butyric acid and trichostatin A (TSA) inhibits the activity of zinc dependent, class I and II HDACs (*133, 142*). VPA did not inhibit the activity of class II HDACs 6 and 10 (*145*), in contrast to the conventional HDACi trichostatin A (TSA), which implies that VPA might be a more selective HDACi than TSA. Controversial data were reported by Kramer and co-workers (*135, 146*). Although they presented further evidence that VPA selectively inhibits the catalytic activity of class I HDACs (*140*), they also demonstrated that VPA induced proteasomal degradation of HDAC2, in contrast to TSA (*146*). To investigate further the mechanism of HDAC inhibition, binding studies with radiolabeled VPA, [^{3}H]VPA, showed that it binds to protein complexes precipitated with antibodies against N-CoR, mSin3 or HDAC2 *in vitro* (*142*). TSA has been shown to bind directly to the active site of a deacetylase (*19*). Since TSA efficiently competes for binding of [^{3}H] VPA, both compounds not only exert similar effects on HDAC activity but also appear to share identical or overlapping binding sites. Therefore, the mechanism of HDAC inhibition by VPA was speculated to involve blocking of substrate access to the catalytic center of the enzyme (*142*). To date, another HDACi (suberoyl anilide hydroxamic acid, SAHA) has also been reported to decrease HDAC protein levels, but is selective for HDAC 3 (*146*).

VPA has been shown to inhibit the proliferation and morphological differentiation of several carcinoma and hematopoetic cell lines. Of special note is the strong antineoplastic activity of VPA in chemoresistant cancer cells. In some cell lines VPA was able to induce apoptosis. The question, why some cancer cells do not

respond in terms of apoptosis, remains to be elucidated. In terms of understanding the molecular underpinnings of the antitumoral VPA action, it becomes increasingly clear that this is closely related to modulation of histone acetylation (*135*).

VPA was shown to directly inhibit HDAC class I and enhance HDAC2 degradation (*142*). Clinical studies are underway and the preliminary results indicate that VPA alone or in combination is promising both in solid and hematopoetic malignancies. VPA is a promising tool to fight cancer and needs further investigation to define its full potential (*135*).

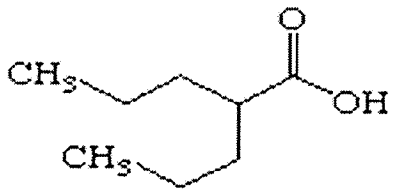


Fig. 5. Chemical structure of valproic acid.

1.13.5 Butyrate

The first encountered HDACis belong to the class of short-chain fatty acid (Fig. 6). The first ones were sodium N-butyrate (*147*), phenylacetate and phenylbutyrate (*148, 149*), but limited efficacy has been observed during the clinical trials (*31*) with some toxicities and fatigue. Phase II clinical trials are ongoing. It was shown that the aliphatic chain of TSA occupies a hydrophobic cleft on the surface of the enzyme (*19*). Possibly two butyrate molecules also could occupy the hydrophobic pocket and inhibit the enzyme. However, butyrate was found to be a noncompetitive inhibitor of HDAC, which argues that butyrate

does not associate with the substrate-binding site (*150*). The binding site and the mechanism by which butyrate inhibits HDAC activity remain unknown.

Butyrate can inhibit HDAC class I and II members except HDAC6 and HDAC10 (*7*). P21Waf1/Cip1 promoter has six Sp1 binding sites. Level of acetylated histones associated with p21 promoter is low which leads to more condensed chromatin structure and inactive promoter. HDAC is recruited to p21Waf1/Cip1 by Sp1 or Sp3. With butyrate, HAT activity of p300 and histone acetylation levels at the promoter will increase which results in more p21Waf1/Cip1 expression and less cell proliferation (*151*). This is a p53- independent process suggesting that the production of butyrate in the colon may be protective against colon carcinogenesis (*152, 153*). Other clinical trial studies showed that other HDAC inhibiters such as TSA and sub-eroylanilide hydroxamic acid are effective drugs in arresting cancer cell proliferation and their use results in cell differentiation or apoptosis (*154*). Therefore by regulating gene expression, cancer may be prevented or cured so that research on HDACis can be considered in the challenge of preventing and treating cancer (*7*).

Fig. 6. Chemical structure of butyrate.

2.0 RESEARCH AIMS AND HYPOTHESIS

Hypothesis

-TSA, butyrate and VPA are competitive inhibitors.

-These HDACis cause the dissociation of HDAC1/2 from different HDAC associated complexes

-Phosphorylation of HDAC1 occurs in the absence of HDAC2

Aims

1- Describe the enzyme kinetics of HDACs in MCF-7 cell lysate in the presence of different inhibitors

2- Characterize the effects of different inhibitors on integrity of HDAC complexes in MCF-7 cell lysate

3- Define the effects of different inhibitors on integrity of HDAC complexes in MCF-7 cells

4- Define the effect of TSA on HDAC2 phosphorylation

5- Examine whether phosphorylation of HDAC1 occurs in the absence of HDAC2 as a default for CK2

6- Re-evaluate HDAC1 phosphorylation in Jurkat cell line

3.0 MATERIALS AND METHODS

3.1 Reagents

Swim's S-77 medium was purchased from Sigma. [^{3}H] acetic acid sodium salt (4.1 Ci/mmol) was from PerkinElmer. Protease inhibitor cocktail was from Roche, and protein A-sepharose was obtained from Pierce. Polyclonal antibodies anti-HDAC1 and anti-Sin3A were from Affinity BioReagents. Anti-Sp1 was from Upstate, and monoclonal anti-HDAC2 was purchased from Sigma. RbAp48 antibody was from Gene Tex.

3.2 Cell culture

Human breast cancer cell lines MCF-7 (T5) [estrogen-receptor (ER) positive and estrogen dependent] were cultured in Dulbecco's modified Eagle's medium (DMEM) from GIBCO supplemented with 5% fetal bovine serum (GIBCO), antibiotics (100U/ml penicillin G sodium, 100mg/ml streptomycin sulfate, 0.25mg/ml amphotericin B) from GIBCO, 2mM L-glutamine (GIBCO) and 5% glucose. Wild type and null for p53 HCT116 human colon tumor cells were a kind gift from Dr. Bert Vogelstein (John Hopkins University). These were cultured in modified McCoy's 5A medium containing L-glutamine from Invitrogen. Jurkat cell lines were grown in RPMI 1640 medium with 5% fetal bovine serum (GIBCO), antibiotics (100U/ml penicillin G sodium, 100mg/ml streptomycin sulfate, 0.25mg/ml amphotericin B) from GIBCO in a 37°C humidified incubator with 5% CO_2.

3.3 Isolation of avian immature erythrocytes

Isolation of avian immature erythrocytes was done as described previously (*155*). Briefly, adult white Leghorn chickens (3 months old) were made anemic by injection of 2.5% (w/v) 1-acetyl 2-phenylhydrazine dissolved in 60% ethanol for 7 days. On the seventh day, blood was collected by severing the vein into 1 to 2 volume of ice-cold blood collection buffer (75mM NaCl, 25mM Tris-Cl pH 7.5, 25mM EDTA pH 8). The cells were then filtered and collected by centrifugation at 3500rpm (SS-34 rotor) for 10min at 4°C. The white buffy coat layer which contained white blood cells was removed by aspiration. The chicken erythrocytes were washed in blood collection buffer and Swim's S-77 medium pH 7.2 (Sigma).

3.4 Incubation of avian immature erythrocyte with radiolabeled acetate

Briefly, cells suspended in Swim's S-77 medium were incubated for 30min with 20μM cycloheximide which inhibited protein synthesis and did not significantly disturb the balance between histone acetyltransferase and histone deacetylase activity in chicken immature erythrocytes so that rates of histone acetylation and deacetylation can be determined. Then cells were labeled for 60min with [^{3}H] sodium acetate at a final concentration of 0.1mCi/ml. Cells were collected by centrifugation and washed with Swim's S-77 medium containing 10mM sodium butyrate. Following this treatment erythrocytes were collected by centrifugation. The cell pellet was then washed with Swim's medium to remove unincorporated free isotope.

3.5 Isolation of radiolabelled core histones from immature chicken erythrocytes

Approximately 20ml of labeled chicken erythrocytes was washed with 100ml reticulocyte standard buffer (RSB: 10mM Tris-HCl pH 7.5, 10mM NaCl, 3mM $MgCl_2$, 5mM sodium butyrate), 1mM PMSF and 0.25% Nonidet P-40. Cells were then homogenized five times and centrifuged at 3500rpm after which nuclei were collected. The nuclei were washed with 100ml of RSB and resuspended in 20ml RSB followed by extraction with 0.4N sulfuric acid. The acid extracted supernatant was collected following centrifugation at 10000rpm Sorvall™ SS-34 rotor (16900 X g) for 20min and then dialyzed against 4 Liters of 0.1M acetic acid for 2h followed by deionized distilled water with one change of water overnight. The dialysate was then lyophilized and the radiolabeled histones resuspended in deionized distilled water. Histone concentration was measured by TCA precipitation protein assay. The specific activity was 2,700 cpm/μg histone.

3.6 Preparation of histone deacetylase

MCF-7 (T5) cells were lysed in lysis buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 50mM NaF, 0.5% Nonidet P-40, 1mM EDTA) containing 1mM PMSF, phosphatase inhibitors (25mM β-glycerophosphate, 10mM sodium fluoride, 1mM sodium orthovanadate) and 1x protease inhibitor cocktail (Roche). The cells were sonicated twice for 15s at 30% output (Sonifer cell disrupter). The supernatant (cell lysate) was collected by centrifugation at 10000X g for 10min at 4°C. Protein

concentration of an enzyme source (MCF-7 cell lysate) for histone deacetylase assay was determined using the Spectra Max 190 (Molecular Devices).

3.7 Histone deacetylase assays

Radiolabeled acetylated histone substrates were used at different concentrations. The test sample was incubated in a 1.5ml tube in a final volume of 0.3ml, 25mM sodium phosphate buffer pH 7, [^{3}H] acetate-labeled histones, and the optimum amount of fresh cell lysate (150μg) which was prepared at the same day. For each sample, a control reaction identical to the test reaction was boiled for 5min to destroy the enzymatic activity before initiating the reaction. All other samples were not boiled. The reaction was incubated for 1h at 37 °C with shaking and then was stopped by adding 30μl of 0.12N/0.72 N glacial acetic acid /HCl mixture. The released radioactive acetate was extracted by adding 0.6ml ethyl acetate with mixing. After centrifugation at 13,000rpm for 1min in a microcentrifuge, 0.3ml of the upper phase was transferred into a scintillation vial containing 5ml scintillation liquid. The amount of [^{3}H] acetate released into the upper phase was determined using scintillation counting. To determine inhibition of HDAC activity, the cell lysate was incubated with 2nM or 10nM TSA, 0.5mM VPA or 10μM or 75μM butyrate for 60min at 37°C with shaking prior to the addition of histones. Lineweaver-Burk graphs were derived from each set of raw data using the linear regression equation provided by the Microsoft Office Excel software.

3.8 Immunoprecipitation and immunoblotting

MCF-7 cells lysates were obtained as described above. Cell lysates (500µg total protein in 1ml final volume) were precleared by incubation with 50µl of 50% protein A-sepharose slurry for 2h at 4°C. Samples subjected to HDAC activity inhibition were incubated with 300nM TSA for 15min on ice prior to immunoprecipitation. Samples were incubated with 2µg of polyclonal antibody specific for HDAC1, mSin3a (Affinity Bioreagents), Sp1 (Upstate) or RbAP48 (GeneTex) overnight at 4°C. A negative control was included in which no antibody was added. Immune complexes were recovered by incubation with 20-30µl of a 50% protein-A-sepharose slurry for 4h at 4°C and washing 5 times with IP buffer. Immunoblotting analysis of bound and unbound fractions was carried out as described previously (*156*). Equal volumes of cell lysate and HDAC1 immunodepleted fractions were used to perform HDAC activity assay.

3.9 Immunoblot analysis

For western blot analysis, the beads were washed five times with 500µl of IP buffer, diluted with 2x SDS buffer, separated by 10% (SDS-PAGE) and transferred onto a nitrocellulose membrane. The total cell lysate and immunodepleted fraction were loaded in the same volume. The gel was analyzed by immunoblotting with antibodies specific for C-terminal residues of polyclonal HDAC1 (Affinity Bioreagents), monoclonal HDAC2 (Sigma), mSin3a (Affinity Bioreagents) and Sp1 (Upstate). A standard curve was generated by running increasing volumes of a known concentration of MCF-7 cell lysate on an SDS-

PAGE gel for 8h at 100-120V and immunoblotting with anti-HDAC1 and HDAC2 antibodies. All blots were imaged by Alpha Innotech Image Station. The net intensity of HDAC1 and HDAC2 bands, as determined using 1D Image Analysis Software (Fluorchem 9900), was plotted against the volume loaded to obtain the best trendline (Microsoft Excel). The intensity of the HDAC1 and HDAC2 bands in total cellular and immunodepleted fractions were then used with the equation for the trendline to determine the relative amounts of HDAC1 and HDAC2 in those fractions.

