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RESEARCH ARTICLE

Selective class IIa HDAC inhibitors: myth or reality

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Abstract The prospect of intervening, through the use of a specific molecule, with a cellular alteration responsible for a disease, is a fundamental ambition of biomedical science. Epigenetic-based therapies appear as a remarkable opportunity to impact on several disorders, including cancer. Many efforts have been made to develop small molecules acting as inhibitors of histone deacetylases (HDACs). These enzymes are key targets to reset altered genetic programs and thus to restore normal cellular activities, including drug responsiveness. Several classes of HDAC inhibitors (HDACis) have been generated, characterized and, in certain cases, approved for the use in clinic. A new frontier is the generation of subtype-specific inhibitors, to increase selectivity and to manage general toxicity. Here we will discuss about a set of molecules, which can interfere with the activity of a specific subclass of HDACs: the class IIa.

Keywords SAHA · HDAC3 · HDAC4 · HDAC5 · HDAC7 · HDAC9 · MEF2 · p21 · Therapy · Apoptosis · Cell cycle · Anti-cancer · Neurodegeneration · Inflammation

Introduction

Why to target HDACs?

Every complex cellular adaptation and behavior is supervised by changes in the transcriptional machinery, which align the gene expression profile of a specific cell type to the general requirements of the organism. The harmonic regulation of genes transcribed in a specific instant is the result of an integrated and complex network of signals that controls the activity of different transcriptional players. Transcription factors (TFs), epigenetic regulators and "structural" proteins, constituting the chromatin are the chief protagonists under the tight influence of the environment. Alterations in the signaling networks or in the transcriptional players are responsible for aberrations in tissue homeostasis and triggering events in several different diseases, from neurodegeneration up to cancer [1, 2]. The opportunity to reset the transcriptional subverted context, with the therapeutic perspective of curing/alleviating diseases, straightway attracted scientist's attention [3, 4].

Perhaps the simplest approach to develop new drugs is the identification of small molecules, acting as inhibitors of an enzymatic activity that is imperative in a specific disease. In the context of gene transcription, post-translational modifications (PTMs) of histones represent realistic targets for the development of epigenetic therapies aimed to amend transcriptional alterations. Acetylation of lysines, placed in histones but also in TFs is an important PTM, exerting both positive (H3K4, 9, 14, 17, 23; H4K5, 8, 12, 16) and negative (in the case of specific TFs) effects on gene expression [5, 6]. Being acetylation reversible and under the scrutiny of different family of enzymes: HATs (histone acetyl transferases) and HDACs (histone deacetylases), it has attracted several interests as a druggable PTM [7]. In particular, during the past decades, many efforts have been made to isolate, synthesize and characterize small molecules targeting HDACs [8]. HDACis are nowadays represented as a considerable fraction of the epigenetic drugs under study and in some circumstances

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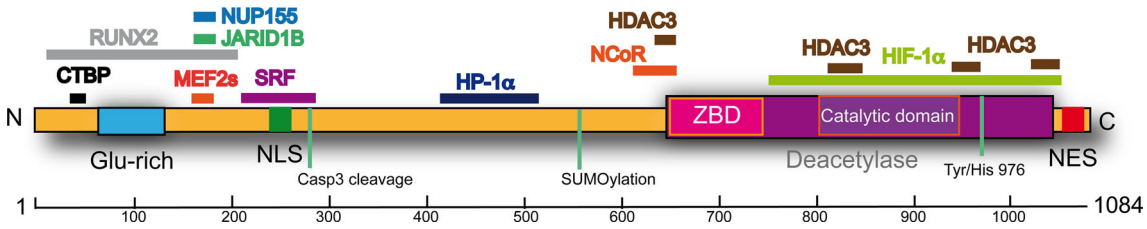


Fig. 1 Schematic representation of class IIa HDACs highlighting the principal domains. As prototype of class IIa we selected HDAC4. Certain interaction partners, as well as the relative HDAC4 sequences involved, are illustrated

these compounds have been approved for the use in clinic (see below). Importantly, epigenetic drugs in cancer therapy represent an opportunity to revert drug-resistance-associated epigenomes and to prevent or reverse non-responsiveness to anti-cancer drugs [2].

