Interpreting clinical assays for histone deacetylase inhibitors

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Abstract: As opposed to genetics, dealing with gene expressions by direct DNA sequence modifications, the term epigenetics applies to all the external influences that target the chromatin structure of cells with impact on gene expression unrelated to the sequence coding of DNA itself. In normal cells, epigenetics modulates gene expression through all development steps. When “imprinted” early by the environment, epigenetic changes influence the organism at an early stage and can be transmitted to the progeny. Together with DNA sequence alterations, DNA aberrant cytosine methylation and microRNA deregulation, epigenetic modifications participate in the malignant transformation of cells. Their reversible nature has led to the emergence of the promising field of epigenetic therapy. The efforts made to inhibit in particular the epigenetic enzyme family called histone deacetylases (HDACs) are described. HDAC inhibitors (HDACi) have been proposed as a viable clinical therapeutic approach for the treatment of leukemia and solid tumors, but also to a lesser degree for noncancerous diseases. Three epigenetic drugs are already arriving at the patient’s bedside, and more than 100 clinical assays for HDACi are registered on the National Cancer Institute website. They explore the eventual additive benefits of combined therapies. In the context of the pleiotropic effects of HDAC isoforms, more specific HDACi and more informative screening tests are being developed for the benefit of the patients.

Keywords: histone deacetylase inhibitors, epigenetic, clinical trials interpretation

Introduction

The transcriptional state of a eukaryotic gene is determined by the surrounding chromatin architecture, the state of DNA cytosine methylation in the promoter/first exons and the associated regulating microRNAs (miRNAs).

In the cell nucleus, the genome is packaged into a superstructure, the chromatin, whose elementary dynamic units are the nucleosomes. Each is made up of four associated dimers of core histones (H2A, H2B, H3, and H4) around which 147 base pairs of DNA are wrapped, the nucleosomes being finally linked together via the linker histone H1 (Figure 1). Chromatin is compacted around the DNA into a so-called “closed state” when cells are resting. It is opened into an “active state” to allow for gene transcription by adenosine triphosphate (ATP)-dependent protein complexes, which remodel the chromatin architecture. These complexes modify the accessibility of DNA regulatory sites through both repositioning (sliding) and ejecting nucleosomes. Modeling complexes include transcription co-activators, transcription factors, and epi-enzymes.¹ Histone acetyltransferases (HATs), for instance, acetylate specific lysine residues of histones and convert them into an amide form, loosening histone contacts with DNA, resulting in exposed binding sites for the transcription

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Figure 1 The nucleosome unit and the histone tail chemical modification.

Abbreviations: HAT, histone acetyltransferase; HDM, histone demethylases; HDAC, histone deacetylase; HMT, histone methyltransferase; UBP, ubiquitin-specific protease.

machinery. On the other hand, other complexes function as gene silencers and deny the same machinery access to DNA. Repressive complexes include histone deacetylases (HDACs), which deacetylate specific lysine residues of the histones tails to induce tighter interactions between the now positively charged lysine (Ne protonated form) and the negatively-charged DNA phosphate groups.² Beside acetylation, several other post-translational lysine modifications in histones have been described: methylation, phosphorylation, SUMOylation, and ATP-ribosylation.^{3,4}

All the modifications incurred by histones form the “histone code”. For example, K9 in H3 turns the chromatin into inactivity when methylated. Phosphorylation of serine 10 in the same H3 is required for methylation of K4 and acetylation of K9 and K14. Similar enzyme crosstalk has also been described, for ubiquitination of K120 in H2B prior to methylation of K79 in H3.⁵ Several epi-enzyme families are involved in the histones modifications. HATs and HDACs² have balancing actions for histones acetylation. Methylation of histones⁶ is controlled by histones methyltransferases (HMTs) and histones demethylases (HDMs), while histones arginine methylation is catalyzed by the protein arginine N-methyltransferases family of enzymes.^{7–9} One, two, or three methylations are possible, with impact on gene expression/repression.¹⁰ The analysis of epigenetic marks at the genome-wide scale has shown that monomethylated H3K4 is associated with transcription factors binding to enhancers, trimethylated H3K4 with transcription start sites, and dimethylated H3K4 with both transcription start sites and enhancers.¹¹

DNA methyltransferases (DNMTs) repress gene expression via DNA cytosine methylation, unfavorable to transcription factor binding. DNMTs are recruited and stabilized, on

DNA, by HMTs and HDMs. Both are also able to recruit HDACs, methyl-binding proteins like methyl CpG binding protein 2 (MECP2), and several co-factors to further tune gene expression.^{12,13} Since DNMT co-factors are lacking in normal tissues, gene re-expression induced by DNMT inhibitors could be limited to tumor tissues to reduce “off-target” effects. DNA methylation in the epigenomes of human embryonic stem cells is an important field of research. The roles of DNA methylation in cancer genesis have also been extensively studied.^{12,13}

Catalyzed by the ten-eleven translocation 1 (TET) family of enzymes, DNA hydroxymethylcytosine has been recently described as a step towards cytosine demethylation.¹⁴ Mutations and translocations of TET are present in myeloid malignancies.¹⁵ The role of hydroxymethylcytosine, if any, is not yet understood, but its existence questions all the results obtained so far when determining the cytosine methylation status.

In a further step of complexity, specialized miRNAs read the epi-code and target effectors genes to modulate their expression. MiRNAs are small non-coding RNAs of 20–22 nucleotides that inhibit gene expression when they engage either in imperfect base-pairing with their target mRNA 3′-untranslated region or affect its stability. MiRNA 29-a, -b and -c target DNMT3a and b directly and cell-specifically. HDAC4 is targeted by both miR-1 and miR-140, while miR-449-a targets HDAC1 in prostate malignant cells.¹⁶ Onco-proteins like promyelocyte leukemia retinoic acid receptor- α (PML-RAR α) in promyelocytic leukemia and B-cell lymphoma 6 in non-Hodgkin’s lymphoma result from translocations. It is near the premiR-223 region that the t(8;21) translocation juxtaposes the Runt-related transcription

factor 1 gene on chromosome 21 with the Cytochrome B Termination 1 gene on chromosome 8, generating the acute myeloid leukemia (AML)1-Eight Twenty One fusion gene.¹⁷ The recruitment, on this chimerical site, of DNMT, MeCP2, and HDAC1 repressor complexes, promotes leukemogenesis. Epi-miRNAs write their own epi-code when their cytosines are methylated. Downregulation of miR-124a induces an up regulation of its target, cyclin-dependent kinase 6 (CDK6), as well as phosphorylation of retinoblastoma, and contributes to the abnormal proliferation of acute lymphoblastic leukemia (ALL) cells both in vitro and in vivo.

Most epigenetic changes translate into either up regulation or silencing of gene expression.¹⁸ When inappropriate, they predispose the organism to more mutational events via increased genomic instability and aberrant cellular signaling. The field of epigenetic being extremely prolific, we have restricted our reference list to the essentials.

HDACs

HDACs remove the acetyl group from an N-ε-acetyl lysine located near the amino terminus of a core histone, cleaving an amide bond and increasing the positive charge of the histone. The removal of acetyl groups from the histones tails stabilizes nucleosomal DNA-histones interactions by its subsequent change in electrostatic charges. It is the basis for HDAC-mediated transcriptional repression via chromatin condensation.¹⁹ HDACs have been categorized into four classes. Class I HDACs (HDAC1, 2, 3, and 8) are nuclear proteins with ubiquitous expression involved in regulating cell proliferation.²⁰ HDAC2 has been shown to suppress apoptosis in tumor cells not only via both the intrinsic/mitochondrial and the extrinsic/death-receptor pathways, but also via mitotic failure and autophagic cell death, while HDAC3 is involved in bone structure and S-phase check point.^{21,22} Class II HDACs have a tissue-specific expression and can shuttle between the nucleus and the cytoplasm. They are divided into two subclasses: IIa with HDAC4, 5, 7, and 9. HDAC4 represses chondrocyte hypertrophy. HDAC7 functions in the down regulation and apoptosis of T-cells.²⁰ HDAC9 is involved in cardiomyocyte differentiation.²³ Class IIb includes HDAC10 and HDAC6. The latter contains two tandem catalytic domains: one is for histones deacetylation and the other for deacetylation of α-tubulin. HDAC6 has also the capacity to bind directly to ubiquitinated proteins through an ubiquitin-binding domain, to target cargo proteins for subsequent processing. HDAC6-specific effects on cell motility and the proteasome are thought to be responsible for much of the toxicity of HDACs inhibitors (HDACi). HDAC 10 and 9

are required for chromosome homologous recombination.²⁴ Class III HDACs include 7 different members of the sirtuin (SIRT) family. They are dependent on nicotinamide adenine dinucleotide (NAD⁺) to remove the acetyl group from lysine residues in histones and nonhistone substrates. Resveratrol from grapes and red wine is a SIRT1 activator.²⁵ HDAC11 is the only member of Class IV.

Thus, it appears that HDAC activity depends on isoform types, sub cellular localization, association into multi-protein complexes and even post-translational modifications. HDACs are also able to deacetylate nonhistone proteins such as transcription factors, chaperone proteins and effectors of DNA repair, cell-signaling and metabolism. The ongoing concept is that deacetylation stabilizes these proteins. HDACs have different developmental functions, as shown by the different phenotypes obtained in knockout mice.²⁶ Disruption of HDAC1 causes early embryonic lethality. HDAC2 knockout mice are viable but present fatal multiple cardiac defects. Germline HDAC3-deficiency causes embryonic lethality. HDAC3 conditional knockout mice gave severe deficits in membranous and endochondral bone formation. Germline deletion of HDAC4 causes premature ossification of the developing bones. HDAC6-deficiency slightly enhances trabecular bone formation. HDAC7 knockout gave vascular defects. HDAC8 is essential for neural crest progenitor cell differentiation and skull bone formation. HDAC9 knockout mice are viable at birth but have a myocardial hypertrophy.