3.10 Gel exclusion chromatography and immunodotblot

Proteins were separated by gel filtration chromatography using the FPLC (fast protein liquid chromatography) system (Amersham Biosciences) equipped with Tricorn™ High Performance columns (Superdex 200 10/300 GL from Amersham Biosciences). A cell extract from untreated or TSA treated MCF-7(T5) cells, equivalent to 6×10^6 cells, was concentrated using a Biomax centrifugal filter with a 10,000 molecular weight cut-off . The column was extensively washed and pre-equilibrated with column buffer (50mM phosphate buffer, 0.15M NaCl, pH 7) before loading the concentrated cell extract. Proteins were eluted using column buffer at a flow rate of 50µl/min, and 50µl fractions collected. The Superdex 200 10/300 GL column has an exclusion limit of 1.3×10^6. Collected fractions were analyzed by immunodotblotting with specific antibodies to HDAC1.

3.11 Cisplatin and formaldehyde DNA cross-linking

MCF-7 cells at a density of 1×10^7cells/ml were resuspended in Hank's buffer containing sodium acetate instead of NaCl at the same concentration and 1mM PMSF, and were incubated with 1mM cisplatin at 37°C for 2h or with 1% formaldehyde at room temperature for 10min with gentle shaking. The cells were resuspended in cross-linking lysis buffer (5M urea, 2M guanidine hydrochloride, 2M NaCl, and 0.2M potassium phosphate buffer, pH 7.5), phosphatase inhibitors (25mM glycerophosphate, 10mM sodium fluoride, 1mM sodium orthovanadate) and protease inhibitor mixture plus 1mM PMSF (Roche). The lysate was incubated with prehydrated hydroxylapatite (4g/20 A_{260} (absorbance at 260 nm) units of lysate; Bio-Rad). The hydroxylapatite resin was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA. DNA-protein cross-links were reversed by incubating them in lysis buffer containing 1M thiourea instead of 5M urea for cisplatin or 65°C for formaldehyde overnight. By doing so, the proteins were released from hydroxylapatite, while the DNA remained bound. The released proteins were dialyzed overnight against double-distilled water and lyophilized. The lyophilized protein preparation was resuspended in 8M urea. Protein concentrations were determined using the Bio-Rad protein assay, as described above. Formaldehyde cross-linked samples were run on a one-dimensional SDS-PAGE gels at 100V for 8h for the purpose of maximum separation.Cisplatin cross-linked samples were run first on one and then two-dimentional gels.

3.12 2D gel electrophoresis with immobilized pH gradients (IPG) strips

Isoelectric focusing (IEF) on immobilized pH gradient (IPG) gels was used for separating complex mixtures of proteins based on their net charge using the technique providing the first dimension of the 2D separation. First dimension of isoelectric focusing is done on a pH 3-10 non-linear using Bio-Rad Ready Prep Strip™ IPG strip from 2-D starter kit and second dimension is done with 10% SDS-PAGE gels.

First, proteins were prepared by isolation from chromatin, using cisplatin cross-linking method as described above. Then 20-50μg protein was mixed with 125μl rehydration buffer (9.8M Urea, 4% CHAPS, 100mM DTT, 0.2% (w/v) Bio-Lytes, 0.001% Bromophenol Blue) and the total solution was added to the IEF focusing tray channels from Bio-Rad.

Second, 7cm Ready Strip pH 3-10 IPG strip gels were applied side down into the tray channels and electrode wicks were placed at both ends of the IPG strips. Each strip was covered with mineral oil to prevent evaporation. The lid was closed and run at 50V at 20°C for 12h until the rehydration was complete.

Third, IEF of proteins was performed using a three-step protocol and the PROTEAN® IEF cell (Bio-Rad) at a temperature of 20°C. The IPG strips were initially conditioned for 20min at 250V (rapid voltage ramping), linearly ramped to 4000V for 2.5h, and focused at 10,000Volt-hours. Focused strips were stored in rehydration/equilibration trays at -80°C until second-dimension separation could be performed.

For second-dimension separation, IPG strips were first equilibrated for 20min in equilibration buffer I (6M urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol) containing 2% DTT and then for 10min in equilibration buffer II containing 2.5% iodoacetamide instead of DTT in buffer I at room temperature. Strips were then transferred down into the 10% Tris-HCl SDS-PAGE gels with the addition of 1% agarose gel on the top of the gel. Electrophoresis of the gel was done first at 50V for 100min to ensure that all the samples entered the phase in the second dimension and then continued at 125V at 4°C overnight in the PROTEAN® IEF Cell (Bio-Rad). At the end the gels were transferred to determine different modification forms of HDAC1 and HDAC2 (phosphorylation) by western blotting.

3.13 Protein phosphatase digestion

50µg Jurkat cell lysate was incubated with and without 20 units of calf intestinal alkaline phosphatase from Amersham Bioscience. The reaction tube was incubated at 30°C and 37°C for 1h in 1X reaction buffer (50mM Tris-HCl, pH 9, 1mM $MgCl_2$).These temperatures previously in the lab were evaluated as the best temperature for alkaline phosphatase reaction. The reaction was terminated upon the addition of SDS sample buffer. The sample was boiled for 5min and separated on 10% SDS-PAGE gels for 8h. After electrophoresis, the gels were transferred to the nitrocellulose membrane for western blotting with HDAC1 antibody.

3.14 Isolation of nuclear matrices

Nuclear matrices (NM) were isolated from MCF-7 cells as described previously (*157*). Briefly, cells were extracted with Triton X-100 to release lipids and soluble proteins. Nuclei were collected, resuspended in DIG buffer (50mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 10mM Tris-HCl, pH 8.0, 1% thiodiglycol) containing Triton X-100, and digested with DNase I. The pellet was washed with ammonium sulfate at a final concentration of 0.25M and then with NaCl at a 2M final concentration to facilitate the removal of chromatin. The resulting pellet contains the nuclear matrix (*108*).

3.15 Nuclear matrix immunofluorescence staining

MCF-7 cells were extracted on poly-l-lysine coated coverslips, using an established procedure (*158*). Briefly, soluble proteins were removed by a 5min incubation in ice-cold CSK buffer (100mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 10mM PIPES pH 6.8) containing 0.5% Triton X-100. The remaining nuclear structure was then cross-linked with 3.7% formaldehyde in CSK buffer for 30min on ice. Chromatin was released by digestion with 0.5mg/ml DNase I (Sigma) for 50min at 32°C and two 5min washes at room temperature. The first wash was done with ammonium sulfate at a final concentration of 0.25M, and the second wash was done with NaCl at a final concentration of 2M. The resulting nuclear matrix preparations were then analyzed by immunofluorescence as described above (*108*).

3.16 Fluorescence microscopy and deconvolution analysis

Fluorescence images were captured on an AxioPhot II microscope (Carl Zeiss, Thornwood, NY) with an AxioHRm camera. The stack images of 100 optical sections with a step size of 200nm were then deconvolved in three dimensions with the AxioVision 4.1 constrained Iterative Algorithm (Carl Zeiss).

4.0 RESULTS

We established the most optimum ranges of enzyme digestion time, enzyme concentration, and substrate concentration for our assay. These were done by keeping two of these parameters constant while testing a range of the third parameter. For example, optimum digestion time was found by using a fixed enzyme concentration and a fixed substrate concentration while changing digestion time. All of these were expressed on graphs as velocity against the parameter being tested (that is, V vs. Time; V vs. Enzyme Concentration; V vs. Substrate Concentration). Enzyme velocity (V) measured 3H-acetyl group released from histone over a set period of time (expressed as mCi per minute). Vmax is the maximal velocity achieved at high substrate concentrations. Vmax (and V) are expressed in units of product formed per time.

4.2 Determination of optimum enzyme concentration

MCF-7 (T5) cells, used as an enzyme source (HDACs) for HDAC assay were lysed in lysis buffer followed by sonication twice for 15s. The cell lysis included sonication to ensure a complete extraction of the proteins under study, including proteins bound to the nuclear matrix. The supernatant (cell lysate) was collected by centrifugation at 10000 *g* for 10min at 4°C. Protein concentration was determined using the Spectra Max 190. We applied different enzyme concentration from 10-210μg while we kept time and labelled substrate which contained all four histone cores constant. The best enzyme concentration is the

lowest required reaching Vmax. In our study, 150μg was the best enzyme concentration (Fig. 7).

4.1 Determination of optimum time for HDAC assay

To establish the best incubation time for our assay, the level of free 3H-acetyl group that had been removed from the histone in different time periods from 15 to 120min were measured. So with the constant enzyme concentration, different time points were applied. The best digestion time is the shortest time required to give the Vmax. In our assay 60min was the appropriate time (Fig. 7).

For the time experiments, if the assay was done over a long period of time, the value would likely decrease. This can be due to depletion of substrate, deterioration of enzyme activity, feedback inhibition by the presence of an excess of product, etc. So when establishing an optimum assay time, a sharp increase in velocity with time (while the enzyme adapted to environment) was seen, then the value increased gradually (enzyme was working at or near Vmax), and then there was a drop in velocity. Where this drop occurred was the longest possible time that the assay could be conducted. After that the value reached a plateau.

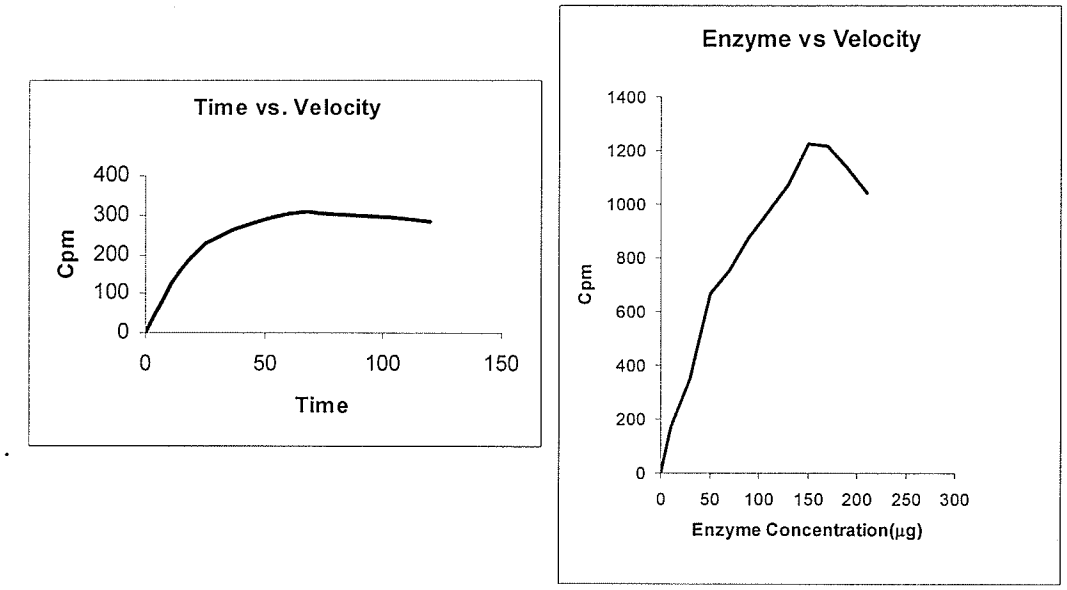


Fig. 7. Optimizing appropriate time and enzyme concentration for HDAC assays. Left, the reaction was incubated across a range of time. One hour was the optimum time in all HDAC assays. Right, the reaction was incubated with different enzyme concentrations (T5 cellular extract) while the other parameter stayed constant. 150µg was the optimum enzyme concentration.

4.3 Determination of appropriate substrate concentration

The substrate [S] graph, if plotted as cpm vs [S], is a Michaelis-Menton type plot. This data could also be expressed as Lineweaver-Burk. This allowed calculation

of Vmax and Km in the absence of any inhibitor (ie. establish positive control). Based on our positive control, we can obtain characteristics of the enzyme under particular conditions of time, enzyme [E] and range of [S]. When assaying the enzyme in the presence of inhibitors, we conducted all our analyses with the same time, [E] and range of [S].