Copious studies on cancer cells' epigenomes have fully justified the rationale of applying HDACis in anti-cancer therapies. Three major intrinsic features of the neoplastic cells could be subject of specific intervention, thanks to HDACis: (1) cancer cells are characterized by an enhanced degree of heterochromatinization compared to normal cells; which makes cancer genomes inaccessible to DNA-damage response enzymes [9]. The treatment of cancer cells with HDACis relaxes chromatin and allows the activation of the DNA-damage response [9]. (2) Several tumor suppressor genes, including some pro-apoptotic genes, are inactivated in cancer cells because of ipo-acetylated promoters [3, 10, 11]. (3) Alterations of the epigenetic machineries embracing HDACs are frequently observed in tumors [12, 13].

Despite the considerable literature debating the use in epigenetic therapies of pan-HDACi and of class I HDACs specific inhibitors [10, 11, 14–22], reviews specifically discussing of molecules acting as inhibitors of class IIa HDACs, are quite rare. In this manuscript we will discuss specifically of them.

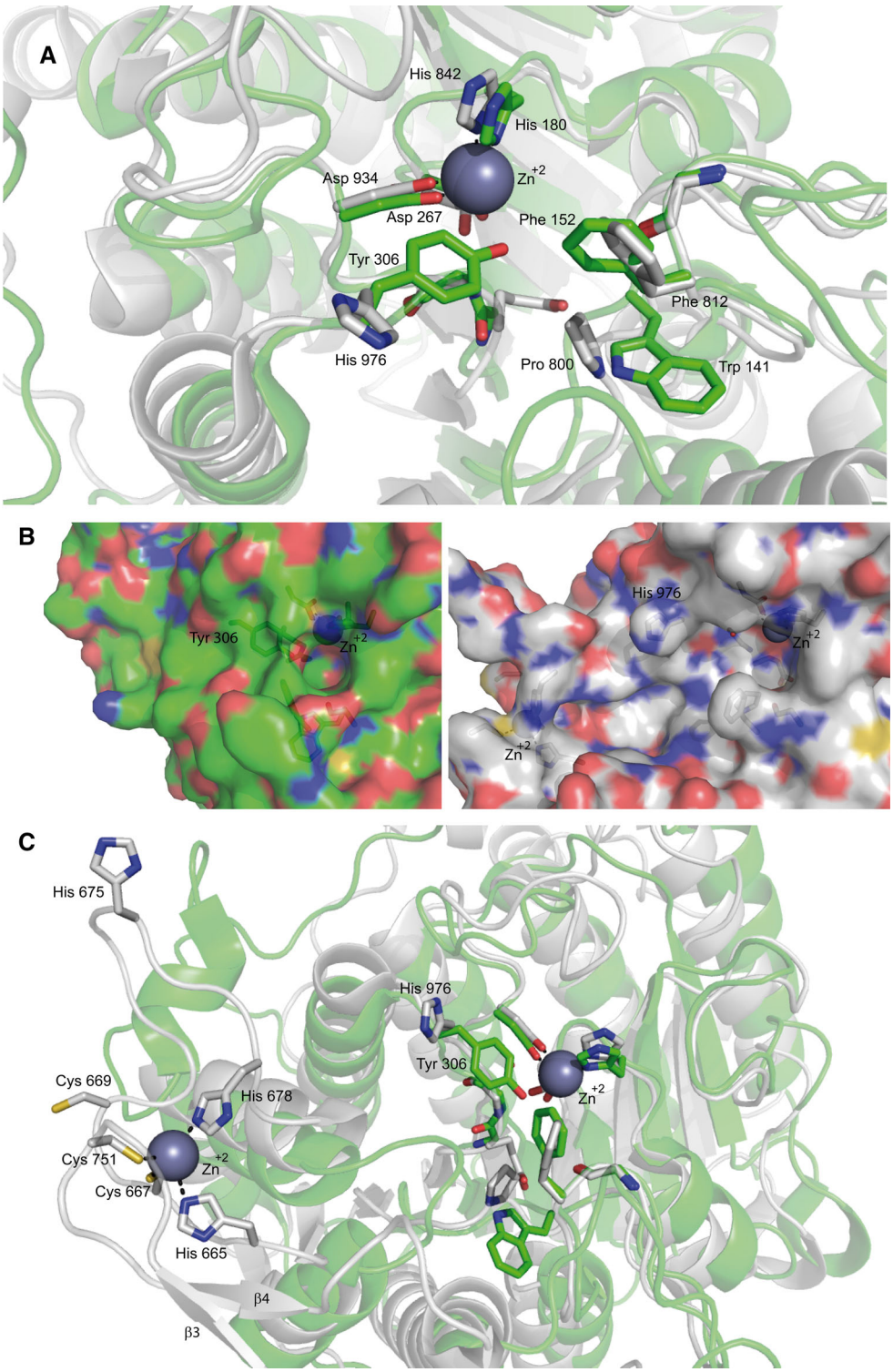
Class IIa HDACs: to be or not to be a lysine deacetylase