HDACs and their inhibitors

In tumor cells, deletion of a single HDAC is not sufficient to induce cell death but leads to nuclear bridging and fragmentation, and 'in fine' to cell mitotic catastrophe. This suggests that inhibition of HDAC may be sufficient for anticancer activity and provides a rational incentive for the development of HDACi.²⁷ In the 1970s, seminal experiments showed that treatment of cells with the short-chain fatty acid NaB (sodium butyrate) caused hyperacetylation of histone octamers and led to the discovery of HDACs.²⁸ The zinc-dependent HDACs of classes I, II, and IV are now known to have a common active site made of a tubular hydrophobic channel with a zinc atom (Zn²⁺) at its end, forming the enzyme catalytic pocket.²⁹ The acetyl part of the lysine substrate in histones/proteins bind to the zinc atom while the protein four carbon lysine chains fits into the catalytic pocket, and deacetylation then follows. An HDACi is designed to block the HDAC catalytic activity. Several possibilities exist: irreversible or reversible binding to the enzyme catalytic site, competition

with the enzymatic substrate, and deformation of the enzyme. Accordingly and as shown in Figure 2, the pharmacophore model for HDACi includes a zinc-binding group, competing somehow with the natural acetyl lysine substrate, a hydrophobic cap interacting with the external surface of the active site (generally aromatic) generating specificity, and a short linker connecting these two elements, which fits in the catalytic pocket. The zinc-binding groups can be a carboxylic acid in valproic acid (VPA), a hydroxamic acid in Vorinostat, benzamide in Entinostat, sulfhydryl in Romidepsin,³⁰ and a ketone in Trapoxin. The linkers can be simple carbon chains, like in Vorinostat, or aromatic groups, like in Entinostat. HDACi in clinical trials are reported in Figure 3. Trichostatine (TSA) from *Streptomyces hygroscopicus* was isolated as an antifungal antibiotic and was incidentally shown to have an anti-proliferative activity on murine leukemia cells. Further studies demonstrated that it was a pan-HDACi. The hydroxamate portion at the end of the molecule acts as a zinc-binding group. Because of toxic side effects, it is not used clinically but participates in the rational conception of HDACi via molecular modeling, as shown in Figure 2.

Besides its HDAC inhibition activity, Vorinostat (Figure 2, left), deriving from TSA, has a complex and not yet fully characterized activity leading to the accumulation of acetylated histones and non histone proteins. First generation HDACi are not selective except for a partial selectivity achieved^{31,32} in rare cases using bulk chemical groups to generate specific interactions with the external surface of the active site of the enzyme, like in tubacin.³³ Sulfur-based zinc-binding groups also showed some selectivity in compounds like Largazole, a potent and selective HDACi for HDAC1 and 2. It is a densely functionalized macrocyclic peptide isolated from the

Cyanobacterium symploca sp. by Luesch and coworkers.³⁴ Entinostat and Mocetinostat have selectivity for HDAC1-3 and also against HDAC11 for the latter. Valproate and sodium butyrate (NaB) better target HDAC I and IIa. For sirtuins³⁵ inhibition is based on NAD⁺ competitive binding with attempts to propose a pharmacophore, according to the various inhibitor structures described.³⁶

SIRT1 activation is the novel therapeutic approach to treat chronic inflammatory diseases, and enzyme activators are therefore sought. Many screening tests to search for HDACi use short histone peptides, capturing baits and engineered cells. All have their limitations because, in vivo, HDAC are parts of mega Daltons modeling chromatin complexes that may change within each cell type.

HDAC inhibitors metabolism

The metabolism of HDACi is an important concern during clinical assays. It is studied to determine the correlation between HDACi blood concentration, effective biological effects and eventual drug interactions. The known metabolisms of some HDACi are reported in Figure 4. TSA is metabolized as the inactive trichostatic acid, which is further demethylated³⁷ for rapid clearance (Figure 4). Phenyl butyrate (PB) metabolism has been described in several contexts.³⁸ PB is β -oxidized to phenylacetate, and cleared out upon glutamine addition. Vorinostat is also oxidized to 4-anilino-4-oxobutanoic acid and glucuronylated.^{39,40} Romidepsin is a disulfide prodrug. The real active form corresponds to the free thiol metabolite,⁴¹ produced in vivo; the butenthio part being thought to be the zinc-binding group. A glutathione conjugate has also been described,⁴² which is metabolized in vivo by the cytochrome P450s⁴³ with slow and high acetylating subjects. Other HDACi stabilities have been investigated.^{44,45}

In-vitro effects of HDAC inhibitors

DNA chips studied the transcriptome of cells treated with Vorinostat and Romidepsin,⁴⁶ revealing that the expression of 40% of all genes was affected over a period of 16 hours. A Belinostat mRNA signature of 25 genes was sufficient to assess the overall gene modulation. Panobinostat modulated cell cycles and angiogenic genes.⁴⁷ Tumor antigen expression modulation and major histo-compatibility antigen (MHC) molecule induction⁴⁸ have been observed with Dacinostat.⁴⁹ Mice bearing human tumor xenografts treated with Belinostat showed a modulation of the expression of genes active in the cellular G2/M phase. This was different from what was seen with 5-fluorouracil (5-FU), Cisplatin, Paclitaxel, or

Figure 2 Left panel: X-ray crystallographic data for SAHA bound to HDAC8. The zinc atom in the HDAC active site is shown in grey. The hydroxamic acid group in SAHA is bound to Zn²⁺, the phenylamide group is outside the enzyme active site, and these two elements are linked by a short carbon chain. **Right panel:** Modeled tubulin bound to HDAC6. Zn²⁺ in the active site is shown in grey. The hydroxamic acid group in tubulin is bound to the Zn²⁺, the phenylamide group is outside the enzyme active site and these two elements are linked by a short carbon chain. A bulk chemical entity has been grafted onto the phenylamide part of SAHA to obtain selectivity towards HDAC6 due to specific S- π interactions from sulfur atom (in yellow).

Abbreviations: HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid.

Figure 3 Histone deacetylase (HDAC) inhibitors used in clinical trials arranged by chemical classes.

Thiotepa. Synergistic effects were obtained when combining HDACi and DNA demethylating agents,⁵⁰ or HDACi and all-trans retinoic acid (ATRA), a cell-differentiating agent used to treat acute promyelocytic leukemia (APL).⁵¹ The influence of epigenetic modulators to modify stem cell fate and its relevance for curing diseases has been reviewed.⁵² Successful therapeutic use of HDACi may thus depend on the cellular environment, the specific HDAC targeted, and the relative dependence of the tumor on the unique set of pathways influenced by a specific HDAC. Results are summarized in Table 1.⁵³

Clinical trials with zinc-dependent HDACi

This part of the review describes the HDACi that have been or are being investigated in clinical trials. In Table 2, all current trials are recapitulated. In Table 3, and for each molecule, some data related to epigenetic measurements are summarized.

PB or its sodium salt

PB or its corresponding sodium salt (NaPB) is a short chain fatty acid approved by the Food and Drug Administration (FDA) for the treatment of hyperammonemia. It stops the

Figure 4 Metabolic processes for some histone deacetylase inhibitors.
Abbreviations: PB, phenyl butyrate; TSA, trichostatin.

cell cycle in its G1–G0 phase. PB is an efficient HDACi at about 0.5 mM.^{54,55} PB induces apoptosis – probably via c-jun N-terminal kinase (JNK) – in lung carcinoma cells,⁵⁶ p21waf1-mediated growth arrest in MCF-7 cells,⁵⁷ tumor necrosis factor (TNF)- α ⁵⁸ or peroxisome proliferator-activated receptor (PPAR) γ -mediated⁵⁹ cell differentiation, and is more potent than phenylacetate in prostate cancer cells,⁶⁰ while increasing MHC class I expression. PB is converted in vivo into the active metabolite phenylacetate (PA) by β -oxidation in the liver and kidney mitochondria.⁶¹ Most dose-limiting toxicities (DLTs) are fatigue, nausea, and somnolence. Preliminary studies have been conducted in patients with recurrent glioblastoma multiform (GBM)⁶² (Table 3, A). Phase I studies have been conducted in patients with hormone refractory prostate cancers,⁶³ refractory solid tumor malignancies⁶⁴ like colon carcinoma, non small cell lung cancer (NSCLC), anaplastic astrocytoma, GBM, bladder carcinoma, sarcoma, ovarian carcinoma, rectal hemangiopericytoma, and pancreatic carcinoma,⁶⁵ mainly as intravenous infusions but also in AML and myelodysplastic syndrome (MDS).⁶⁶ DLTs were neuro-cortical with milder fatigue and nausea/vomiting, light-headedness, short-term memory loss, sedation, confusion, and hypocalcemia. Although central nervous system

(CNS) toxicity was observed, infusions were well tolerated (Table 3, B). The active metabolite PA accumulated.