So for setting optimum substrate concentration, first different histone concentrations from 0 to 40 with 150µg enzyme were incubated for 1hour. The best substrate concentrations would range between the values needed to give ~half Vmax and the lowest value needed to give Vmax. K_m is defined as the concentration at which the rate of the enzyme reaction is at half $V_{max.}$

The rationale here is that when expressing data in a Lineweaver-Burk (LB) (1/V vs. 1/S) we are relying on the data giving us a linear regression. Nonlinear curves such as Michaelis-Menten equation make it difficult to estimate K_m and V_{max} accurately. Therefore, several researchers developed linearization of the Michaelis-Menten equation, such as the Lineweaver-Burk plot and the Eadie-Hofstee diagram. Therefore linear regression line was used to determine values for Vmax and Km. Unfortunately, this may not necessarily be the case when working with very high substrate concentaration (because all these values will tend to have an associated V very close to Vmax) or with a very low substrate concentration (because these values will tend to have the most error of all the points analyze of). They should also be high enough to give you readings that are strong enough to make error due to the measuring equipment relatively small, since when deriving Ki, error should be as low as possible

relative to signal. So from 0 to 30μg substrate concentration was plotted to obtain information regarding the optimum concentrations to be used. The below graphs are the resulting graphs (Fig. 8).

The most linear LB graph was the one that was generated when using substrate concentrations around the Km. As such, we used 7, 9, 11, 13 and 15μg histones which ranged from some value lower than Km to some value higher than Km in our standard graphs.

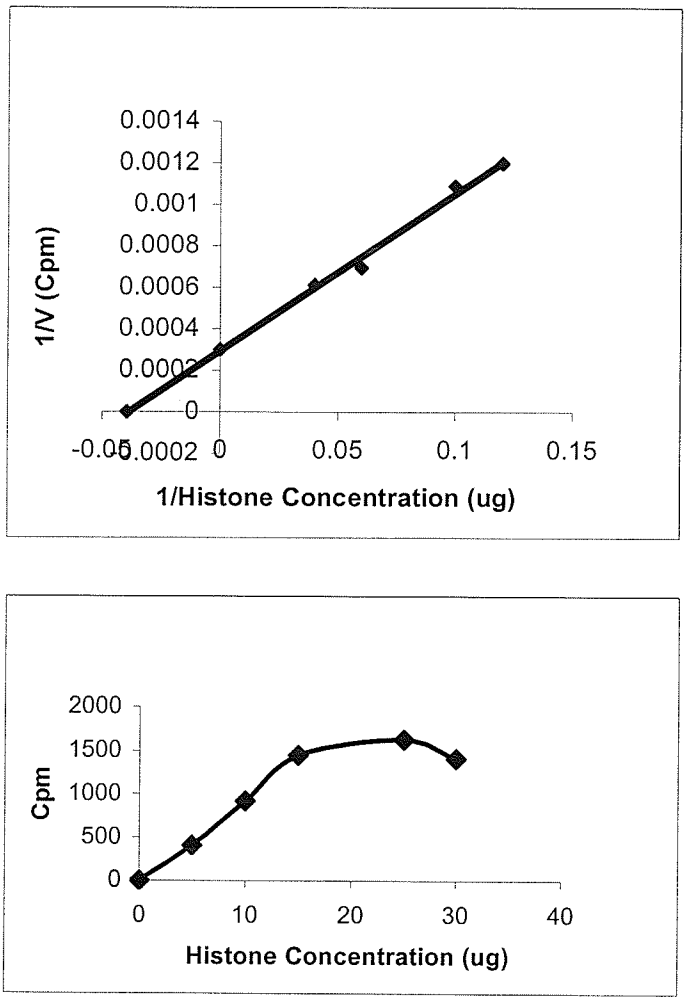


Fig. 8. Optimizing appropriate substrate concentrations. Top, Lineweaver-Burk graphs plotted as 1/cpm vs [1/S] Below, Michaelis-Menton, plotted as cpm vs [S], is a Michaelis-Menton type plot: we incubated different histone concentration from 0 to 40 with 150μg enzyme for 1h. The best substrate concentrations would be in the linearity range of Lineweaver-Burk graph around Km.

4.4 Determination of appropriate inhibitor concentration

Optimum conditions (time, enzyme concentration, and substrate concentration) were established and 5-10 inhibitor concentrations were tested each with 10-20 substrate concentrations. The rationale here was that enough datasets (inhibitor concentrations) and data points (substrate concentrations) were created to provide confidence in data analyses. Note that each combination of inhibitor and substrate concentration was tested at least in triplicate, and the experiment was replicated at least in triplicate as well. The number of inhibitor concentrations was reduced to a maximum of 3, and the number of substrate concentrations to a minimum of 5.

So we have done 2*5*3*3=90 individual assays for butyrate or VPA and 3*5*3*3=135 assays for TSA.

4.5 Kinetic analysis of HDAC activity inhibition

According to the crystal structure of TSA bound to a HDAC1 homologue and docking studies of TSA to HDAC 1, 2, 3 and 8 models, TSA would be expected to act as a competitive inhibitor since this HDACi occupied the catalytic site of the enzymes (*12, 159*). However, this hypothesis is not supported by the published kinetic data reporting that the inhibition of HDAC by TSA was non-competitive (*12*), and is also challenged by a structural study of HDAC8-TSA complex crystals showing that HDAC8 can bind two TSA molecules, one in the catalytic site and another in a cavity nearby (*160*). Thus, we set out to reevaluate the kinetic characteristics of HDAC inhibition by TSA.

Class I HDACs are associated with the nuclear matrix, consequently these HDAC proteins resist extraction from cells or nuclei by detergent containing buffers (*161*). To maximize the extraction of HDAC from cells and maintain the integrity of the HDAC multiprotein complexes, cells were sonicated in a low stringency buffer (*97*). We determined that sonication of cells was effective in solubilizing 98% of the HDACs (data not shown). Furthermore, all HDAC activity assays were performed using freshly (same day) prepared MCF-7 cell lysates, in order to avoid degradation of HDAC complexes, as it is known that HDAC activity is modulated by other protein interactions (*162*). Before analyzing the nature of the inhibition exerted on HDAC activity, the assay was optimized by testing a range of different values for each parameter (time of incubation, amount of MCF-7 cell extract and amount of substrate). Figure 9A shows that the MCF-7 cell lysate HDAC activity was inhibited by TSA with an IC_{50} value of 8nM, falling within the range of published values (*163-166*). A kinetic analysis of the TSA inhibition of HDAC activity was then performed and is shown as a Lineweaver-Burk plot (1/V versus 1/S) in Figure 9B. The y intercept did not vary with increasing TSA concentrations, illustrating that TSA did not affect V_{max} thus behaving as a competitive inhibitor. The TSA binding constant, Ki, was calculated to be 1.0 nM. The results of competitive binding experiments between the two HDACis, TSA and VPA, suggested that TSA and VPA were bound to the same site or overlapping HDAC sites (*142*). Hence, it was assumed that the mode of action of VPA was also to block the substrate access to the catalytic site of HDAC enzymes. To test this assumption, we analyzed the kinetics of MCF-7 cell lysate

HDAC activity inhibition by VPA, as described above. The IC_{50} value for inhibition by VPA was 0.35mM (Fig. 10A), comparable to previously reported values (*133, 142, 145*). In the Lineweaver-Burk plot of Figure 10B, the y intercept or V_{max} was not affected by the presence of 0.5mM VPA, indicating that VPA indeed acted as a competitive inhibitor. The Ki value for VPA was determined to be 0.31mM.

A detailed kinetic study of another HDACi, butyrate, led to the conclusion that butyrate was a non-competitive inhibitor as it had no effect on the enzymatic reaction's apparent K_m, but decreased V_{max} (*150*). Our butyrate inhibition study is shown in Figure 11A with an IC_{50} value of 101μM, similar to published values (*163, 164*) and in Figure 11B as a Lineweaver-Burk plot. In contradiction with published results, our results demonstrate competitive inhibition, as V_{max} was not altered in the presence of 10μM or 75μM butyrate.The calculated Ki value was 52 μM for butyrate.

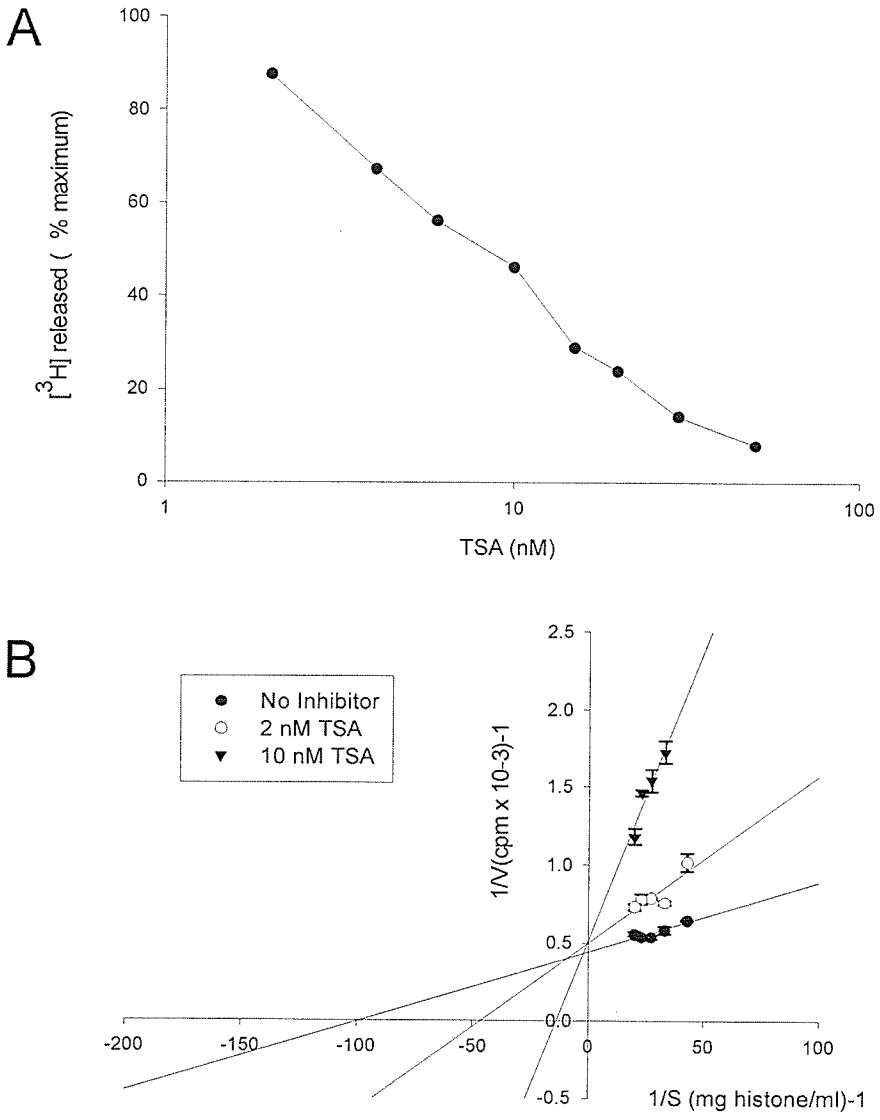


Fig. 9. Kinetic analysis of MCF-7 HDAC inhibition by TSA. (A) MCF-7 HDAC activity was determined in the presence of increasing concentrations of TSA. Results are presented as presences of 0 (●), 2 (○) or 10 (▼) nM TSA, is presented as a Lineweaver-Burk plot.

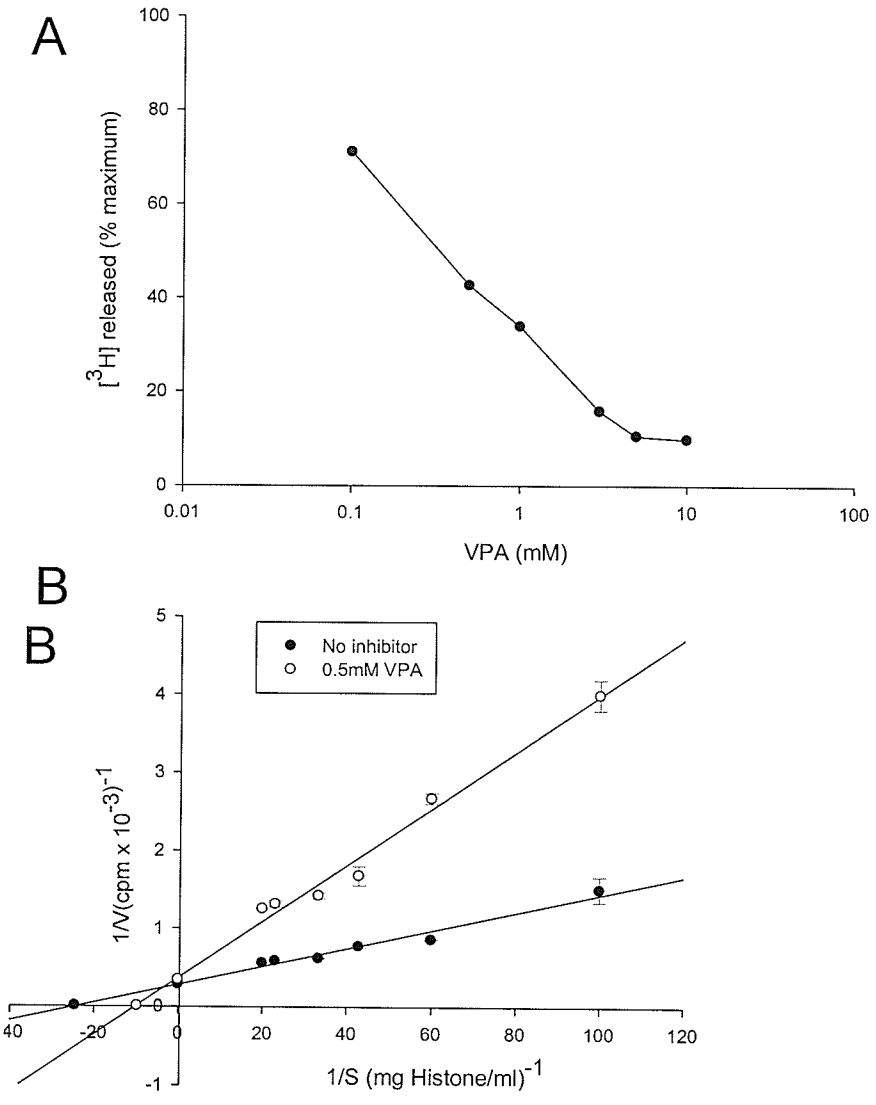


Fig. 10. Kinetic analysis of MCF-7 HDAC inhibition by VPA. (A) MCF-7 HDAC activity was determined in the presence of increasing concentrations of VPA. Results are presented as percentages of the activity measured in the absence of inhibitor. (B) MCF-7 HDAC activity ([^{3}H] acetate released from purified labeled histones), in the presence of 0 (●) or 0.5 (○) mM VPA, is presented as a Lineweaver-Burk plot.