In humans there are 18 HDACs grouped into five different classes according to phylogenesis and sequence homology [7]. Class I HDACs (including HDAC1, 2, 3 and 8), class IIb HDACs (including HDAC6 and 10), class III HDACs or Sirtuins (including all Sirtuins from 1 to 7) and class IV (HDAC11) all displaying enzymatic activities [23]. By contrast, when we discuss about class IIa HDACs (HDAC4, 5, 7 and 9) as histone deacetylases, it should be taken into account that these proteins show an extremely low enzymatic activity against acetylated lysines [24, 25] and are rarely associated with histone tails [26].

Structurally, class IIa HDACs can be divided into two parts: the N- and the C-terminal regions (Fig. 1). The N-terminal regulates the nuclear import and contains a coiled-coil glutamine-rich domain that is peculiar of the family. This region is highly devoted to protein–protein interactions both in terms of homo- and of heterotypic partners. The C-terminal region contains the catalytic "deacetylase" domain and the nuclear export sequence (Fig. 1). These enzymes are under the control of different signaling pathways, which operate through specific PTMs to influence peculiar aspects of the class IIa biology, including the nuclear/cytoplasmic shuttling (for reviews [7, 12, 27, 28]).

The deacetylase domain is made up of approximately 400 residues (aa) arranged into 21 α-helices and 10 β-strands organized in a single domain, structured around a central "catalytic" Zn^{2+} ion [29]. Likewise to class I HDACs, 2 aspartates and an histidine coordinate this Zn^{2+} while 2 other aspartates (Fig. 2a), another histidine, a serine and a leucine coordinate two potassium ions [29–31]. Despite this high similarity, in vertebrates class IIa possess a bigger active site than class I HDACs (Fig. 2b), which impacts on their druggability [26, 29]. The evolution-related event responsible for this structural peculiarity is the mutation of a tyrosine into a histidine, Y967H in HDAC4 [25]. Histidine is sterically less cumbersome and induces the relaxation of the structure. As a consequence, this histidine is far from the central Zn^{2+} and not able to form hydrogen bonds with the intermediate of the enzymatic reaction (Fig. 2a). The intermediate is, therefore, very unstable, thus resulting in an ineffective reaction. Nevertheless, class IIa can efficiently process alternative substrates such as trifluoroacetyl-lysine. Mechanistically, the presence of the trifluoro group should destabilize the amide bond, hence favoring the reaction even in the absence of transition-state stabilization [25].

Importantly, replacing back the His with Tyr generates class IIa HDACs with a catalytic efficiency 1,000-fold higher compared to the wild-type (wt) form [25, 31]. Nonetheless, this mutant does not show enhanced repression respect to the wt, at least in the instance of MEF2-dependent transcription, a well-known class IIa partner [25].



Another distinctive feature of class IIa HDAC catalytic site is the existence of a Zinc Binding Domain (ZBD). This ZBD consists in a β-hairpin surrounded by two antiparallel β-strands, forming a pocket-like structure that accommodates a second "structural" zinc ion [29]. In the case of HDAC4 three cysteines (667, 669, 751) and one histidine

◀**Fig. 2** Representation of class I and class IIa catalytic sites (**a**, **b**) and the zinc binding domain (**c**). **a** Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (*green*) and of class IIa HDAC4 (*white*) catalytic sites. As mentioned in the text the His 976 is rotated away from the active site differently from Tyr 308 in HDAC8. **b** Surface representation of class I HDAC8 (*green*) and class IIa HDAC4 (*white*) catalytic sites. The figure shows the hydrophilic tunnel necessary for the release of the reaction product in HDAC8 (*green*), while in HDAC4 (*white*) the His/Tyr substitution prevents tunnel formation. **c** Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (*green*) and of class IIa HDAC4 (*white*) catalytic site (*right*) and zinc binding domain of HDAC4 (*left*). β3 and β4 are the two antiparallel β-strands involved in the formation of the pocket-like structure in the zinc binding domain. Importantly, His 665 and His 678 in this inhibitor-bound structure are replaced by Cys 669 and His 675 in the coordination of the zinc ion in the Apo-structure. Unfortunately the crystallization of Apo-HDAC4 was unsuccessful and these differences are deduced from crystallographic studies of the mutant GOF (H976Y) of HDAC4 [31]. The coordinates of the protein structures were retrieved from the protein data bank. Amino acids discussed in the text are labeled and shown in stick representation. The accession codes for the protein structures are: 2VQJ (HDAC4) and 1T69 (HDAC8). Figures are edited using PyMOL Molecular graphics system, Schrödinger, LLC