In the AML/MDS study,⁶⁷ with sequential administration of 5-aza-cytidine (5-aza) (Table 3, C), partial remissions or stable diseases were obtained. Targeting different biological mechanisms is feasible with acceptable toxicity. Phase I trials in combination with several drugs have been reported. Prostate, colorectal, leiomyosarcoma, and esophageal cancers were treated in combination with 5-aza (Table 3, C),⁶⁸ metastatic colorectal cancer with fluorouracil 5-FU as a 24-hour continuous intravenous infusion (CIV).⁶⁹ With 5-aza, no re-expression of E-cadherin, endothelin B, and glutathione S transferase (GST) pi was observed, a result explained by the lack of dose effect or by the fact that DNA methylation is an S-phase-dependent process while in-vivo prostatic cells may be in S-phase at any given time. Stable disease was the best response. Combining 5-FU appeared also feasible.

Pivaloyloxymethyl butyrate

Pivaloyloxymethyl butyrate (AN)-9, is an ester prodrug of butyric acid (BA)⁷⁰ but with a greater potency at inducing malignant cell differentiation and tumor growth inhibition. It showed more favorable toxicological, pharmacological,

Table 1 In-vitro modulation of gene expression by HDACi

HDACi	Gene expression	
	Upregulated (cell type)	Downregulated (cell type)
Vorinostat	TOBI, BTG1, BTG2, MX11, MAD, MLX, TIEG, ID2, ID3,	MYC, MCM-3, MCM-5, MCM-7.
Romidepsin	CDKs, DRAK1, DRAK2, DAPK3, GADD45 β , GADD153.	
Vorinostat (in CDK2 expressing cells)	PKCA, PAK1, TRAF1.	ZAK, BCL2, B-MYC, GRIM19, PDC4, P2RX1, CD27.
Belinostat	CTGF, DHRS2, DNAJB1, HIF0, MAP1 LC3B, ODC, SAT, TACCI.	ABLI, CTPS, EIF4G2, KPNBI, CAD, RAN, TP53, TYMS, TD-60.
Panobinostat		p-FLT-3, FLT-3, Bcr-Abl, p-AKT, phospho-ERK1/2. TS (in HCT116 cells).
Apicidin	CDKN1A, gelsolin.	

Abbreviations: TOBI, transducer of ERBB2; BTG1, B-cell translocation gene 1; BTG2, B-cell translocation gene 2; MX11, MAX dimerization protein 1; MAD, mitotic arrest deficient-like; MLX, MAX-like protein X; TIEG, Kruppel-like factor 10; ID2, inhibitor of DNA binding 2; ID3, inhibitor of DNA binding 3; CDKs, cyclin-dependent kinase; DRAK1, serine/threonine kinase; DRAK2, serine/threonine kinase 17b; DAPK3, death-associated protein kinase 3; GADD45 β , growth arrest and DNA-damage-inducible 45 beta; GADD153, DNA-damage-inducible transcript 3; MYC, v-myc myelocytomatosis viral oncogene homolog tumor necrosis factor; MCM-3-7, minichromosome maintenance complex component 3-5-7; PRKCA, Protein kinase C α ; PAK1, p21 protein (Cdc42/Rac)-activated kinase 1; TRAF1, TNF receptor-associated factor 1; ZAK, zinc finger protein 33A; BCL2, B-cell CLL/lymphoma 2; B-MYC, c-myc binding protein; GRIM19, NADH dehydrogenase 1 alpha subcomplex; PDC4, DEP domain containing 4; P2RX1, purinergic receptor; P2X, ligand-gated ion channel; D27, CD27 molecule; CTGF, connective tissue growth factor; DHRS2, dehydrogenase/reductase S2; DNAJB1, DnaJ (Hsp40) homolog; subfamily B; member 1; HIF0, H1 histone family; member 0; MAP1LC3B, microtubule-associated protein 1 light chain 3 beta; ODC, ornithine decarboxylase 1; SAT, spermidine/spermine N1-acetyltransferase 1; TACCI, transforming acidic coiled-coil containing protein 1; ABL1, c-abl oncogene 1; CTPS, CTP synthase; EIF4G2, eukaryotic translation initiation factor 4 gamma; KPNBI, karyopherin beta; CAD, carbamoyl-phosphate synthetase 2; RAN, member RAS oncogene family; TP53, tumor protein p53; TYMS, thymidylate synthetase; TD-60, regulator of chromosome condensation 2; FLT-3, fms-related tyrosine kinase 3; Bcr-Abl, c-abl oncogene 1; non-receptor tyrosine kinase; p-AKT, v-akt murine thymoma viral oncogene homolog 1; TS, Thymidylate synthase; CDKN1A, cyclin-dependent kinase inhibitor 1A; CDK2, cyclin-dependent kinase inhibitor 2A.

and pharmaceutical properties than BA in preclinical studies. BA itself induces p16 expression and growth arrest of colon cancer cells,⁷¹ and modifies caspase distribution during apoptosis.⁷² AN-9 down regulates c-jun and c-myc and induces differentiation in leukemia cells.⁷³ It is decomposed by esterases in vivo to yield butyric and pivaloyl acids and a formaldehyde molecule, responsible for toxicity resulting in visual acuity disorders. It has demonstrated a synergistic effect with other anticancer agents by reducing bcl-2 levels.⁷⁴ Initial study⁷⁵ with I.V. of AN-9 in advanced solid malignancies (Table 3, E) gave partial responses, and stable diseases as best responses. Later, a multicenter trial of pivaloyloxymethyl butyrate⁷⁶ in refractory NSCLC (Table 3, F), administered as a continuous I.V. infusion, gave partial responses.

VPA

VPA is a nontoxic short-chain carboxylic acid used for the treatment of epilepsy with a long clinical history and well known pharmacokinetics (PKs) and pharmacodynamics (PDs).^{77,78} VPA induces chromatin decondensation,⁷⁹ and differentiation in neural progenitor cells,⁸⁰ and inhibits HDAC activity⁸¹ in the mM range (preferentially HDAC1, 2).⁸² The antiproliferative activity was associated with aberrant cyclin D3 functionality during the C6 glioma G1 phase.⁸³ Activation of PPAR δ was present in F9 cells.⁸⁴ VPA induces caspase-dependent and -independent apoptosis in leukemia cells,⁸⁵ and in AML cells expressing P-gp and multidrug resistance protein 1 (MRP1),⁸⁶ inhibits production of TNF- α

and interleukin (IL)-6 and activates nuclear factor kappa B (NF- κ B).⁸⁷ VPA has been evaluated in combination with other anticancer compounds. For AML, increased 5-aza cytotoxicity was associated to cyclin D1 and p27(Kip1) expression,⁸⁸ while sequential VPA/ATRA treatment reprograms differentiation.⁸⁹ VPA induces p16INK4A upregulation and apoptosis and sensitizes melanoma cells to chemotherapy.⁹⁰ Interestingly, most of the clinical trials reported are for combination therapies.

A Phase I was conducted⁹¹ for refractory advanced cancer (colorectal, melanoma, NSCLC, and others) (Table 3, G). VPA/ATRA combination was evaluated for several diseases. Poor risk AML⁹² (Table 3, H), MDS and relapsed or refractory AML⁹³ (Table 3, I) have also been investigated. A 52% response rate was observed in MDS patients. ATRA exerted no additional effect in patients receiving the combination, but could be used to induce a second response in relapsing VPA-treated patients. In recurrent or refractory AML or MDS in a Phase II protocol⁹⁴ (Table 3, J), ATRA was administered when VPA reached the target serum concentration. The differentiation therapy with VPA was effective in 30% of patients. In 11 elderly patients, de novo AML⁹⁵ (Table 3, K) was also treated with theophyllin to increase cAMP levels and major cell differentiation.⁹⁶ Complete marrow response was observed in three patients, including one complete remission. Two additional patients had hematologic improvement. Patients with AML-M6 were found particularly⁹⁷ responsive, probably due to T-cell acute lymphocytic leukemia 1