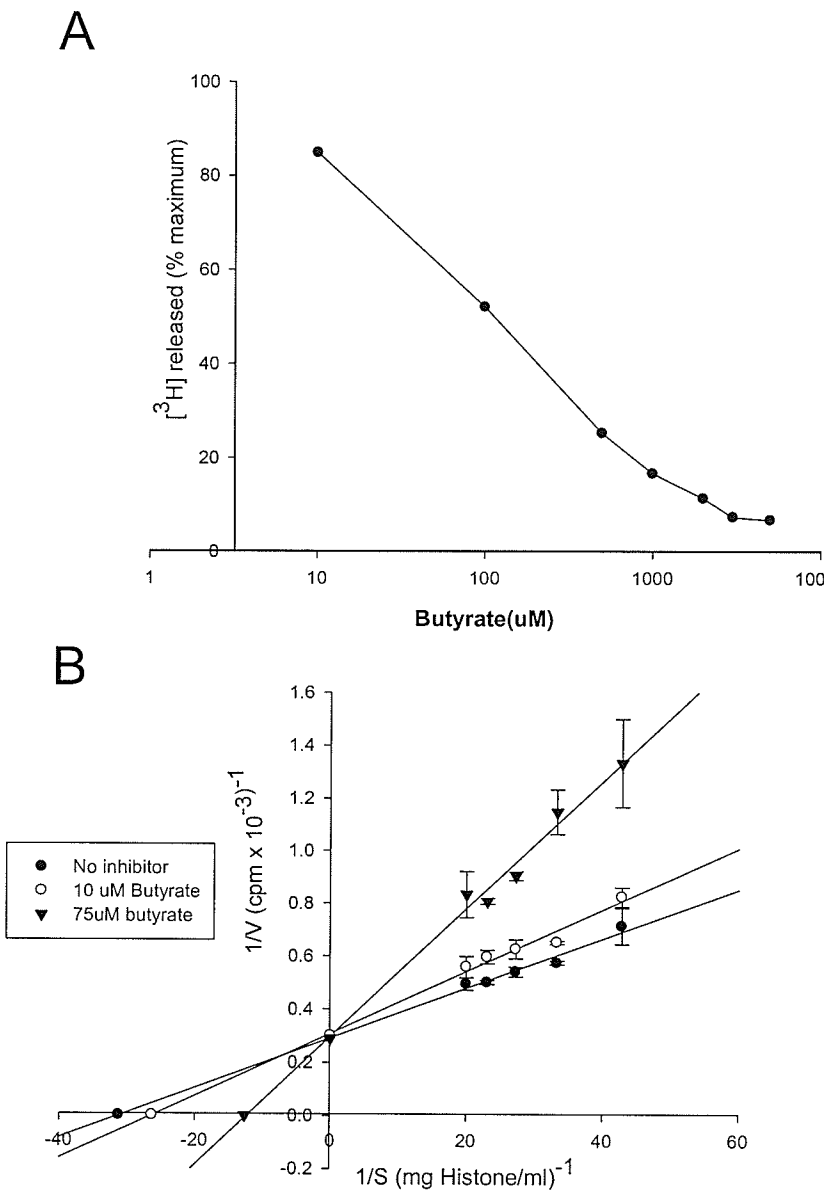


Fig. 11. Kinetic analysis of MCF-7 HDAC inhibition by butyrate. (A) MCF-7 HDAC activity was determined in the presence of increasing concentrations of butyrate. Results are presented as percentages of the activity measured in the absence of inhibitor. (B) MCF-7 HDAC activity ([^{3}H] acetate released from purified labeled histones), in the presence of 0 (●), 10 (○) or 75 (▼) μM sodium butyrate, is presented as a Lineweaver-Burk plot.

4.6 Effect of TSA inhibition on the interaction between HDAC1 and HDAC2

Not only are HDAC1 and HDAC2 core components of multi-protein complexes such as Sin3, NuRD or Co-Rest complexes (*4*), they also display an extensive level of association throughout the nucleus. The degree of HDAC1/HDAC2 interaction was previously demonstrated by Co-IP data as well as immunofluorescence microscopy studies showing that the two HDACs are co-localized in the nucleus of MCF-7 cells (*108*). Histone deacetylase activity before and after HDAC1 and associated HDAC2 removal was measured in a sonicated MCF-7 cell lysate prepared as described in Materials and Methods. Since our HDAC assay was performed by total cell lysate, HDAC activity in the total cell lysate and in the HDAC1 Immunodepled (ID) fraction was measured. This experiment showed that our total cell lysate has most of the HDAC activity and it is suitable for performing HDAC assay. Figure 12 shows that the HDAC activity in the cellular extract was reduced to about 48% of the initial activity, providing evidence that slightly greater than half of the HDAC activity in the cellular extract was due to HDAC1 and associated proteins (e.g. HDAC2).

We studied the effect of TSA on complex integrity. The MCF-7 cell lysate, with or without TSA added, was incubated with anti-HDAC1 antibodies under low stringency conditions. Figure 13 shows that all of the HDAC1 was immunoprecipitated by the antibodies with or without TSA. Most of the HDAC2 (80.8 ± 3.7%, n=2) was co-immunoprecipitated by anti-HDAC1 antibodies in the absence of TSA, confirming our previous results. A similar amount of HDAC2

(80.0 ± 11.5%, n=2) was co-immunoprecipitated in the presence of TSA, indicating that HDAC activity inhibition by TSA did not affect the extent of interaction between HDAC1 and HDAC2.

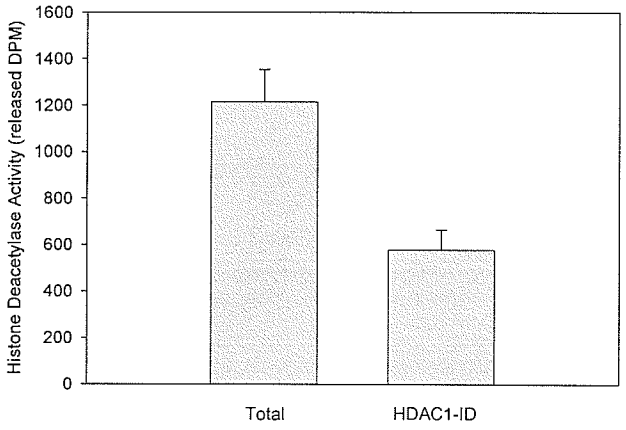


Fig. 12. Determination of HDAC activity in MCF-7 HDAC1-immunodepleted fraction. HDAC activity ([^{3}H] acetate released from purified labeled histones) was measured in total and HDAC1-immunodepleted (HDAC1-ID) MCF-7 cell lysate.

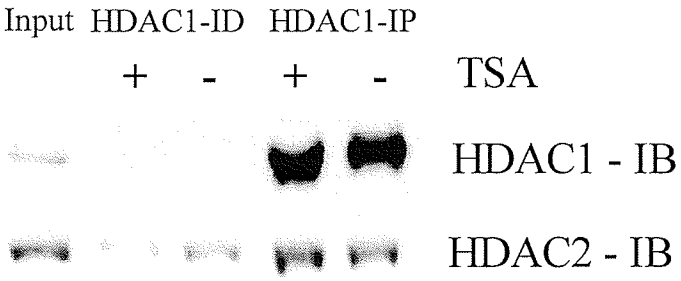


Fig. 13. Co-immunoprecipitation of HDAC1 and HDAC2 in the presence of TSA. MCF-7 cells were lysed in IP buffer containing protease and phosphatase inhibitors. Two cell lysate samples (500μg protein), with (+) or without (-) TSA, were incubated with 2μg of anti-HDAC1 antibodies. The whole immunoprecipitated (IP) fractions and equivalent volumes of lysate (input) and immunodepleted (ID) fractions, corresponding to 20μg of lysate, were loaded onto SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunochemically stained with anti-HDAC1 or anti-HDAC2 antibodies.The antibody used for the immuno-blot (IB) is indicated on the right hand side.

4.7 Effect of TSA on the integrity of HDAC1 associated complexes using gel exclusion chromatography and immunodotblotting

Most HATs and HDACs have been described in more than one macromolecular complex, reflecting the potential of these proteins to interact with different binding partners in a mutually exclusive manner. The release of the HATs and HDACs following addition of HDACi, TSA, may reflect general disruption of all complexes that contain these proteins. To evaluate whether or not TSA liberated a specific subset of proteins from the nucleus, we incubated MCF-7 (T5) cellular extract for 15min with TSA. Then we characterized the cellular extracts from both

TSA-treated and control cell lysate using gel filtration chromatography. This approach requires gel filtration standard (Bio-Rad) which is a calibration standard for size exclusion columns used in protein purification (Fig. 14). Therefore when the cytoplasmic fraction was applied to a Superdex 200 column, two peaks containing HDAC activity were observed (Fig. 15). Our aim was to investigate whether or not there are specific differences after TSA addition in the components of the complexes found in the two extracts. Hence, column fractions were analyzed by immuno-dot blotting using primary antibodies specific for HDAC1 to identify the corresponding protein that eluted from the column and was present in the gel filtration fractions. Immunodot blot results in (Fig. 16) show that HDAC1 is consistently detected in both the control and TSA-treated extracts in the first chromatogram peak eluting from the column. As a control we detected HDAC1 from the known sample and not from the fractions. Also, there was no difference in HDAC1 detection from different fractions between treated and untreated samples, suggesting that TSA did not disrupt HDAC1 associated complexes and did not have any effect on complex integrity. This confirms the previous Co-IP results for HDAC1 and HDAC2 complexes with and without TSA treatment.

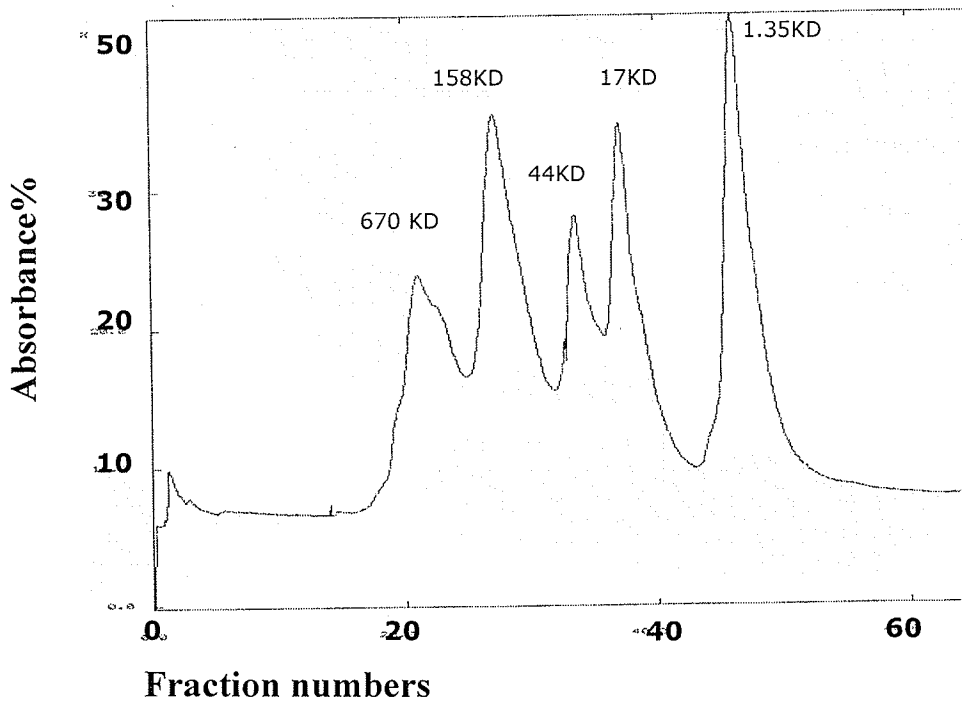


Fig. 14. Profile of gel filtration standard. The gel filtration standard is a lyophilized mixture of five molecular weight markers, ranging from 1.35 to 670kDa. The mixture includes vitamin B_{12} and myoglobin, which are visible when eluting from glass or clear plastic columns and can be used to ensure that the column is properly packed and the sample is eluting evenly.