(675), conserved only among class IIa HDACs, coordinate this Zn^{2+} and made the so-called "core" of the domain [31] (Fig. 2c). Importantly, the inhibitor-bound structure is shown in this figure, where, respect to the Apo-structure, Cys 669 and His 675 replace His 665 and His 678 in the coordination of the Zn^{2+}.

This domain is extremely flexible and the oxidation of the cysteines involved in Zn^{2+} coordination (667 and 669 in HDAC4) is sufficient to free the metal, with the consequent opening and deconstruction of the ZBD [31]. Because this domain is head-to-head to the active site (Fig. 2c), it contributes to make the class IIa HDACs' catalytic site more accessible than that of class I HDACs (Fig. 2b) and does not allow the formation of an efficient hydrophilic tunnel necessary for the release of the acetate reaction product [30, 31].

Old structures and new functions

The enzymatic ineptitude of vertebrates' class IIa deacetylase domain raises several questions and opens the door to different hypothesis. First, they are not completely silenced enzymes. Because class IIa is capable of processing trifluoroacetyl-lysine with high efficiency, still undiscovered new natural substrates could exist [25]. Alternatively, the described enzymatic activity could simply mark a lab finding, without biological implications. Second, as anticipated above, the absence of improved repressive influence in the case of the gain of function His/Tyr substitution in HDAC4, further demonstrates that class IIa HDACs can repress transcription independently from the deacetylase domain [25]. The relevance of the deacetylase-independent repression is testified by MITR, a splice variant of HDAC9 lacking the deacetylase domain [32]. The existence of MITR supports the possibility that the HDAC domain is of little relevance for the functions of class IIa HDACs and may lead to believe that it is an evolutionary heritage intended to being missed. However, since class IIa deacetylase domain has been preserved behind two duplication events occurred during evolution of vertebrates, evolutionists deny the hypothesis that this domain would be subjected to a negative purifying selection [33].

Although there are evidences pointing to deacetylase-independent activities of class IIa, generation of a mouse model in which, mutated versions of this domain can be analyzed in a physiological context will help our understanding. This point is of crucial relevance for the design and development of class IIa inhibitors.

Along with the enzymatic activity, the deacetylase domain can operate as a scaffold for the recruitment of multi-protein complexes containing class I HDAC3 and other co-repressors [31]. HDAC4 interacts with the RD3 domain of N-CoR [24, 34], while HDAC3 binds the SAINT domain [35] and, as a matter of fact, HDAC4 binds N-CoR/SMRT regardless of HDAC3 and only in a second time the deacetylase is recruited [36]. However, the precise order of the sequential molecular interactions driving the assembly of the multi-protein complex is still waiting for a final verification.

When class IIa HDACs are isolated under native conditions, a lysine deacetylase activity can be measured. This activity is due to class I HDACs co-purified with class IIa [24, 37, 38]. The existence of a heterogeneous repressive complex complicates the assessment of effectiveness and specificity of HDACis, when tested on proteins purified from cells or tissues.

A final consideration refers to a fascinating hypothesis, which attributes to class IIa deacetylase domain the function of acetylated lysine reader [26]. In this view, class IIa could act as readers and interpreters of the histone code, thus orchestrating the epigenetic status thanks to their capability of recruiting additional enzymes, such as methylases [39] or deacetylases [24, 36]. A scenario where class IIa HDACs, acting as molecular scaffolds supervise the introduction of different epigenetic markers, onto specific regions of chromatin or in proximity of different acetylated cellular protein. In this context inhibitors of the deacetylase domain could in principle both interfere with the reading activity or, by promoting structural changes, with the possibility of recruiting additional co-repressors.