Table 2 Clinical trials for epigenetic drugs

Safety study of CHR-3996, in patients with advanced solid tumours
Safety and Tolerability of CHR-2845 to treat hematological diseases or lymphoid malignancies
A safety and dose-finding study of JNJ-26481585 for patients with advanced refractory leukemia or myelodysplastic syndrome
Phase II study of Givinostat in very high-risk relapsed/refractory Hodgkin's lymphoma patients
Phase II study of Givinostat followed by Mechlorethamine in relapsed/refractory Hodgkin's lymphoma patients
Phase II study of Givinostat in refractory/relapsed lymphocytic leukemia
Phase II study of Givinostat in combination with hydroxyurea in polycythemia vera
Clinical trial of Belinostat in patients with advanced multiple myeloma
Belinostat to treat tumors of the thymus at an advanced stage
Belinostat in relapsed or refractory peripheral T-cell lymphoma
Belinostat in treating patients with MSD
Safety and efficacy of Belinostat when used with standard of care chemotherapy for untreated NSCLC
A Phase I study of Belinostat in combination with Cisplatin and Etoposide in adults with SCLC and other advanced cancers
Vorinostat for locally advanced NSCLC
Vorinostat in treating patients with metastatic and/or locally advanced or locally recurrent thyroid cancer
A Study of the efficacy of Vorinostat in patients with polycythemia verae and essential thrombocythemia
Study of Vorinostat Plus Capecitabine and Cisplatin for 1st Line Treatment of metastatic or recurrent gastric cancer
Vorinostat with Capecitabine Using a new weekly dose regimen for advanced breast cancer
Study of Vorinostat combination with Bortezomib in patients with multiple myeloma
Study of Vorinostat and Gefitinib in relapsed/or refractory patients with advanced NSCLC
Proteasome Inhibitor NPI-0052 (marizomlib, salinosporamide A) and Vorinostat in patients with NSCLC, pancreatic cancer, melanoma or lymphoma
Vorinostat combined with Gemtuzumab Ozogamicin, Idarubicin and Cytarabine in acute myeloid leukemia
Trial for locally advanced Her2 positive breast cancer using Vorinostat and Paclitaxel, Trastuzumab, Doxorubicin and Cyclophosphamide on a weekly basis
Sirolimus and Vorinostat in advanced cancer
Temsirolimus and Vorinostat in treating patients with metastatic prostate cancer
Vorinostat, Carboplatin and Gemcitabine plus Vorinostat maintenance in women with recurrent, Platinum-sensitive epithelial ovarian, Fallopian tube, or peritoneal cancer
Vorinostat and Gemcitabine in treating patients with metastatic or unresectable solid tumors
Vorinostat and Lenalidomide in treating patients with relapsed or refractory Hodgkin lymphoma or non-Hodgkin lymphoma
Hydroxychloroquine + Vorinostat in advanced solid tumors
Vorinostat in combination with palliative radiotherapy for patients with NSCLC
Vorinostat in combination with radiation therapy and infusional Fluorouracil (5-FU) in patients with locally advanced adenocarcinoma of the pancreas
Study of 5-azacytidine in combination with Vorinostat in patients with relapsed or refractory diffuse large B cell lymphoma
An Investigational Study of Vorinostat Plus Targretin (Bexaroten) in cutaneous T-cell lymphoma patients
Phase II Trial of Vorinostat and Tamoxifen for patients with breast cancer
Oral Panobinostat in relapsed or refractory CLL and MCL (non-Hodgkin's lymphoma)
Panobinostat in Phase II in SCLC
Panobinostat in treating patients with relapsed or refractory acute lymphoblastic leukemia or acute myeloid leukemia
Study of Oral Panobinostat in adult patients with refractory/resistant cutaneous T-cell lymphoma
Study of Bortezomib and Panobinostat in treating patients with relapsed/refractory peripheral T-cell lymphoma or NK/T-cell Lymphoma
Study of Panobinostat to treat malignant brain tumors
Study of Oral Panobinostat in patients with cutaneous T-cell lymphoma and adult T-cell leukemia/lymphoma
Panobinostat in adult patients with advanced solid tumors or cutaneous T-cell lymphoma
A study of Panobinostat as second-line therapy in patients with chronic graft-versus-host disease
Panobinostat treatment for refractory clear cell renal carcinoma
A Study to investigate the effect of food on oral Panobinostat absorption in patients with advanced solid tumors
ERB-B4 after treatment with Panobinostat in ER+ Tamoxifen refractory breast cancer
Panobinostat in addition to corticosteroids in patients with acute graft versus host disease
Panobinostat and Imatinib Mesylate in treating patients with previously treated chronic phase chronic myelogenous leukemia
Study of Imatinib, a Platelet-derived Growth Factor Receptor Inhibitor, and Panobinostat, in the treatment of newly diagnosed and recurrent chordoma
Oral Panobinostat in combination with Carboplatin and Paclitaxel in advanced solid tumors
Safety and efficacy studies of Panobinostat and Bicalutamide in patients with recurrent prostate cancer after castration
Panobinostat and Everolimus in treating patients with recurrent multiple myeloma, non-Hodgkin lymphoma, or Hodgkin lymphoma
Panobinostat and Fluorouracil followed by Leucovorin Calcium in treating patients with stage IV colorectal cancer who did not respond to previous Fluorouracil-based chemotherapy
Sorafenib and Panobinostat in hepatocellular carcinoma
A Safety study of Panobinostat and Everolimus to stabilize kidney cancer
Phase I/II Study of Panobinostat and Erlotinib for advanced aerodigestive tract cancers

(Continued)

Table 2 (Continued)

Use of Panobinostat with or without Rituximab to treat B-cell non-Hodgkin lymphoma
Entinostat in treating patients with advanced solid tumors or lymphoma
Entinostat in treating patients with hematologic cancer
Safety and efficacy study of Entinostat a new chemotherapy agent to treat metastatic melanoma
Entinostat and Sorafenib Tosylate in treating patients with advanced or metastatic solid tumors or refractory or relapsed acute myeloid leukemia
Entinostat and Isotretinoin in treating patients with metastatic or advanced solid tumors or lymphoma
Entinostat and Azacitidine in treating patients with myelodysplastic syndromes, chronic myelomonocytic leukemia, or acute myeloid leukemia
Entinostat and Clofarabine in treating patients with newly diagnosed, relapsed, or refractory poor-risk acute lymphoblastic leukemia or bilineage/biphenotypic leukemia
A Phase II Study of Entinostat, in combination with GM-CSF treating relapsed and refractory myeloid malignancies
Azacitidine with or without Entinostat in treating patients with MSD, chronic myelomonocytic leukemia, or acute myeloid leukemia
Interleukin 2, Aldesleukin and Entinostat for kidney cancer
Safety and Efficiency Study of Valproic Acid In HAM/TSP
Valproic Acid as an effective therapy for chronic lymphocytic leukemia
Valproic Acid and Its effects on HIV latent reservoirs
Valproic Acid in treating patients with previously treated non-Hodgkin lymphoma, Hodgkin lymphoma, or chronic lymphocytic Leukemia
Bevacizumab, chemotherapy and Valproic Acid in advanced sarcoma
Combined therapy with Valproic Acid, All-trans Retinoic Acid (ATRA) and Cytarabine in acute myelogenous leukemia
Valproic Acid with Temozolomide and radiation therapy to treat brain tumors
Azacitidine and Valproic Acid in patients with advanced cancers
Phase II study of 5-Azacitidine Plus Valproic Acid and eventually ATRA in intermediate II and high risk MDS
Phase I/II Trial of Valproic Acid and Karenitecin for melanoma
5-azacitidine, Valproic Acid and ATRA in acute myeloid leukemia and high risk MDS
Hydralazine and Valproate added to chemotherapy for breast cancer
Hydralazine and Valproate plus Cisplatin chemoradiation in cervical cancer
A Pilot study of Pivanex in patients with chronic lymphocytic leukemia
A Pilot study of Pivanex in patients with malignant melanoma
Comparative trial of Pivanex and Docetaxel vs Docetaxel Monotherapy in Patients with advanced NSCLC
Study of SB939 in Subjects with myelofibrosis
SB939 in treating patients with locally advanced or metastatic solid tumors
SB939 in treating patients with recurrent or metastatic prostate cancer
Phase 2 study of Azacitidine vs MGCD0103 vs combination in elderly subjects with newly diagnosed AML or MDS
AR-42 (OSU-HDAC42) in treating patients with advanced or relapsed multiple myeloma, chronic lymphocytic leukemia, or Lymphoma
Resminostat in relapsed or refractory Hodgkin's lymphoma
Study of the safety and tolerability of PCI-2478 in patients with lymphoma
Safety and tolerability study of PCI-2478I in subjects with cancer
Phase I Study of gene induction mediated by sequential Decitabine/Depsipeptide infusion with or without concurrent Celecoxib in subjects with pulmonary and pleural malignancies
Romidepsin in treating patients with relapsed or refractory non-Hodgkin's Lymphoma

Data from US National Cancer Institute

(TAL1)⁹⁸ and GATA1⁹⁹ interactions with HDACi, inducing differentiation in murine erythroleukemia (MEL) cells. Siitonen et al¹⁰⁰ reported a negative study trying VPA, in combination with 13-cis-retinoic acid (13-cis-RA)¹⁰¹ and 1,25-dihydroxyvitamin D3, in 19 naive patients with MDS or chronic myelomonocytic leukemia (CMML) (Table 3, L). Combinations with demethylating agents have been reported. Phase I/II¹⁰² study with 5-aza-2'-deoxycytidine (5-azaDc) in leukemia (Table 3, M) included gene expression analysis (p57kip2, p15, p73, MDR1 and THBS2). Initial DNMT1 levels were too low to be informative. A Phase I study¹⁰³ with 5-azaDc in AML (Table 3, N) gave partial to complete remissions, warranting further studies of 5-azaDc alone or with alternative HDACi. A Phase I¹⁰⁴ study of epigenetic

modulation with 5-aza for advanced cancers (Table 3, O, colon, skin melanoma, breast, other) gave stable diseases. A Phase I/II study¹⁰⁵ with 5-aza and ATRA for AML and MDS (Table 3, P) gave 42% positive overall responses.