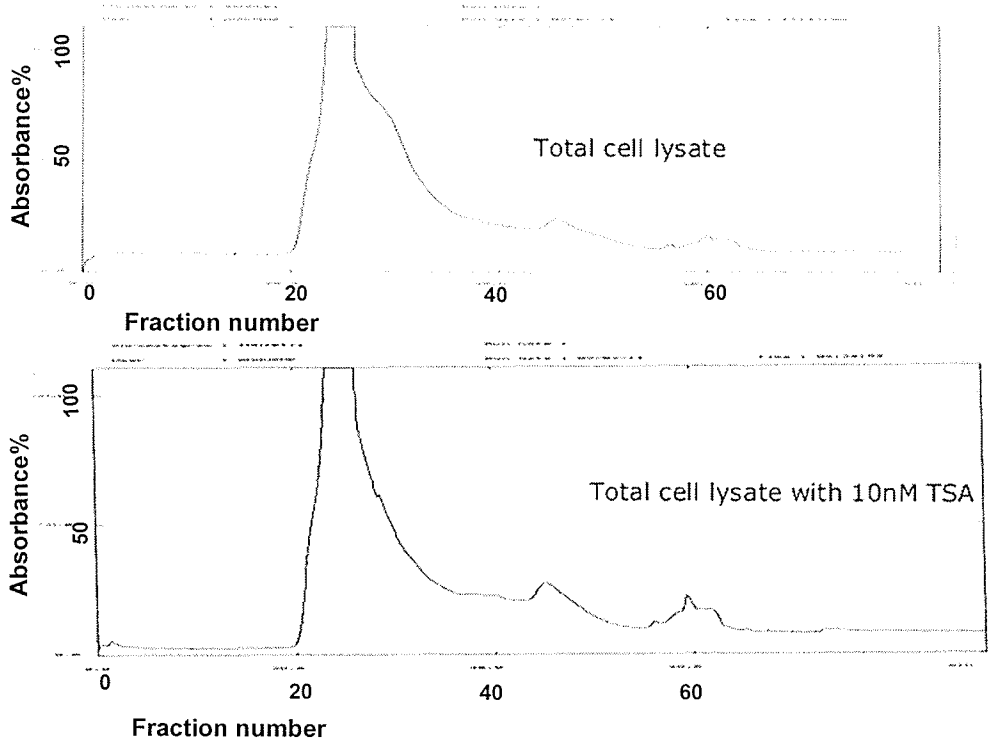
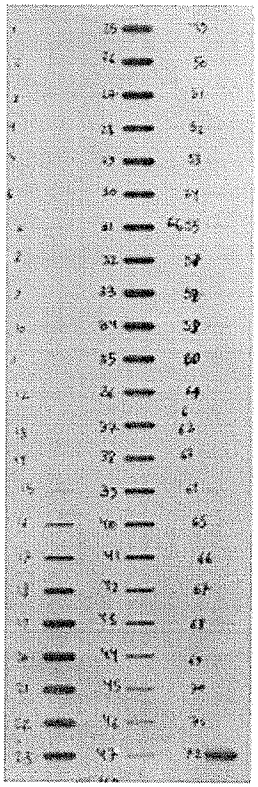
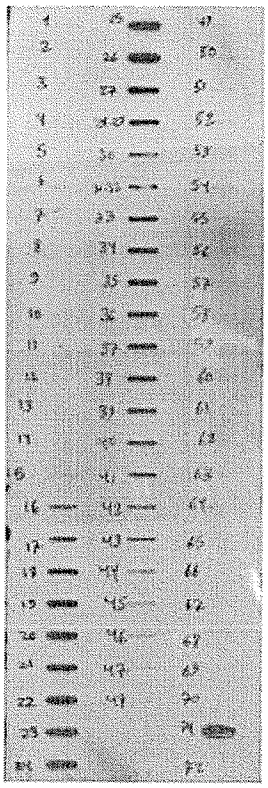


Fig. 15. Gel–exclusion chromatography of control and 10nM TSA-treated MCF-7 cellular extract. Since the sample is separated in the column, different peaks on the chromatogram correspond to different components in the sample mixture. 0.1-0.2ml cytoplasmic fractions were applied directly to Superdex 200 column (Pharmacia) chromatography with 25mM sodium phosphate/citric acid buffer. Fractions were collected and used for Immunodot-blotting.

A



Untreated



10nM TSA treated

B

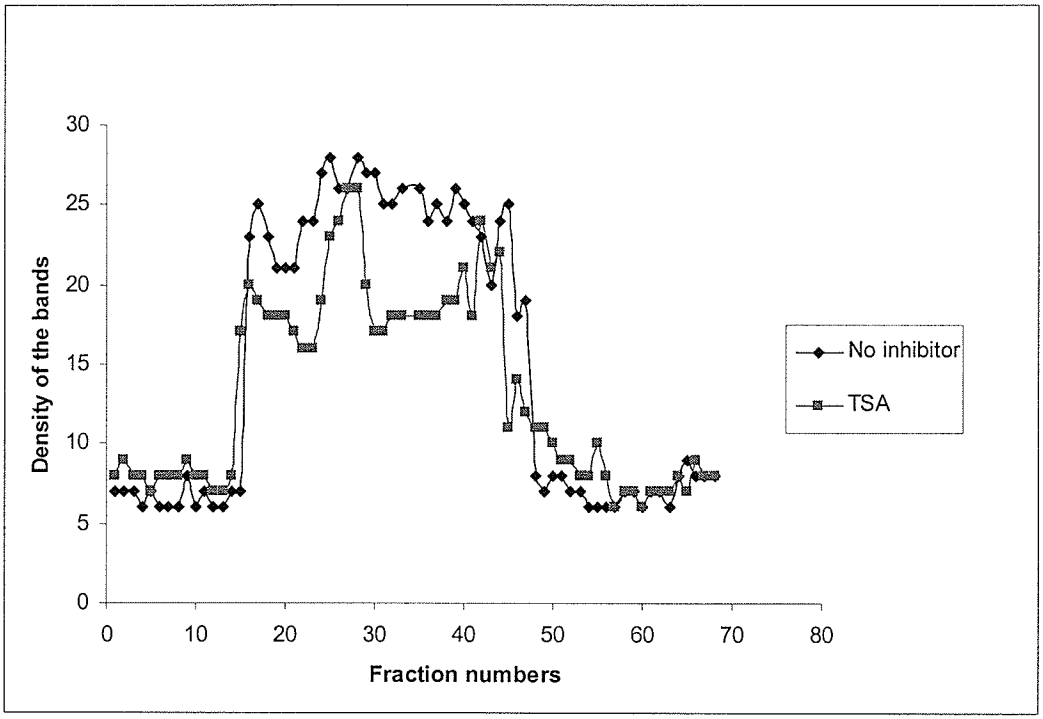


Fig. 16. Profile of fraction numbers versus dentisty of the bands corresponding to HDAC1 proteins present in immuno-dot blotting. The identification of corresponding individual HDAC1 proteins present in gel exclusion fractions from control and TSA-treated samples was done by immunodotblotting of numbered fractions with specific antibodies to HDAC1. All immunodotblots were performed in triplicate (A). density of the bands corresponding to different fraction were measured by using 1D Image Analysis Software (Fluorchem 9900) (B).

4.8 Effect of TSA inhibition on Sin3 complex formation

We next tested whether TSA would result in the dissociation of HDAC1 or -2 from the Sin3 complex. Immunoprecipitates isolated with anti-Sin3A antibodies from cellular extracts of MCF-7 cells incubated without or with TSA were analyzed by immunoblotting with anti-HDAC1 or 2 antibodies. Figure 17 illustrates that TSA had no effect on the extent of Co-IP of HDAC1 or HDAC2 by anti-Sin3A antibodies. Quantification of the immunoblot signals in immunodepleted fractions and input samples established that in the absence of TSA, 12.2 ± 3.5% of the HDAC1 population was immunoprecipitated by anti-Sin3A antibodies, compared to 9.8 ± 3.7% in the presence of TSA. Similarly, 12.2 ± 3.1% and 10.7 ± 4.4% of the HDAC2 population was found associated with Sin3A without and with TSA, respectively. These results are the average of three independent experiments.

4.9 Effect of TSA inhibition on HDAC1 and HDAC2 interaction with transcription factor Sp1

Several chromatin immunoprecipitation studies have found that TSA treatment of cells promoted dissociation of HDAC1 and HDAC2 from promoters (*98, 167*). HDAC1 and HDAC2 complexes are recruited to regulatory regions through the interaction of HDAC1 and HDAC2 with transcription factors (*4*). One such transcription factor is Sp1 (*168, 169*) Hence, the levels of HDAC1 and HDAC2 co-immunoprecipitated by anti-Sp1 antibodies from MCF-7 cell lysates without and with TSA were compared. The results, presented in Figure 18, indicate no

change in the amounts of HDAC2 associated with Sp1 when TSA was added. Without TSA, 12.1 ± 0.6% of HDAC2 were co-immunoprecipitated with anti-Sp1 antibodies, compared to 15.4 ± 2.0% HDAC2 with TSA. Similar results were obtained for HDAC1 (not shown), with 10.5 ± 3.2% and 14.6 ± 0.4% precipitated without and with TSA, respectively. These results are the average of three independent experiments.

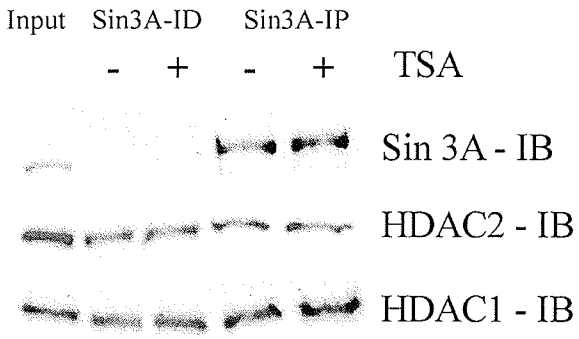


Fig. 17. TSA had no effect on HDAC1 and HDAC2 association with Sin3A. MCF-7 cells were lysed in IP buffer containing protease and phosphatase inhibitors. Two cell lysate samples (500μg protein), with (+) or without (-) TSA, were incubated with 2 μg of anti-Sin3A antibodies. The whole immunoprecipitated (IP) fractions and equivalent volumes of lysate (input) and immunodepleted (ID) fractions, corresponding to 20μg of lysate, were loaded onto SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblot immunochemically stained with anti-Sin3A, anti-HDAC1 or anti-HDAC2 antibodies.The antibody used for the immuno-blot (IB) is indicated on the right hand site of the figure.

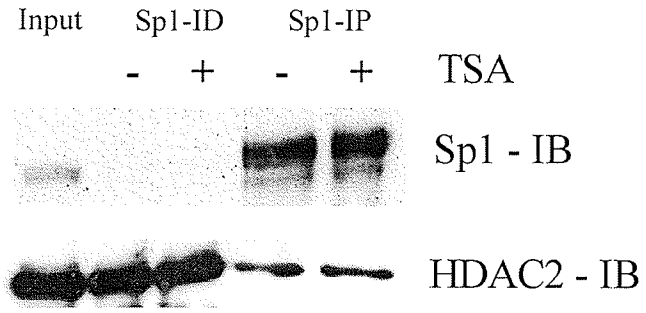


Fig. 18. TSA had no effect on HDAC1 and HDAC2 interaction with transcription factor Sp1. MCF-7 cells were lysed in IP buffer containing protease and phosphatase inhibitors. Two cell lysate samples (500μg protein), with (+) or without (-) TSA, were incubated with 2μg of anti-Sp1 antibodies. The whole immunoprecipitated (IP) fractions and equivalent volumes of lysate (input) and immunodepleted (ID) fractions, corresponding to 20μg of lysate, were loaded onto SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunochemically stained with anti-Sp1, anti-HDAC1 or anti-HDAC2 antibodies.The antibody used for the immuno-blot (IB) is indicated on the right hand side of the figure.