Unresolved issues

Biochemically, the enzymatic activity associated to class IIa HDACs could be explained by the recruitment of class I enzymes [24]. Moreover, all the point mutants of the HDAC4 deacetylase domain which, accordingly to Finnin model [40], abrogate its enzymatic activity (H803A, G811A, D838A, D840A, H842A, N845D, D934 N, E973G) demonstrate a perfect correlation between enzymatic activity and the ability to recruit HDAC3 [24]. Classic deacetylase activity is not associated with a cytoplasmic HDAC7 or HDAC4 immunoprecipitated from HEK293 cells and therefore, weakly associated to the mainly nuclear HDAC3 [24, 36]. Similarly, HDAC4 mutants that have lost the ability of binding to N-CoR/SMRT drop the deacetylase activity [24]. Despite in vitro binding experiments prove that the fraction of HDAC3 in complex with HDAC4 is relevant, in vivo HDAC3 preferentially forms homodimers, rather than heterodimers with HDAC4 [41]. Furthermore, the fraction of HDAC4 co-purified with HDAC3 in mammalian cells is extremely low [24, 35, 37].

As aforementioned, another peculiar feature of class IIa deacetylase domain is its sensitivity to redox conditions [31, 42]. Particularly, in HDAC4 the oxidation of cysteines 667 and 669 induces the formation of a disulphide bond that causes the exposition of the NES, the export in the cytoplasm and also the detachment of HDAC3 [31, 42, 43]. This oxidation causes the de-structuration of the HDAC domain because Cys 667 and Cys 669 are directly involved in the "structural" Zn^{2+} coordination and substrate binding [29, 31] (Fig. 2c). These findings show that researchers should be extremely cautious in verifying the redox status when studying class IIa deacetylase domain.

In addition to nuclear roles of class IIa HDACs, recently, a cytoplasmic enzymatic activity has been reported towards non-histone substrates [reviewed in 44]. During muscle denervation HDAC4, which plays a pro-atrophic role in this context [45, 46] can deacetylate and activate MEKK2 [47]. Kinase engagement culminates in AP-1 activation and cytokines production that stimulate muscle remodeling [47]. Interestingly only the wild-type form, capable of shuttling between the nucleus and the cytoplasm and not a nuclear resident mutant of HDAC4 deacetylated MEKK2. Importantly, this activity is independent from HDAC3 and is not shared with HDAC5 [47]. Paradoxically, MEKK2 activation should activate ERK5 and therefore MEF2s, thus pointing to a positive rather than repressive influence of HDAC4 versus MEF2s [48, 49]. A similar cytoplasmic KDAC (lysine deacetylase) activity of class IIa HDACs was reported towards HIF-1α and STAT-1. Also in these circumstances class IIa deacetylase activity seems to be independent from class I HDACs [44].

Another unresolved issue is the requirement of additional factors to exert the full enzymatic activity. Class I HDACs require particular cofactors both for histone and non-histone substrates [35, 41, 50]. For the enzymatic activity of class IIa HDACs towards the synthetic trifluoroacetyl-lysine or against these cytoplasmic partners, any cofactor seems to be dispensable [25].

The rationale for developing class IIa HDACs inhibitors

HDACis have entered multiple clinical trials principally in virtue of their anti-neoplastic properties [10]. Much more emphasis has been pushed on the identification, synthesis and characterization of class I HDACis. Commonly HDACis show a selective cytotoxicity against tumor cells and weak effects on normal ones [11, 51, 52]. These molecules display cytostatic effects, especially through the induction of p21 and blockage of the cell cycle [53, 54] or by triggering apoptosis via multiple mechanisms [11, 53, 55, 56]. Some HDACis in vivo stimulate also the clearance of tumor cells from the immune system [57, 58] or block angiogenesis [59, 60]. Despite these promising anti-neoplastic properties, entering of HDACis in clinic is slower than expected, principally due to some side effects and toxicity displayed during early-phase clinical trials [14, 61]. In fact, up to now only two HDACis have been approved for the treatment of cutaneous T cell lymphoma: SAHA (Zolinza) in 2006 and Romidepsin/FK-228 in 2009. In 2011 the depsipeptide FK-228 has been further approved for the treatment of peripheral T-cell lymphoma [15]. Considering the recent evidences about a pro-oncogenic potential of class IIa HDACs [12, 37, 38, 62–64] and their impact on epigenetics [65], a stratagem to circumvent the side effects of class I HDACs inhibitors might consist in targeting class IIa HDACs.