Other combinations were investigated: a Phase I dose escalation combination trial with epirubicin, 5-FU, and cyclophosphamide¹⁰⁶ in breast cancer (Table 3, Q), and a Phase I trial¹⁰⁷ with epirubicin for solid tumors (Table 3, R). The rationale for the combination was to facilitate epirubicin access to DNA to potentiate its strand breaks activity as a topoisomerase II inhibitor. Intrinsic epirubicin toxicity was not exacerbated. Reverse combination was found inadequate by the same group. The same group investigated combination with the topoisomerase I inhibitor karenitecin (KTN)

Table 3 In vivo HDACi effects from clinical data

HDACi (metabolism, half-life, bioavailability) and combinations		MTD, cancer target, DLTs	Biological analyses (source)	Remarks or recommendations
PB converted in vivo to the active metabolite PA, not indicated, 78% at 0.5 mM	A	Phase II, 27 g/day, GBM, common DLTs		Contradictory with P450-inducing anticonvulsants. One complete response for 5 years
	B	300–410 mg/kg/day, various cancers, CNS		
PB+5-aza	C	AML/MDS, skin reaction (5-aza)	H4 acetylation increased (not correlated with response)	
	D	25 mg/m ² /d SQ d. 1–14, Several cancers, common DLTs + confusion, hearing loss, triglyceridemia and hyperuricemia	Low DNMT activity	
AN-9 Is converted in vivo to the active metabolite BA, half-life <2 minutes	E	3.3 g/m ² /day for the solubility limits, advanced solid malignancies, common DLTs + visual complaints ^a		Well tolerated, active alone and usable for NSCLC with chemotherapy, 1-year survival around 30%
	F	Phase II refractory NSCLC, common DLTs and dysgeusia		
VPA	G	60 mg/kg/d, refractory advanced cancer, neurological side effects (Grade 3/4)	H3, H4 acetylation increased, HDAC2 decreased (PMBC)	VPA should be used alone for low risk MDS and with other chemotherapeutics for high risk MDS Platelet transfusion independence should reduce palliative care and improve the quality of life Particular response from patients with AML-M6 Near 50% patients had to end the treatment
Glucuronylation, glutamination, half-life: 9–18 hours	H	AML, neurologic and cardiovascular toxicity	HDAC2 decreased (PMBC)	
VPA+ATRA	I	MDS and relapsed or refractory AML	Bone marrow blast count correlated with response	
	J	Recurrent or refractory AML or MDS, neurocortical, severe bone pain (Grade 3/4)	No significant blast count reduction, cytogenetic analysis of patients is described	
	K	AML		
VPA+13-cis RA or vitamin D3	L	MDS or CMML	No relation between VPA serum level, H3 acetylation (PMBC, BMMC) or clinical response	Objective responses rate: 22%, complete remissions: 19%, safety and efficacy correlated with reversal of epigenetics marks
VPA + 5-azaDc	M	Phase I/II leukemia	DNA demethylation decreased, H3 and H4 acetylation increased, p15 reactivation, p21 cip1 not stimulated	
	N	AML, limited non hematologic toxicity (5-azaDc), encephalopathy (VPA)	Correlation with re-expression of ER mRNA and clinical response, p15 promoter methylation decreased DNA methylation decreased, DNMT1 decreased, histone acetylation increased	

(Continued)

Table 3 (Continued)

HDACi (metabolism, half-life, bioavailability) and combinations		MTD, cancer target, DLTs	Biological analyses (source)	Remarks or recommendations
VPA + 5-aza	O	Advanced cancers	DNA methylation decreased (not significant), H3 acetylation increased (PMBC)	Safe at doses up to 75 mg/m ² for 5-aza
VPA + 5-aza + ATRA	P	50 mg/kg/d for 7 days, AML and MDS, neurotoxicity	DNA methylation decreased, H3 and H4 deacetylation increased, p21 cip1 and p15 mRNA expression not associated with clinical response	Combination safe with significant clinical activity
VPA + epirubicin or 5-FU or cyclophosphamide	Q	Breast cancers	H3 and H4 acetylation increased (PMBC), strong correlation for HDAC2 decreased in MCF-7 cells, no correlation for HDAC6	Objective responses for 64% (9/15) of the patients
VPA + epirubicin	R	Solid tumors, confusion, hallucinations, hearing loss and dizziness (due to VPA half-life)	48 hours exposure VPA for chromatin decondensation prior to epirubicin exposure. Histone acetylation increased (PBMN)	Responses were obtained for anthracycline-resistant cancers (breast, cervical and NSCLC).
VPA + KTN	S	Melanoma, no VPA/KTN synergistic toxicity	H3 and H4 acetylation increased (PMBC, apparent plateau for 60 mg/kg/day VPA)	Potential use in randomized trials where topoisomerase I inhibitors are involved
VPA + dacarbazine + interferon- α	T	Advanced inoperable or metastatic melanoma, high doses VPA side effects	Histone acetylation increased (PMBC) with adjusted VPA doses.	Modification of the schedule for further evaluation of VPA with chemo-immunotherapy
VPA Mg salt	U	Cervical cancer, depressed level of consciousness	H3 and H4 acetylation increased (PMBC and tumors).	
VPA Mg salt + hydralazine	V	Chemotherapy resistant refractory solid tumors, hematologic toxicity	DNA methylation decreased, histone deacetylase activity decreased, promoter methylation decreased for RAR- α and DPK. 1091 genes upregulated, 89 genes downregulated.	Patients from cisplatin, carboplatin, paclitaxel, vinorelbine, gemcitabine, pemetrexed, topotecan, doxorubicin, cyclophosphamide, and anastrozole treatments. Supports epigenetic-driven tumor-cell chemoresistance hypothesis.
Vorinostat, glucuronylated, β -oxidized, half-life <2 hours, 43% oral bioavailability	W	Solid tumors and hematological malignancies, leukopenia, thrombocytopenia, respiratory distress (Grade 3/4)	Histones acetylation increased (v)	A randomized Phase III study can be proposed for patients already treated unsuccessfully with pemetrexed
	X	Mesothelioma, common ^b DLTs.		

(Continued)

Table 3 (Continued)

HDACi (metabolism, half-life, bioavailability) and combinations		MTD, cancer target, DLTs	Biological analyses (source)	Remarks or recommendations
Vorinostat + carboplatin and paclitaxel	Y	Hematologic malignancies and solid, common DLTs + anorexia	Histone acetylation (PMBC, 200 to 600 mg).	Safe when administered chronically, broad range of antitumor activity.
	Z	Advanced leukemias and MDS (AML, CLL, MDS, ALL, CML), common DLTs (Grade 3/4)	Incomplete blood count recovery (AML), histone acetylation increased at all doses	None of the responding patients have a specific mRNA signature for antioxidant genes to be used as a biomarker for further studies.
	AA	Recurrent or persistent epithelial ovarian or primary peritoneal carcinoma platinum-resistant/refractory, common Grade 3 DLTs + leucopenia and neutropenia (Grade 4).		SAHA is well tolerated but had minimal activity as a single agent
	AB	Measurable, relapsed or refractory breast cancer or NSCLC or colorectal cancer, common DLTs (300–400 mg) No DLTs at 200 mg.		The limited patient exposure was not sufficient to assess SAHA efficacy.
	AC	Recurrent and/or metastatic head and neck tumors, thrombocytopenia, anorexia, and dehydration		
	AD	GBM, nonhematologic, and hematologic toxicities (Grade 3).	H2B, H4 and H3 acetylation increased. Upregulation of E-cadherin.	Enzyme-inducing anticonvulsants gave lower SAHA concentrations, well tolerated, modest activity.
	AE	Metastatic radioiodine-refractory thyroid carcinoma, common DLTs (Grade 3), pneumonia, severe thrombocytopenia	Tg (DTC) and calcitonin (MTC) are not convenient biomarkers.	Lack of therapeutic effect
	AF	Advanced solid malignancies, common DLTs + emesis (Grade 3), neutropenia (Grade 4)	SAHA metabolite 4-anilino-4-oxobutanoic acid used as a marker to monitor for adherence to SAHA therapy.	Combinations well tolerated and increased SAHA half-life, paclitaxel PKs not altered
	AG	1000 mg/m ² /day, refractory solid tumors, common DLTs + atrial fibrillation	H4 acetylation (PMBC), IL-6 expression levels proposed as a marker for HDACi toxicity ²⁰⁹	50% of the patients achieve stable disease
	AH	1000 mg/m ² /day, heavily pre-treated patients with advanced hematological neoplasia, common DLTs	Histone acetylation increased (PMBC) up to 24 hours post injection.	No bone marrow toxicity as a parameter for combination therapies.
Belinostat, half-life 1–2 hours	AI	Relapsed malignant pleural mesothelioma, common DLTs		No objective responses. One death from cardiac arrhythmia, possibly related to therapy.

(Continued)

Table 3 (Continued)

HDACi (metabolism, half-life, bioavailability) and combinations		MTD, cancer target, DLTs	Biological analyses (source)	Remarks or recommendations
	AJ	Resistant micro papillary ovarian tumors (LMP) and epithelial ovarian cancer, common DLTs + thrombosis (Grade 3)	H3 and H4 acetylation increased (PMBC and tumor tissue). Disease.	Diseases with poor prognostic and scarce studies, well tolerated, promising results for LMP
Givinostat	AK	Relapsed/refractory HL, thrombocytopenia and prolongation of QTc interval	QTc interval in some cases prompting drug discontinuation	
Givinostat, alone or + dexamethasone	AL	Twice daily 100 mg/4 days/week, 12 weeks, MM, one death, cardiac toxicities (givinostat)	Already treated patients, one death was reported	Unlikely to play a significant role for MM, other combination may be investigated
Givinostat + meclizethamine	AM	Relapsed/refractory HL	TARC decrease in serum as an easy-to-detect biomarker predicting response to therapy, five (15%) complete remissions and eight (23%) partial remissions, median survival 28 months, projected 2-year survival 52%	15% complete remissions, median survival at 28 months, projected 2-year survival of 52%.
Panobinostat, half-life 11–16 hours	AN	< 11.5 mg/m ² , AML, ALL, MDS, common minor DLTs, and cardiac toxicity (Grade 3)	H3 acetylation increased (B-cells (CD19+) and blasts (CD34+)), H2B acetylation increased (CD19+ and CD34+ cells), apoptosis increased for CD34+.	
	AO	CTCL, classical DLTs at 20 mg + thrombocytopenia, toxic at 30 mg	RNA profiling: 23 genes regulated in all patients. H3 acetylation increased (PMBC and tumors)	Complete remission
Panobinostat + docetaxel	AP	Castration resistant prostate cancer, neutropenia and dyspnea (Grade 3)	Progressive disease despite histone acetylation increased (PMBC) in first regimen, PSA decreased in second regimen	Intravenous administration suggested for further investigations
Dacinostat, half-life 9–18 hours	AQ	Advanced solid tumors, common DLTs and transaminitis, fibrillation, raised serum creatinine, and hyperbilirubinemia.	Histones acetylation increased (PMBC), HSP90 ²¹⁰ inhibition measured by HSP72 levels increased; H3 and H4 acetylation increased for >24 hours, Hsp70 increased and c-Raf decreased.	Nonhistone-mediated effects requires further study
	AR	ALL, AML, CLL, CML, MDS, dose dependant DLTs: cerebral bleed secondary to thrombocytopenia (CLL), reversible hyperbilirubinemia	Histone acetylation increased >24 hours, nonlinear PK	Rematologic improvement observed, mean terminal half-lives 9–18 hours (maximum plasma concentrations 1.5 hours after the beginning of infusion).
PCI-24781 half-life 5.9 hours, oral bioavailability 34%	AS	Refractory advanced solid tumors, common and cardiac DLTs	Acetylation levels increased at 1.5 hours post dose sustained ≥24 hours (oral).	