4.10 HCT116 p53 null cells do not express HDAC2 but do express HDAC1

Schriber reported that total HDAC1 were phosphorylated by CK2 in T-Ag Jurkat lysates which contained low amount of HDAC2 by using calf intestinal phosphatase digestion assay. To examine whether phosphorylation of total

HDAC1 occurs in the absence of HDAC2 as a default for CK2, we used a unique model consisting of p53 null and wild type HCT116 cell lines. In this model HCT116/53 null cell lines contain no HDAC2 expression in contrast to HCT116/53 wild type. Therefore no endogenous expression of HDAC2 will interfere with our experiment. The null cell lines were a generous gift from Dr Burt Vogelstein and were made by homologous recombinant approach (Fig. 19).

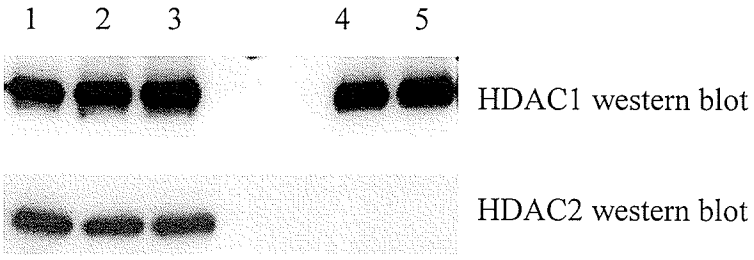


Fig. 19. Western blot analysis of HDAC1 and HDAC2 in HCT116 p53 null and wt cells. HCT116 p53 null cells contain no HDAC2 but express HDAC1. HCT116 p53 wt cells present with HDAC2 as well as HDAC1. As a control MCF-7 cell lines (T5) which has been shown previously to have HDAC1 and HDAC2 was loaded as well.Lane 1: MCF-7 (T5) cell extract. Lane1:MCF-7 cell extract, Lane 2, 3:HCT116/p53 wt cell extract. Lane 4, 5: HCT116/p53 null cell extract.

4.11 HDAC2 cross linked to DNA with formaldehyde is preferentially phosphorylated in HCT116 p53 wt cells

HDAC1 was shown to be phosphorylated by CK2(*27, 106*). The CK2 phosphorylation sites located in the C-terminal region of HDAC1 are conserved in HDAC2(*27*). In our lab it was illustrated that incubation of HDAC2 in the cell lysate with CK2 and ATP generates the slower migrating HDAC2 form in MCF-7 cells. He determined that the slower migrating form of HDAC2 was phosphorylated by incubating the proteins cross-linked to DNA with alkaline phosphatase. Therefore after incubation of the protein sample with alkaline phosphatase the slower migrating form of HDAC2 disappeared. It has been shown that chromatin is preferentially crosslinked to phosphorylated HDAC2 and not total HDAC2. This was proven by the slower migrating form of HDAC2 with the transcription factors Sp1 and Sp3, suggesting that the phosphorylated form of HDAC2 may be selectively in contact with chromatin (*97*).

Therefore to evaluate HDAC2 phosphorylation without using anti-phosphoHDAC2 antibody, MCF-7 (T5), p53 null and *wt* HCT116 cells were incubated with the cross-linker formaldehyde and the proteins cross-linked to nuclear DNA were isolated and ran on SDS-10% polyacrylamide gel for 8h (previously described in *Materia*l and *Method)*. Fig. 20shows that the slower migrating form of HDAC2 in HCT116/p53 wt is enriched in the proteins cross linked to DNA and therefore HDAC2 can get phosphorylated in this cell line as well as in T5 cells. For negative control, HCT116/p53 null cells which did not express HDAC2 were used. MCF7 (T5) cells which have been reported before to

have phosphorylated HDAC2 associated with chromatin were used as positive control. The next step would be confirming HDAC2 phosphorylation in HCT wt cells with 2D gel electrophoresis with immobilized pH gradients (IPG) strips.

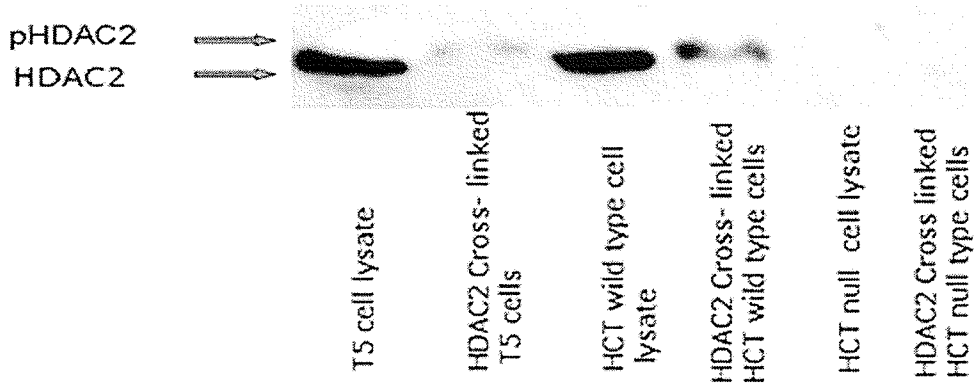
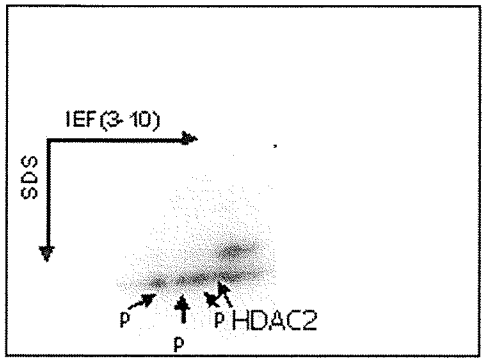


Fig. 20. Phosphorylated HDAC2 is preferentially associated with chromatin. T5 cells, and p53 null and wt HCT116 cells were incubated with 1% formaldehyde at room temperature for 10min, and proteins cross-linked to DNA were isolated. 10μg of protein was separated on a SDS-10% polyacrylamide gel at 100V for 8h, transferred onto nitrocellulose membranes, and detected with anti-HDAC2 antibody.

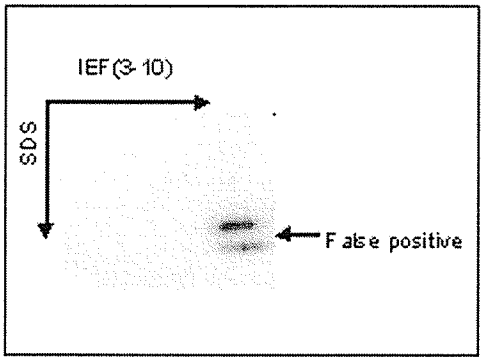
4.12 HDAC2 is phosphorylated in HCT116 p53 wt cells

To determine HDAC2 phosphorylation in HCT116 wt cells, we used 2D gel electrophoresis with Immobilized pH Gradients (IPG) strips for separating complex mixtures of proteins based on their net charge. Using this technique

provides the first dimension of the 2D separation on a pH range from 3 to10 which goes toward 3 when phosphorylation happens. Second dimension SDS-PAGE is done with 10% SDS gels. Therefore, HCT116/53 null and wt cells were incubated with the cross-linker cisplatin and proteins were prepared by isolation from chromatin as described in *Materials* and *Method*. In this experiment we had HCT116 /p53 null as negative control. Since these cells do not express any HDAC2, any signals on the gel would be considered as false positive. Consistent with the reports of Seto and Davie in T5 and Hela cells (*104*), HDAC2 can get phosphorylated in HCT 116 wt at three sites (Fig. 21).



HCT116p53 wt



HCT116p53null

Fig. 21. Two-dimensional electrophoresis and western blotting of HDAC2 phospho-forms. p53 null and wt HCT116 cells were incubated with 1mM cisplatin for 1h at 37°C and proteins bound to DNA were isolated from chromatin. 20-50µg proteins mixed with 125µl rehydration buffer were added to the IEF focusing tray channels. IPG strip gels (pH 3-10) were applied to the tray channels and were run at the optimum voltage for separating complex mixtures of proteins based on their net charge. IPG strips were then transferred down into the 10% second dimension SDS-PAGE gels. In the end the gels were transferred for HDAC2 western blotting.

4.13 Effect of TSA on HDAC2 phosphorylation in MCF-7 cells

HDAC2 typically coexists in the same protein complexes as HDAC1 such as Sin3 complex and the NuRD/NRD/Mi2 complex. It has been proven by Co-IP experiments that phosphorylation of HDAC2 changes the ability of protein to interact with other proteins. For example by using antibody against HDAC1, mSin3, and Mi2 in the cells that expressed wild-type or phosphosite mutant HDAC2 on phosphorylation sites, neither mSin3 nor Mi2 coprecipitated with the mutant HDAC2 suggesting that mSin3 and Mi2 preferentially bind phosphorylated HDAC2 (*104*).

We showed previously that HDAC2 is phosphorylated in T5 and HCT116/p53 wt cells by using 2D gel approach.

Our laboratory also showed by using dithiobis(succinimidyl)propionate (DSP) cross linkers that HDAC2 phosphorylation is important for complex formation via RbAp48 in Sin3 and NuRD complexes (data not shown). In order to examine the effect of TSA on HDAC2 phosphorylation, critical for formation of HDAC associated complexes, we did 2D gel electrophoresis with IPG strips for separating complex mixtures of proteins based on their net charge. MCF-7 (T5) cells were incubated with cisplatin following treatment with 400nM TSA for 2h. TSA did not have significant effect on HDAC2 phosphorylation MCF-7 cells (Fig. 22).

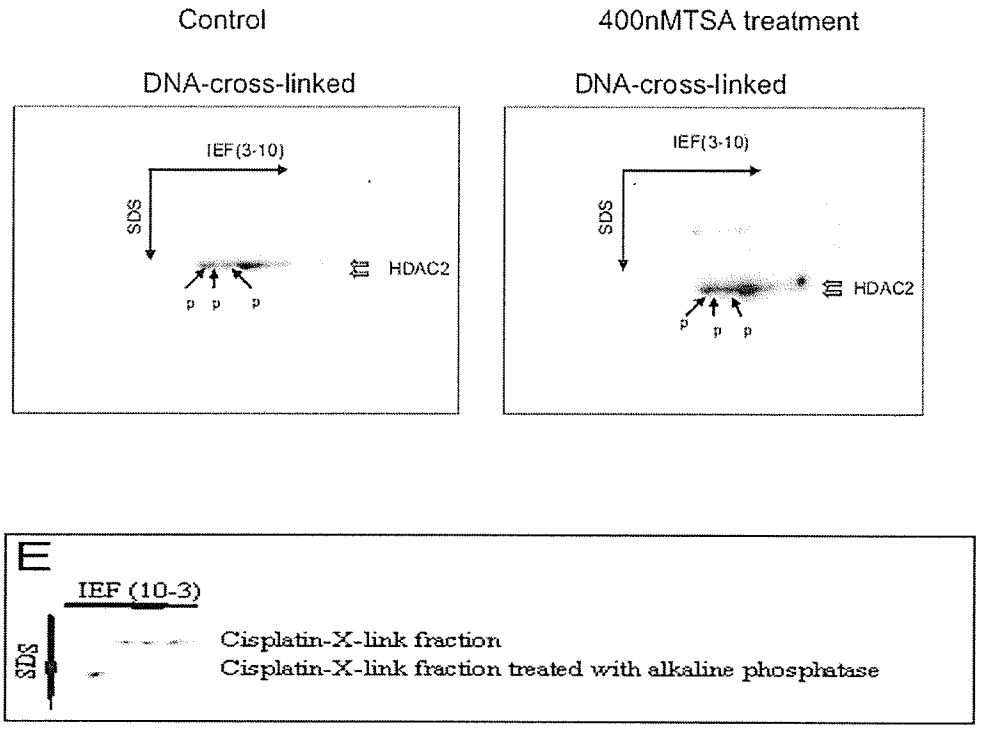


Fig. 22. Two-dimensional electrophoresis and western blotting of HDAC2 phospho-forms with and without 400nM TSA treatment. MCF-7 cells were treated with (+) or without (-) TSA for 2h. Cells were incubated with 1mM cisplatin for 1h at 37°C and proteins isolated from chromatin. 2D gel electrophoresis with immobilized pH gradients (IPG) strips was performed. IPG stripes were applied onto SDS-10% polyacrylamide gels which would transfer to nitrocellulose membranes, and were then immunochemically stained with anti-HDAC2 antibody. E, Cisplatin cross-linked MCF-7 cells were treated with (+) and without (-) alkaline phosphatase. HDAC2 phosphorylated forms disappeared after alkaline phosphatase treatment. One spot represents parental HDAC2 and the 3 other spots present HDAC2 phosphorylated forms in all 2D gel western blots with HDAC2 antibody (Panel E performed by JM Sun).

4.14 HDAC1 phosphorylation in different cell lines

Control of HDAC1 and HDAC2 function by protein interaction and recruitment has been well studied, and we were interested in exploring post-translational modifications such as phosphorylation in HDAC1 and HDAC2 and its effect in complex formation. Schreiber and Cohen (*27, 106*) showed that all HDAC1 is phosphorylated in Jurkat cell lines. However, in Schreiber's experiment, HDAC1 was from Jurkat cell lysates which contain only small amounts of endogenous HDAC2. HDAC1 phosphorylation in MCF-7 (T5) and HCT116/p53 wt cells, both of which contain HDAC2, was compared with Jurkat and HCT116/p53 null cells both of which do not contain HDAC2 or contain low amounts only. In all cases, a second band was seen on top of the original HDAC1 signal, indicating the possibility of total HDAC1 phosphorylation. Loading equality was checked using actin (Fig. 23). In addition, total HDAC2 level in Jurkat cells was compared with T5 cells by western blotting. In agreement with Schreiber's data (*27*), HDAC2 expression in Jurkat cells was low in comparison with T5 cells which are known to express HDAC2 (Fig. 23).

To reevaluate Schreiber's data and to clarify the identity of the higher molecular weight bands from the original HDAC1 signal, Jurkat cell lysates were incubated at 30°C and 37°C for an hour in the presence and absence of calf intestinal phosphatase and separated by SDS-PAGE (Fig. 24). In the presence of phosphatase, the slower migerating band on the top of HDAC1 disappeared, suggesting the possibility that HDAC1 is phosphorylated. However we could not conclude that all HDAC1 is phosphorylated in the cells in contrast with the two

previous studies (*27, 106*). HDAC1 phosphorylation is indicative of association with RbAp48 in HDAC1's C-terminal domain in Jurkat cells which contain low amount of HDAC2 (*27, 106*). However, in Seiser et al.'s paper (*10*) HDAC1's N-terminal domain was reported to be responsible for associations with protein such as with RbAp48 (*10*).

It can be concluded from Figures 23-24 that only a portion of HDAC1 is phosphorylated in Jurkat cells which express a very low amount of HDAC2. This data is different from Schreiber et al's data (*27*) who reported that all of HDAC1 is phosphorylated.

The next step was to determine whether HDAC1 phosphorylation is increased when the cells do not contain HDAC2 in HCT116/p53 null cell lines.

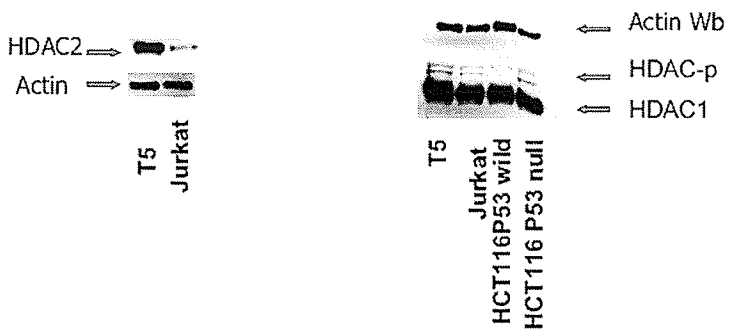


Fig. 23. HDAC1 phosphorylation in different cell lines. Cellular extracts from T5, Jurkat, HCT116 p53 null and wt cells were loaded onto 10% SDS-PAGE gel and transferred to nitrocellulose membranes, and immunochemically stained with anti-HDAC1 antibody (right). The same amount of cellular extract from Jurkat and T5 cells were loaded onto a 10% SDS-PAGE gel and probed for HDAC2. Jurkat cells contain low amounts of HDAC2 (left).

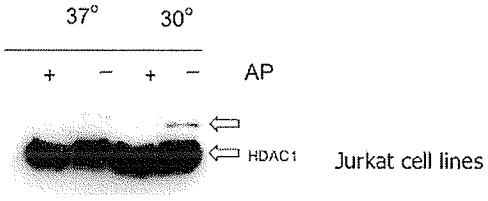


Fig. 24. HDAC1 is phosphorylated in Jurkat cell line. Jurkat cell lysates were incubated at 30°C and 37°C for an hour with (+) and without (-) calf intestinal phosphatase and were separated by 10% SDS-PAGE for 8h at 100V. In the presence of phosphatase, the second band on the top of HDAC1 disappeared.

4.15 HDAC1 cross linked to DNA is phosphorylated in HCT116 p53 wt cells

To determine HDAC1 phosphorylation in HCT116/p53 null cells, we used 2D gel electrophoresis with IPG strips to separate complex mixtures of proteins based on their net charge. HCT116/p53 null and wt cells were incubated with the cross-linker cisplatin and proteins bound to DNA were isolation as described above in *Materials and Method.* In this experiment, we compared HCT116 /p53 null and wt cells to understand whether phosphorylated HDAC1 in cells with no HDAC2 can form HDAC associated complexes by binding to RbAp48. From Figure 25 it seems HDAC1 may get phosphorylated in HCT116 p53 wt and null cells in the same manner. The key experiment then was to find out whether an IP of RbAp46/48 preferentially pulled down phosphorylated HDAC1 in Jurkat and

HCT 116 p53 null cells. Such a finding would suggest that most of HDAC1 gets phosphorylated and is associated with RbAp48 for complex formation in the absence of HDAC2.

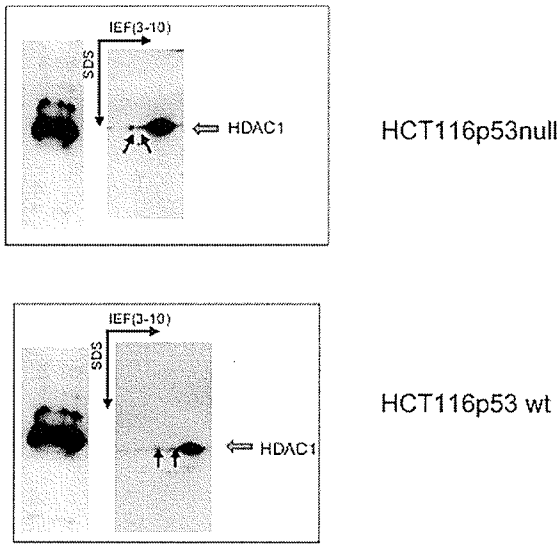


Fig. 25. Two-dimensional electrophoresis and western blotting of HDAC1 phospho-forms. HCT116 p53 null and wt cells were incubated with 1mM cisplatin for 1h at 37°C and proteins were prepared by isolation from chromatin. 20-50µg protein mixed with 125µl rehydration buffer was added to the IEF focusing tray channels. IPG strip gels (pH 3-10) were applied to the tray channels and were run at the optimum voltage to separate complex mixtures of proteins based on their net charge. IPG Strips were then transferred down into the 10% second dimension SDS-PAGE gels. In the end the gels were transferred for HDAC1 western blotting.

4.16 RbAp48, HDAC1 and HDAC2 co-immunoprecipitation in HCT116 p53 null and wt and Jurkat cell lines

To determine HDAC1 phosphorylation in the absence of HDAC2, we tested the hypothesis that RbAp46/48 does preferentially associates with phosphorylated HDAC1 in Jurkat and HCT116 p53 null cell lines, in comparison to HCT116 p53 wt cell which contain HDAC2. The amount of HDAC1 bound to RbAp48 that co-immunoprecipitated through anti-RbAp48 antibodies from Jurkat and HCT116/p53 wt and null cell lysates was determined. The results, presented in Fig. 26 indicate no change in the amount of low migrating bands of HDAC1 associated with RbAp48 in input and IP fractions.This suggests that HDAC1 is not phosphorylated more in the absence of HDAC2 since RbAp48 does not pull down more the phosphorylated form of HDAC1 than unmodifeied form.

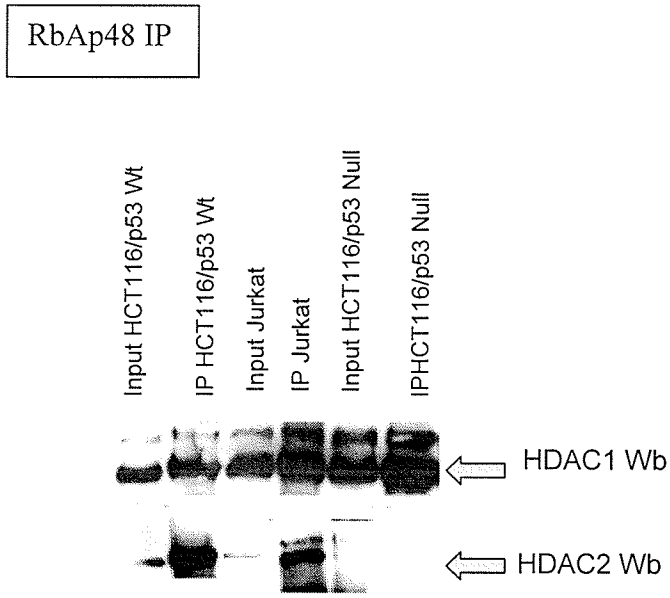


Fig. 26. Co-immunoprecipitation of HDAC1, HDAC2 and RbAp48. Jurkat cells as well as HCT116 p53 null and wt cells were lysed in IP buffer containing protease and phosphatase inhibitors. Cell lysate samples (500μg protein) were incubated with 2μg of anti-RbAp48 antibodies. The whole immunoprecipitated (IP) fractions and equivalent volumes of lysate (input) and immunodepleted (ID) fractions, corresponding to 20μg of lysate were loaded onto SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunochemically stained with anti-HDAC1 or anti-HDAC2 antibodies.

4.17 Effect of HDACis on the association of HDAC1/2 with nuclear matrix

Our research group has shown that HDAC1 and HDAC2 are colocalized in the nucleus of MCF-7 cells (*108*). Further, these HDACs are co-localized on the nuclear matrix, which is the nuclear substructure thought to be involved in the organization and function of nuclear DNA (*108*). Based on the above knowledge, we tested the hypothesis that HDACi disrupt the interaction of HDAC1 and 2 with nuclear matrix in the nuclei. Disruption of HDAC nuclear position may compromise the enzyme's role in deacetylating specific gene loci.

To study the effect of HDACis on association of HDAC1/2 complexes with nuclear matrix, the human breast cancer cell line MCF-7 was grown on poly-L lysine coated cover slips and incubated with 300nM TSA and 10mM butyrate for 2 hours. Next, soluble proteins were removed by 5min incubation with 0.5% Triton X-100. The remaining nuclear structures then were cross-linked with 3.7% formaldehyde. Chromatin was released by digestion with DNaseI and salt extraction. The resulting nuclear matrix preparations were then analyzed by immunofluorescence with HDAC1 and HDAC2 antibodies as described in *Material* and *Method* (*108*). Figures 27 and 28 show no significant loss of colocalization of HDAC1 and HDAC2 in treated samples in comparison with the control samples which suggests TSA or butyrate do not cause re-localization of HDAC1/2 complexes.

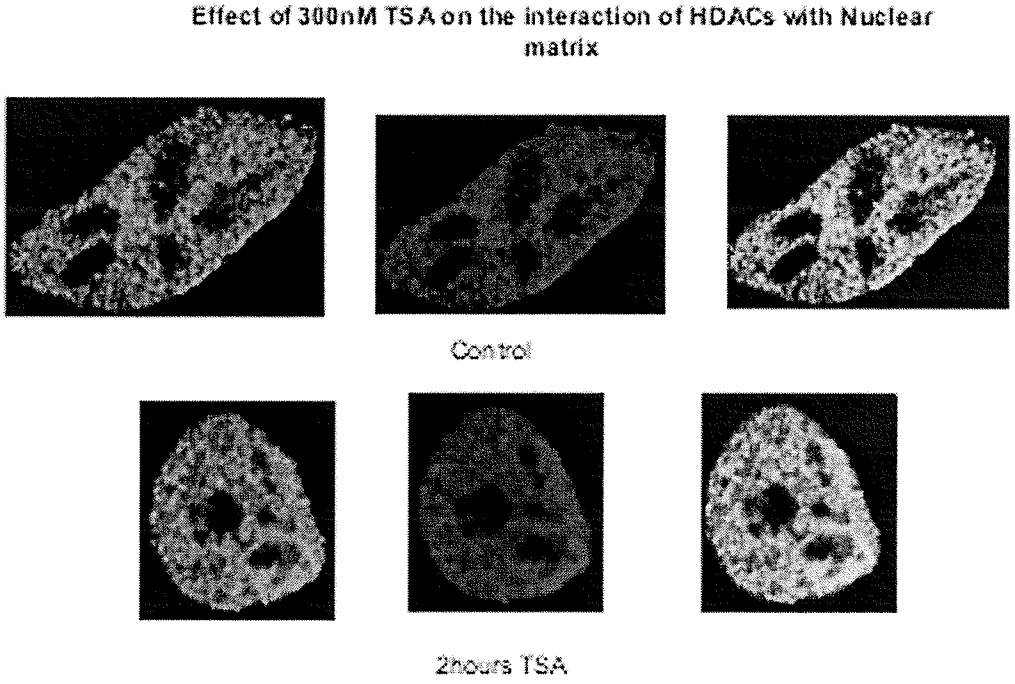


Fig. 27. The effect of 300nM TSA treatment on nuclear matrix interaction with HDAC1 and HDAC2. MCF-7 cells were grown on coverslips in DMEM complete medium, fixed, and double labeled with anti-HDAC1 and anti-HDAC2 antibodies. Spatial distribution was visualized by fluorescence microscopy and image deconvolution as described in *Materials and Methods*. Single optical sections are shown.Red indicates HDAC1, Green indicates HDAC2. Yellow in the merged images signifies colocalization.