(Continued)

Table 3 (Continued)

HDACi (metabolism, half-life, bioavailability) and combinations		MTD, cancer target, DLTs	Biological analyses (source)	Remarks or recommendations
Entinostat, half-life 34–50 hours, highly protein bound, apparent linear PKs	AT	10 mg/m ² , advanced solid tumors or lymphoma, common DLTs	HDAC inhibition (PMBC)	More frequent dosing proposed for evaluation from linear PKs data, elimination half-life dose-independent clearance
	AU	Refractory solid tumors and human lymphoid malignancies, reversible DLTs (Grade 3, hypophosphatemia, hyponatremia, and hypoalbuminemia)	Protein acetylation increased (by multivariable flow cytometry in PMBC (T cells (most robust response), B cells, and monocytes))	Well tolerated administered weekly with food
	AV	8 mg/m ² weekly for 4 weeks every 6 weeks, AML, infections and neurologic toxicity	Protein and histone H3/H4 acetylation increased (PMBC, BMMC), p21 expression increased, and caspase-3 activation (BMMC)	Detailed cytogenetic analysis performed on patients, inherent resistance to MS-275 for advanced leukemia with complex karyotype
	AW	Advanced solid malignancies and lymphomas, hypophosphatemia, and asthenia	High degree of interpatient variations in H3 and H4 acetylation increased (PMBC)	Patients with pretreated metastatic, melanoma, treatment well tolerated, no objective responses, median time-to-progression was 51–56 days
	AX	Metastatic melanoma, toxicity mild to moderate		
Mocetinostat, half-life 6.7–12.2 hours	AY	45 mg/m ² /d, advanced solid tumors, rare common DLTs	H3 acetylation increased (PWBC, measured with the BOC-Lys(ε-Ac)-AMC fluorophore), IL-6 induction Histone acetylation increased (PWBC, measured with the BOC-Lys(ε-Ac)-AMC fluorophore).	Interpatient variability improved with low pH beverages Three complete bone marrow response, cytogenetic analysis of patients not correlated with responses, safe and anti-leukemia activity for advanced leukemia
	AZ	60 mg/m ² , AML, MDS, ALL, and CML, common DLTs (Grade 3)		
Mocetinostat continued	BA	Advanced leukemias or MDS, common DLTs	HDAC inhibition (PMBC)	Four patients with stable disease
	BB	85 mg dose exhibited meaningful activity, HL	TARC levels correlated with clinical response	Two complete responses (10%), six partial responses (29%)
	BC	FL, common Grade 3 DLTs + anorexia, thrombocytopenia, pericardial serious adverse event		No clear relationships with schedules, cardiac diseases, pathologies, and biomarkers such as HDAC activity.
Tacedinaline	BD	8 mg/m ² /day for 8 weeks, repeated after a 2-week drug-free interval, solid tumors, common DLTs + thrombocytopenia, anemia, mucositis		

(Continued)

Table 3 (Continued)

HDACi (metabolism, half-life, bioavailability) and combinations		MTD, cancer target, DLTs	Biological analyses (source)	Remarks or recommendations
Tacedinaline + capecitabine	BE	6 mg/m ² Tacedinaline and 2000 mg/m ² /day capecitabine, for 2 weeks of a 3-week cycle, advanced solid malignancy, DLT was thrombocytopenia		No overlapping toxicity
Tacedinaline + gemcitabine	BF	Advanced pancreatic cancers, neutropenia, and thrombocytopenia		Combination does not improve treatment
Depsipeptide, natural disulfide prodrug, half-life 8 hours	BG	13.3 mg/m ² , incurable cancers, common DLTs + thrombocytopenia, and fatigue.	Histone acetylation increased (MNPB), PC3 cell cycle arrest induction, MDR-1 induced, functional PgP.	4-hour infusion safe
	BH	17.8 mg/m ² /4 h, advanced or refractory neoplasms, common DLTs + grade-4 thrombocytopenia and cardiac arrhythmia.	Histone acetylation increased (PMBC), MDR-1 induced.	Continuous cardiac monitoring, one partial response, 472.6 ng/mL mean maximum plasma concentration at MTD
	BI	CLL, AML, common DLTs	HDAC inhibition increased, histone and p21 promoter H4 acetylation increased, p21 protein and ID10 antigen expression increased, acetylation increased for H4 K5, K12, K8, K16, and H3 K9, K14	
	BJ	17 mg/m ² , refractory or recurrent solid tumors, reversible, asymptomatic T-wave inversions, transient asymptomatic sick sinus syndrome, and hypocalcemia	HDAC inhibition increased (PMBC)	Depsipeptide is well tolerated but no objective responses
	BK	MDS and AML, common DLTs (Grade 3/4 asymptomatic hypophosphatemia)	Apoptosis increased and changes in myeloid maturation marker expression. No changes in H3 and H4 acetylation, CD34/C13 stimulation.	One complete remission (AML) acceptable toxicity, limited activity in unselected AML/MDS patients
	BL	Refractory renal cell cancer, cardiac side effects		One complete response but not active enough in this population, one sudden death
	BM	Metastatic neuroendocrine tumors, common DLTs, serious cardiac adverse events		One sudden death
	BN	Lung cancers	H4 acetylation increased, p21 expression increased. 16 gene expressions stimulated ≥ 2 -fold, >1000 genes repressed ≥ 2 -fold.	Depsipeptide not appropriate but renormalize lung cancer cells to normal bronchial epithelia

Notes: *Resulted from formaldehyde released after AN-9 metabolism; ^bCommon DLTs are considered to be fatigue, nausea, vomiting.

Abbreviations: 5-aza, 5-aza-cytidine; 5-azaDc, 5-aza-2'-deoxycytidine; 5-FU, 5-fluorouracil; 13-cis-RA, 13-cis-retinoic acid; ALL, acute lymphoblastic leukemia; AMC, 7-amino-4-methylcoumarin; AML, acute myeloid leukemia; AN-9, pivaloyloxymethyl butyrate 9; ATRA, all-trans retinoic acid; BA, butyric acid; BMBC, bone marrow mononuclear cell; CLL, chronic lymphocytic leukemia; CML, chronic myelocytic leukemia; CMML, chronic myelomonocytic leukemia; CNS, central nervous system; CTCL, cutaneous T-cell lymphoma; DLT, dose-limiting toxicity; DNMT, DNA methyltransferases; DTC, differentiated thyroid carcinoma; ER, estrogen receptor; FL, follicular lymphoma; GBM, glioblastoma multiforme; HDAC, histone deacetylase; HDACi, HDAC inhibitor; HL, Hodgkin lymphoma; IL-6, interleukin-6; KTN, karenectin; LMP, low malignant potential; MDS, myelodysplastic syndrome; MM, multiple myeloma; MTC, medullary thyroid cancer; MTD, maximum tolerated dose; NSCLC, nonsmall cell lung cancer; PA, phenylacetate; PB, phenylbutyrate; PgP, P-glycoprotein; PK, pharmacokinetic; PMBC, peripheral mononuclear blood cell; PSA, prostate specific antigen; PWBC, peripheral white blood cell; QTc, QT interval corrected for heart rate; RAR- α , retinoic acid receptor- α ; SAHA, suberoylanilide hydroxamic acid; TARC, thymus and activation regulated chemokine; Tg, thyroglobulin; VPA, valproic acid.

(Table 3, S) for treating melanoma with both Phase I/II trials.¹⁰⁸ No VPA/KTN synergistic toxicity was observed. The best response was disease stabilization. VPA plus chem-immunotherapy was investigated in a Phase II study¹⁰⁹ for advanced inoperable or metastatic melanoma (Table 3, T), HDACi having been previously found to have a tumorigenic potential in melanoma.¹¹⁰ Some patients then received dacarbazine plus interferon- α with VPA.

The magnesium salt of VPA has been tested in phase I¹¹¹ for cervical cancer (squamous and in adenocarcinoma) and Phase II¹¹² clinical trials. In the Phase I study (Table 3, U), VPA was given per os, and the authors emphasized the requirement for new endpoint trials based on biomarker analysis^{113,114} with, in this particular case, H3 and H4 acetylation and in vivo HDAC inhibition detection. The Phase II study was conducted with hydralazine, a demethylating agent,¹¹⁵ (Table 3, V) to overcome chemotherapy resistance in refractory solid tumors (cervix, breast, ovarian, and others). Partial responses and disease stabilization were the best responses.

Vorinostat

Vorinostat (suberoylanilide hydroxamic acid [SAHA], Zolinza[®]) has probably been the most studied compound in clinical trials on several cancer types. SAHA induces differentiation,¹¹⁶ growth arrest,¹¹⁷ or apoptosis at micromolar concentrations. Vorinostat is an unselective zinc-binding¹¹⁸ hydroxamic-acid-type inhibitor of HDAC1, 2, 3, 6, and 8. In glioma cells, SAHA induced expression of DR5, TNF α , p21Waf1, and p27Kip1 and reduced expression of CDK2, CDK4, cyclin D1, and cyclin D2.¹¹⁹ SAHA can induce thyroid cancer cell death by caspase-mediated pathways,¹²⁰ and induces G1 and G2-M arrest and apoptosis in several types of breast cancer cell lines,^{121,122} NSCLC,¹²³ and prostate cancer cells.¹²⁴ It potentiates the activity of other molecules like Paclitaxel in ovarian cancers.¹²⁵

Phase I trials have been described for both oral and I.V. administrations. Escalating I.V. administration¹²⁶ in solid tumors and hematological malignancies (Table 3, W) gave hypotension for one schedule. In mesothelioma, with I.V. or oral formulations,¹²⁷ the best responses were partial (Table 3, X). An oral formulation for hematologic malignancies (Hodgkin's and others) (Table 3, Y) and solid tumors (mainly mesothelioma, prostate, urothelial, thyroid, and renal)¹²⁸ gave one complete response while others were incomplete. Oral twice- or thrice-daily administrations in advanced leukemias and MDS (AML, CLL [chronic lymphocytic leukemia], MDS, ALL and CML [chronic myelocytic leukemia]) (Table 3, Z)¹²⁹

gave two complete responses and two complete responses with incomplete blood-count recovery (all with AML treated at/below maximum tolerated dose [MTD]).

Phase II clinical trials were mainly proposed with oral formulations. A multi-institutional trial¹³⁰ in women with recurrent or persistent epithelial ovarian (Table 3, AA) or primary peritoneal carcinoma platinum-resistant/refractory gave one partial response. Another multicenter open-label oral trial¹³¹ investigated measurable, relapsed, or refractory breast cancer, NSCLC, or colorectal cancer (Table 3, AB). Disease stabilization was observed in eight patients. SAHA is tolerated at 200 mg only, in a daily oral schedule for 14 days–3 weeks. In recurrent and/or metastatic head and neck cancer (400 mg every day) (Table AC)¹³² no confirmed responses have been observed. In patients with metastatic breast cancer,¹³³ there were no complete or partial responses, and the heterogeneity of the recruited patients did not allow production of significant statistical results. Eight patients were positive for estrogen and/or progesterone receptors, four had amplified *Cerb-2*. Fatigue, nausea, diarrhea, and lymphopenia were the most frequent clinically significant adverse effects. In GBM¹³⁴ (Table 3 AD), an oral dose of 200 mg followed by a 7-day rest period showed that SAHA monotherapy is well tolerated with modest single-agent activity. Although HDACi were shown to induce cell death and sensitize cells to cytotoxic chemotherapy in thyroid cancer cell lines, Woyach et al¹³⁵ described the lack of therapeutic effect of SAHA in patients with metastatic radioiodine-refractory thyroid carcinoma in a Phase II study (Table 3, AE). A Phase II oral combination therapy was proposed with carboplatin (I.V.) and Paclitaxel (I.V.) for advanced solid malignancies¹³⁶ (Table 3, AF). Eleven partial responses occurred and seven disease stabilizations. The regimen requires drug–drug interaction to be determined. Encouraging results were obtained in patients with previously untreated NSCLC.

Belinostat

Belinostat (PXD101) is a recent hydroxamic acid HDACi that has growth-inhibitory and pro-apoptotic activity in several cancer types at submicromolar concentrations,^{137,138} and that has been investigated in ovarian cancers.¹³⁹ It down regulates thymidilate synthase, vascular endothelial growth factor (VEGF), aurora kinase, and epidermal growth factor receptor (EGFR), and up regulates cyclin A. It has been used in combination. A gene expression-signature profiling has been reported for Belinostat.¹⁴⁰ According to publications¹⁴¹ PKs gave a general 1–2-hour half-life. In early trials, DNA fragmentation increased with a combination of 5-FU in

HCT116 colon cancer cells in vitro and in both HT-29 and HCT116 in xenograft models,¹⁴² and also a poly(ADP-ribose) polymerase (PARP) cleavage and down regulation of thymidylate synthase expression in HCT116. Improved tumor reduction was obtained in vivo with mouse HT29 and HCT116 xenograft models compared with single compounds, validating a rationale for the clinical schedule.

In Phase I treatment of refractory solid tumors by I.V. administration (Table 3, AG)¹⁴³ the caspase-dependent cleavage of cytokeratin-18 was determined to measure the level of apoptosis.¹⁴⁴ Heavily pre-treated patients with advanced hematological neoplasia¹⁴⁵ (Table 3, AH) were also treated. In Phase II trials, investigations of I.V. administration of relapsed malignant pleural mesothelioma¹⁴⁶ (Table 3, AI) indicated that combination strategies or alternate dosing schedules might be necessary. In resistant micro-papillary ovarian tumors (low malignant potential [LMP]) and epithelial ovarian cancers (EOC) (Table 3, AJ),¹⁴⁷ a Phase II trial with Belinostat gave partial responses or stable diseases for LMP, and stabilized diseases for EOC.

Givinostat

Givinostat (ITF2357) belongs to the hydroxamic acid family of HDACi which is very similar to SAHA. It inhibits IL-6 and VEGF production in stromal cells.¹⁴⁸

Two Phase II studies were described for relapsed/refractory Hodgkin lymphoma (HL) (Table 3, AK). A first oral one, gave stable diseases by computed tomography (CT) scan that have been associated with a significant reduction in fluorodeoxyglucose-positron emission tomography scan uptake.¹⁴⁹ Galli et al¹⁵⁰ developed a Phase II multicenter trial in 19 heavily treated patients that were relapsing from progressive multiple myeloma (MM) (Table 3, AL). The best responses were disease stabilization. This regimen appears as unlikely to play a significant role for advanced MM, and other combinations with currently used drugs should be investigated. A combination with the alkylating agent mechlorethamine¹⁵¹ (Table 3, AM) was investigated in relapsed/refractory HL.

Panobinostat

Panobinostat (LBH589) is a hydroxamic acid HDACi, which has demonstrated anti-angiogenic and anti-proliferative activities in human prostate carcinoma cell PC-3 xenografts in vivo, inducing H3 and tubulin acetylation¹⁵² in human umbilical vascular endothelial cells (HUVEC), which corresponded to G2-M cell cycle arrest and inhibition of HUVEC cell proliferation and viability. Non cytotoxic concentrations

of Panobinostat inhibited endothelial tube formation, matrix invasion, AKT, extracellular signal-regulated kinase 1/2 phosphorylation, and chemokine receptor CXCR4 expression. Association with anti-VEGF therapies should be considered. Prince et al have discussed preclinical data on Panobinostat and emerging data from Phase I and II studies in cancer patients.¹⁵³

A Phase I study in refractory hematologic malignancies (AML, ALL, and MDS) (Table 3, AN)¹⁵⁴ with I.V. administration appeared convenient to obtain anti-leukemic and biological effects. In cutaneous T-cell lymphoma (CTCL)¹⁴⁷ with oral formulation (Table 3, AO), the responses ranged from disease stabilization to complete remission, showing the potential of this molecule in CTCL. In combination with Docetaxel¹⁵⁵ (Table 3, AP), a microtubule interacting agent for castration-resistant prostate cancer, Panobinostat inhibited LnCAP androgen receptor positive prostate cancer cell proliferation, potentiated by Docetaxel. Single or combined treatments were administered with oral Panobinostat.

Dacinostat

Dacinostat (NVPLAQ824, LAQ) is a hydroxamic acid derivative similar to Panobinostat.¹⁵⁶ It showed anti-neoplastic activity and can activate genes that produce cell cycle arrest. It acetylates hsp90, inducing proteosomal degradation of Bcr-Abl and HER-2. Combination of Dacinostat with 5-azaDe¹⁵⁷ in human MDA-MB-231 and MCF-7 breast carcinoma cells showed a synergic anti-neoplastic activity for the MDA-MB-231. For the MCF-7 tumor cells, simultaneous 5-azaDe and Dacinostat administration were antagonistic, unseen when used in a sequential schedule (5-azaDe first). This is probably due to interference in the S-phase of Dacinostat since 5-azaDe is a S-phase specific interfering molecule. Dacinostat appeared to be well tolerated in clinical trials. Phase I investigations¹⁵⁸ in advanced solid tumors included measure of HSP72 levels and was consistent with HSP90 inhibition (Table 3, AQ). Another group¹⁵⁹ reported the same results with increased expression of Hsp70 and decreased c-Raf levels. The biological importance of these non histones mediated effects requires further study. I.V. administration for ALL, AML, CLL, CML¹⁶⁰ (Table 3, AR) in blast crisis or advanced MDS gave some stable diseases.