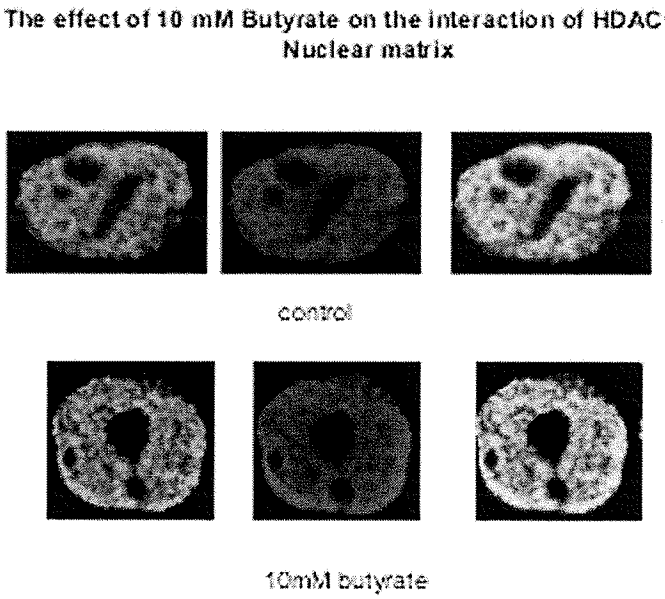


Fig. 28. Nuclear matrix interaction with HDAC1 and HDAC2 after 10mM butyrate treatment. MCF-7 cells were grown on coverslips in DMEM complete medium, fixed, and double labeled with anti-HDAC1 and -HDAC2 antibodies with and without (control) 10mM butyrate. HDAC1 and HDAC2 distributions were visualized by fluorescence microscopy and image deconvolution as described in *Materials and Methods*. Single optical sections are shown.Red indicates HDAC1, Green indicates HDAC2. Yellow in the merged images signifies colocalization.

5.0 DISCUSSION

In this study, we determined that the mode of inhibition of butyrate, VPA and TSA is competitive. Although these results contradict data previously published by others (*12, 150*), they are compatible with the interaction of TSA or SAHA with the active site of the mammalian HDAC homolog HDLP, as elucidated by crystallographic analysis (*19*) or with the interaction of TSA and other active inhibitors with the catalytic site of HDAC1, 2, 3 or 8 as determined by crystallography or homology modeling studies (*160, 170, 171*). In this study we measured total HDAC activity in MCF-7 cell lysate; however, greater than 50% of the measured activity is accounted for by HDAC1 and HDAC2. It is also of note that MCF-7 cells have an elevated expression of HDAC 1, 2 and 3 but not 8 relative to normal breast epithelial cells (*172*). While TSA is believed to inhibit all class I and II HDAC activities, it has been reported that class II HDAC activities, more particularly HDAC6 and 10 activities, are less susceptible to VPA and butyrate inhibition (*114, 142, 145, 173*). We also found that TSA, when added to MCF-7 cell extract, did not promote the dissociation of HDAC1 and 2 from the Sin3 complex. Likewise, the substitution of a conserved amino acid residue located in the catalytic site of HDAC1 resulted in the loss of most of the enzymatic activity but did not abolish the interaction of HDAC1 with Sin3A or RbAp48 (*174*). This result was confirmed by gel exclusion chromatography. It has been shown that dissociation of HDAC1/2 complexes may result in the change of substrate preference and catalytic activity (*162*). We note that previous kinetic studies of HDAC inhibition were done with HDACs isolated in the

presence of 1M $(NH_4)_2SO_4$ (*12, 150*). Such conditions may have resulted in the dissociation of HDAC complexes as Li *et al* demonstrated that 1.6M NaCl promoted the dissociation of HDAC complexes (*162*). It is possible that the dissociation of the HDAC complexes affects HDAC tertiary structure in such a way that it exposes binding cavities for inhibitors that would be unavailable in the complexes. It should also be noted that the histone substrate specific activity in our assays was 5-7 times higher than those reported in previous studies (*12, 150*). Thus experimental errors in our assay will have a lower impact, increasing the accuracy of the assay.

Our results indicate that HDACis do not disrupt HDAC complexes and do not result in the dissociation of HDAC1 or 2 from Sp1, at least not directly. HDACis would first affect the rapid turnover of acetylation of histones associated with active and competent genes (*175, 176*). Yet, HDACis were previously reported to release HDACs from promoters (*98, 167, 177*). Moreover, published data on the association of HDAC1 and Sp1 after cell treatment by HDACis for a 24h period are conflicting, with two studies reporting no effect by HDACis (*178, 179*) and two studies reporting a disruption of the interaction between HDAC1 and Sp1 (*180, 181*). To explain the loss of HDAC1 and 2 from promoters or the disruption of their interaction with Sp1 following treatment with HDACis, it is possible that transcription factors and/or architectural proteins (e.g. high-mobility group protein A, HMGA, previously known as HMGI(Y)) would become acetylated by surrounding HATs resulting in the loss of DNA binding or the change of structure of regulatory complexes (*182*).

Post-translational modifications especially phosphorylation control the activity of a wide range of regulatory proteins. For example, phosphorylation in class II histone deacetylases regulates their subcellular localization and their enzymatic activities (*4*). In 2001, Schreiber (*27*) and Cohen (*106*) reported that HDAC1 is a phosphoprotein in Jurkat and COS-7 cells and may be regulated by phosphorylation. Schreiber et al.'s findings indicated HDAC1 phosphorylation sites were at Ser^{421} and Ser^{423} (corresponding to Ser^{422} and Ser^{424} of HDAC2) which promoted enzymatic activity (*27*). At the same time Cohen et al.'s data showed that a phosphorylation site exists between residues 387 and 409 and did not influence enzymatic activity (*27, 106*). Both groups were in agreement with Seto et al.'s studies in the case of HDAC1 phosphorylation by CK2, PKA and PKG *in vitro* but not by PKC (*104*).

From the above studies, the significance of HDAC1 phosphorylation for its activity was not clear. We showed that only a portion of HDAC1 is phosphorylated in Jurkat cell lines (expressed very low amount of HDAC2). These data are different from Schreiber's data who reported that all of HDAC1 is phosphorylated (*27*).

In 2002, Seto and his group by using an HDAC2 deletion approach *in vivo* found that Ser^{394} (which corresponds to Ser^{393} of HDAC1), Ser^{411}, Ser^{422}, and Ser^{424} of HDAC2 are phosphorylated. However, unlike HDAC1, HDAC2 is phosphorylated *in vitro* only by CK2 but not by PKA or PKG. Because Ser^{422} and Ser^{424}, but not Ser^{411}, lie within CK2 recognition sequences, HDAC2 contains three phosphorylated residues on Ser^{394}, Ser^{422}, and Ser^{424} (*104*). There is

homology between HDAC1 and HDAC2 proteins and similar residues of HDAC1 and HDAC2 may be targeted for phosphorylation, although the kinases and the time and situation of phosphorylation may be different between these two different enzymes. Thus we determined whether HDAC1 phosphorylation is a default for HDAC2 when the cell does not contain HDAC2 (in HCT116/p53 null cell lines). Our co-immunoprecipitation study did not show HDAC1 phosphorylation as a default for HDAC2 since RbAp48 did not preferentially pull down phosphorylated HDAC1 in Jurkat and HCT116 p53 null cells. This means HDAC1 did not get phosphorylated for association with RbAp48 and complex formation in the absence of HDAC2.

It is possible that according to Seiser et al. instead of the C-terminal of HDAC1, the N-terminal HDAC1 association domain is necessary for *in vitro* binding to HDAC2, and association with RbAp48 and for catalytic activity of the enzyme. Seiser reported that the C-terminal domain of HDAC1 is only for nuclear localization of mouse HDAC1 (*10*).

Moreover, in our lab HDAC2 phosphorylation was shown before in T5 cells by using a 2D gel approach. This phosphorylation is important for complex formation with RbAp48 in Sin3 and NuRD complexes (data not published). Thus our data using 2D gels showed no significant effect of TSA on HDAC2 phosphorylation leading to complex formation.

Our data show that HDAC1 binding to RbAp48 is not hyper-phosphorylated in the absence or with only low amounts of HDAC2 expression. Co-IP experiment

with RbAp48 and HDAC1 did not demonstrate an increase of phospho-HDAC1 pulled down with RbAp48 antibody.

Co-IP studies with RbAp48 and HDAC2 showed that the majority of HDAC2 associated with RbAp48 was phosphorylated. Current evidence shows that RbAp48 does not interact with the phosphorylated region in HDAC1 or HDAC2. In GST pull down experiments it was demonstrated that RbAp48 interacted with avian HDAC2 regions encompassing amino acids 82 to 180 and 245 to 314 (*183*). It is interesting that one of the HDAC2 regions interacting with RbAp48 is next to the region containing the three phosphorylation sites. As the phosphorylation of HDAC2 appears to be necessary for RbAp48 to bind to this HDAC, we propose that the phosphorylation event leads to a change in HDAC2 conformation that results in exposing the binding site on HDAC2 to RbAp48.

In contrast to HDAC2, our results show that HDAC1 that is associated with RbAp48 is mostly unphosphorylated with small amount of the RbAp48 associated HDAC1 being phosphorylated. Analyses of the binding of RbAp48 to HDAC1 revealed that RbAp48 bound to the N-terminal section (first 51 amino acids) of HDAC1 *in situ* in HeLa cells (*10*). To explain these observations as well as Schreiber's study that HDAC1 phosphorylation is required for RbAp48 to bind to HDAC1, we propose first, phospho-HDAC1 undergoes a conformational change exposing the RbAp48 binding site. However, unlike HDAC2, the HDAC1 can be subsequently dephosphorylated without the loss of RbAp48 binding. Therefore, this model explains why most of HDAC1 associated with RbAp48 is not phosphorylated.

6.0 CONCLUSIONS

Abnormal HDAC activity at critical genes through the recruitment of HDAC by altered protein partners is a common theme in hematological cancers. HDAC inhibitors are an exciting new class of drugs that are targeted as anti-cancer agents. These compounds can induce growth arrest, apoptosis and/or terminal differentiation in a variety of solid and hematological neoplasms in patients with advanced disease. Therefore, a wide-ranging knowledge of the role of HDACs in tumorigenesis, and of the action of HDACi, has been achieved. However, several basic aspects such as mechanisms of HDAC inhibition by HDACi and characteristics of HDAC complexes are not yet fully understood. Investigating these aspects in the context of what we now know about HDACi both *in vitro* and *in vivo* will further improve therapeutics of HDAC inhibitors. Also, HDAC1 and HDAC2 interact with cellular proteins implicated in cancer, such as Rb, p53 and MTA2. Therefore, understanding the role of HDAC1 and HDAC2 phosphorylation in regulating their interaction with RbAp48 and other proteins might contribute to the over role of HDACs in cancer.

Our data showed three HDACis, TSA, VPA and butyrate were competitive inhibitors. Their Ki demonstrated the potency of these inhibitors with TSA being the most potent inhibitor, followed by butyrate and VPA, respectively. Co-IP experiments proved that the integrity of HDAC1/HDAC2 interaction in MCF-7 cells was not disturbed by TSA. In addition, HDAC1 and HDAC2 interaction with Sin3A was not disrupted with TSA inhibition. Thus TSA did not have an

effect on the integrity of Sin3A complex. Moreover, TSA did not show any effect on the association of HDAC1 and HDAC2 with transcription factor Sp1.

Our results also demonstrated that HDAC2 phosphorylation was not affected by TSA. In summary our results show that the most potent of the HDACi we tested (TSA) was a competitive inhibitor that did not disrupt HDAC1/2 complexes. It remains to be investigated whether the other HDACi will lead to the immediate disruption of the HDAC1/2 complexes. With this said, a more potent HDACi may be one that inhibits both enzyme activity as well as complex formation.

More over, our data demonstrated that HDAC1 and HDAC2 both can get phosphorylated. HDAC2 is associated with RbAp48 in a phosphorylated form. However HDAC1 is not entirely phosphorylated and it is associated with RbAp48 mainly in an unmodified form.

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