PCI-24781

PCI-24781 is a broad-spectrum hydroxamic acid-based HDACi. PCI-24781 reverses drug resistance in four multi-drug resistant sarcoma cell lines and synergizes with chemotherapeutic agents to enhance caspase-3/7 activity.¹⁶¹

In refractory advanced solid tumors¹⁶² (Table 3, AS), I.V. administration followed by dose escalation was well tolerated. Electro cardiac monitoring revealed grade ≤ 1 QTcF (QT interval corrected for heart rate using Fridericia's formula) prolongation and asymptomatic nonspecific ST and T wave changes leading to discontinuation.

Entinostat

Entinostat (MS-275, SNX-275) is a benzamide HDACi, which promotes expression of genes involved in growth arrest and differentiation, like p21 and the maturation marker: gelsolin,¹⁶³ inducing caspase-dependant apoptosis in CLL B-cells,¹⁶⁴ p21CIP1/WAF1 differentiation or apoptosis in human leukemia cells,¹⁶⁵ and also tissue growth factor (TGF) β II receptor expression in human breast cancer.¹⁶⁶ Reported half-life in animals is about 1 hour, and species-variable protein binding was reported.¹⁶⁷ Half-life in human plasma was higher than in animals, which is supposedly to be linked to protein binding, as Entinostat was found to be 80% bound.¹⁶⁷

Phase I study in advanced solid tumors or lymphoma by oral route¹⁶⁸ (Table 3, AT) was reasonably well tolerated. In refractory solid tumors and human lymphoid malignancies¹⁶⁹ (Table 3, AU), drug exposure increases linearly with dose. In AML¹⁷⁰ (Table 3, AV), results showed that Entinostat effectively inhibits HDAC in vivo in patients with AML and should be further tested, preferably in patients with less-advanced disease. Several protocols were designed for patients with advanced solid malignancies and lymphomas¹⁷¹ (Table 3, AW). PKs revealed dose-dependent and dose-proportional increases. Responses were partial remissions and prolonged disease stabilization. In a Phase II study for metastatic melanoma (Table 3, AX) with low efficacy treatment,¹⁷² long-term tumor stabilizations have been observed, but no objective responses was assessed.

Mocetinostat

Mocetinostat (MGCD0103)¹⁷³ is a benzamide selective class I/IV HDACi (1, 2, 3, and 11). It inhibits neoplastic growth in multiple human tumor xenograft models including colon (HCT116, SW48, and Colo205), NSCLC (A549), prostate (DU145), pancreatic (PANC1), and vulval epidermal (A431) cancer models and does not interact with the potassium voltage-gated channel, subfamily H (eag-related), member 2 (HERG) channel. Gene expression induced by Mocetinostat is modest compared with other hydroxamic HDACi.¹⁷⁴

In patients with advanced solid tumors¹⁷⁵ (Table 3, AY), a phase I study gave disease stabilization as the best response.

IL-6 induction related to HDACi activity has been postulated but not confirmed. At the tested doses, Mocetinostat appeared tolerable and exhibited favorable PK and PD profiles, as well as evidence of target inhibition in surrogate tissues. Cytogenetically analyzed patients with AML, MDS, ALL, and CML¹⁷⁶ (Table 3, AZ) were treated orally. A total of 18 of the 29 patients had abnormal cytogenetics. PK analyses indicated rapid absorption of Mocetinostat. Several administration schedules have been proposed for advanced leukemias or MDS¹⁷⁷ (Table 3, BA). A Phase II study for HL¹⁷⁸ (Table 3, BB) demonstrated significant anti-tumor activity in relapsed/refractory post-transplant HL. For 437 patients¹⁷⁹ (Table 3, BC), partial responses were obtained. Extended studies are ongoing.

Tacedinaline

The long-known molecule Tacedinaline (CI-994) is a benzamide HDACi similar to MS-275¹⁸⁰ with anti-tumor activities in HCT-8 colon carcinoma.¹⁸¹ Following Tacedinaline administration, inhibition of both histone deacetylation and cellular proliferation at the G1 to S transition phase of the cell cycle are observed.

Oral administration with food intake for solid tumors¹⁸² (Table 3, BD) did not affect the rate or extent of the drug absorption. Best responses were partial or stable diseases. Advanced solid malignancies (mainly colorectal, pancreatic and mesothelioma) were treated in combination with Capecitabine¹⁸³ (Table 3, BE), an FDA-approved compound used to treat a variety of cancer. Three treatment protocols were implemented. A combination Phase II study with Gemcitabine¹⁸⁴ for advanced pancreatic carcinoma (Table 3, BF) gave no improvement.

Depsipeptide

Depsipeptide (Romidepsin, FK228, FR901228) is a cyclic tetrapeptide isolated from *Chromobacterium violaceum* which has demonstrated anti tumor activities (A549 lung adenocarcinoma, MCF-7 and ZR-75-1 breast adenocarcinoma, and LOX IMVI melanoma cell lines) and is postulated as a Pg-p substrate.^{185,186} It is considered as a natural HDACi prodrug, as its disulfide bond is reduced in vivo to give the active species⁴⁰ and is the only reported sulfur-based HDACi used in clinical trials. It received FDA approval for cutaneous T-cell lymphoma in 2009. Romidepsin induces growth arrest and apoptosis in lung cancer cells.¹⁸⁷ Romidepsin induces p21-dependent G1 arrest and p21-independent G2 arrest¹⁸⁸ by downregulating cyclin D1 and upregulating cyclin E.¹⁸⁹ It inhibits c-Myc and Fas ligand expression,¹⁹⁰ modulates p53,

ErbB1, HER2, and Raf-1 expression in lung cancer cells,¹⁹¹ increases p21, phosphorylation of Bcl2, and apoptosis in human breast cancer cells,¹⁹² increases expression of a NaI symporter in thyroid carcinoma cells for possible resensitization of radio resistant thyroid cancer,¹⁹³ and activates the caspase 8-mediated apoptosis and down regulates the c-FLIP protein.¹⁹⁴ Sequential treatments with 5-azaDe facilitates cancer cell recognition by T lymphocytes specific for cancer/testis antigen 1B (NY-ESO-1) as a possible option for immunotherapy¹⁹⁵ or induces tissue factor pathway inhibitor (TFPI)-2 expression in cancer cells.¹⁹⁶ Initial cardiac toxicity was resolved by convenient administration schedules but cardiac monitoring is most of the time implemented during clinical investigations. Concentrations studies in CLL and AML have correlated apoptosis induction and HDAC inhibition. Combination of Romidepsin and DNA demethylating agents is potentiated in ETO positive leukemia cells.¹⁹⁷ A gene signature specific for Romidepsin sensitivity has been reported.¹⁹⁸ Due to the recent approval of Romidepsin in CTCL, all early published clinical trials for this disease are not discussed.^{199,200} Romidepsin induced MDR-1 gene expression in several cancer cell lines.

A Phase I study in advanced, incurable cancers²⁰¹ (Table 3, BG) indicated that further clinical trials are warranted. Used for advanced or refractory neoplasms²⁰² (Table 3, BH), elimination half-life was 8.1 hours. In CCL and AML²⁰³ (Table 3, BI), intravenous treatment gave no responses. Romidepsin effectively inhibits HDAC in vivo in patients with CLL and AML, but future studies should examine alternative administration routes. In refractory or recurrent solid tumors²⁰⁴ (Table 3, BJ), DLTs were not associated with changes in troponin levels or evidence of ventricular dysfunction, transient asymptomatic sick sinus syndrome and hypocalcemia. For MDS and AML²⁰⁵ (Table 3, BK), intravenous administration gave no significant cardiac toxicity. Romidepsin therapy can be administered with acceptable short-term toxicity. Gastrointestinal symptoms and fatigue seemed to be treatment-limiting after multiple cycles. Phase II performed on refractory renal cell cancer²⁰⁶ (Table 3, BL), I.V., gave classical but serious toxicities. Two patients developed a prolonged QT interval, one patient developed grade 3 atrial fibrillation and tachycardia, and there was one sudden death. In metastatic neuroendocrine tumors²⁰⁷ (Table 3, BM), adverse events were ventricular tachycardia and prolonged QT, possibly resulting in a sudden death, terminating the study prematurely. Romidepsin has serious cardiac adverse events, and risks need to be comprehensively evaluated. In lung cancers (NSCLC and SCLC)

(Table 3, BN),²⁰⁸ Romidepsin was not appropriate. This study presented an in depth gene expression profiling.

Conclusion

A number of clinical trials have been completed and many others are ongoing using HDACi as single agents and in combination with radiotherapy and/or chemotherapy for the treatment of various hematological and solid malignancies with some promising early results. Vorinostat is the most established HDACi, and was approved in October 2006 by the FDA for the treatment of advanced forms of cutaneous T-cell lymphoma that have failed multiple other systemic treatment options. Significant single agent activity for Romidepsin has also been demonstrated in peripheral cutaneous T-cell lymphoma, and encouraging results have also been seen in HL with Mocetinostat. From the trials conducted, it is also clear that a major clinical advantage is that HDACi are well tolerated in the majority of patients. The future of HDACi lies in designing rational combination therapies. The sequence of drug administration may be of paramount importance to avoid antagonistic effects. The possibility of drug–drug interactions and enhanced toxicities is to be considered. HDACi are also evaluated in non cancerous pathologies like AIDS, graft versus host diseases, and polycythemia verae. Very soon, SIRT activators could find therapeutic applications in lung interstitial diseases. Like for the kinase inhibitors, more selective third generation HDACi are sought, yet specific tests remain to be designed to screen for bioactivity in vitro and in vivo.²¹¹

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Disclosure

The authors report no conflicts of interest in this work.

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