Theoretically, targeting class IIa HDACs with specific inhibitors has three major drawbacks:

1. The high similarity of the catalytic site of these proteins to class I HDACs, which makes selective targeting rather difficult to achieve;
2. The formal question about the legitimacy of hitting the catalytic site of proteins that are almost enzymatically inactive against acetylated lysines. About this consideration the work of Bottomley et al. [31] explains how targeting of the catalytic site of class IIa HDACs and in particular the Zn^{2+} atom could impact on the structure of the C-terminus of the proteins, thus compromising their capability to interact with the super complex HDAC3/N-CoR/SMRT. Therefore, targeting class IIa HDAC domain could be an indirect strategy to impact on class I HDACs. By releasing

only class IIa driven deacetylation, a more selected transcriptional re-setting can be achieved, which could favor a drop in toxicity.

3. The methodological approach to measure class IIa HDAC inhibition. Up to now the best-characterized substrate for probing the elusive catalytic activity of vertebrate class IIa histone deacetylases is trifluoroacetyl-lysine [25, 66]. The activity of class I HDACs towards this molecule is indiscernible. Its use as a substrate for the validation of an inhibitor efficiency could exclude all class I HDACs as off-targets. Class IIa HDAC enzymatic activity measured with other methods or with classical substrates (e.g., acetylated H3) or commercial assays, generally based on acetyl-Lys, is extremely low when recombinant proteins are used [24]. Instead, when class IIa are purified from vertebrates the enzymatic activity can be provided by associated class I or IIb enzymes [24, 25, 31, 67]. Therefore, a double check approach should be used to test the potency and specificity of a class IIa HDACis. The potency of the compound should be evaluated by employing trifluoroacetyl-lysine, as a class IIa specific substrate, while its inhibitory activity against other HDAC classes should be excluded using "classical" substrates, such as acetylated lysines. A simplified screening could take advantage from the recently developed trifluoroacetyl-lysine derivative, a trifluoro acetyl-lysine tripeptide named substrate 6, which can be processed by all HDACs, with the exclusion of HDAC10 and 11. This molecule looks like a promising tool for single-run screening aimed to isolate/characterize subtype specific HDACis [68].

Class IIa inhibitors

Three different peculiarities of class IIa HDACs have been exploited to design specific inhibitors:

a. The catalytic site, and in particular the Zn^{2+} atoms.
b. The nuclear/cytoplasmic shuttling.
c. The N-terminal region and the binding to specific partners, such as the MEF2 family of TFs.

Targeting the Zn^{2+} binding domain

In accordance to the connecting unit (CU) linker chelator pharmacophore model [16, 69], a classical HDACi is composed of three parts [17]:

1. The MBG (metal binding group or zinc binding group ZBG), which is a group capable of chelating the Zn^{2+} in the catalytic site of HDACs (with the exception of sirtuins).
2. The connecting unit (CU), generally a linker hydrophobic region of five or more carbons, that mimics the acetyl-lysine. It could be linear or aromatic and it perfectly fits to the hydrophobic catalytic site of the targeted HDAC.
3. The CAP hydrophobic domain (usually aromatic) that interacts with aminoacids delimiting the border of the deacetylase catalytic site.

Slight modifications of the described structure impact both on the specificity and potency of the inhibitor.

The availability of the crystal structure of the class IIa deacetylase domain [29, 31] has encouraged the development and synthesis of many hydroxamates stemmed from SAHA, with the purpose of selectively influencing class IIa HDACs. In particular to improve specificity, many efforts have been spent in the modification of the CAP and of the ZBG of SAHA. In principle, the selective targeting of class IIa HDACs would require only some changes in the linker region, to better fit the peculiar catalytic site of class IIa HDACs. A recent study effectively demonstrated that slight modifications only in the linker region of SAHA increase the selectivity towards class IIa and class IIb HDACs [70]. However, the achieved results were not as promising as those obtained after modification of both the CAP and the linker region of SAHA [71]. This double tuning seems to be the better strategy to produce SAHA derivatives specific for class IIa HDACs. In a next future, new generation class IIa HDACis could stem from Tasquinimod (described below) that selectively targets the "structural" and not the "catalytic" Zn^{2+}. This peculiarity should increase the specificity because, as discussed above, this "structural zinc" is unique of class IIa HDACs. A summary of the literature data is shown in Fig. 3.

The most characterized of these hydroxamate-like drugs, are:

– MC1568 and MC1575 (Fig. 3, please note that in Fig. 3 we provide for MC1568 the recently reassigned structure [72]) are two class II HDACs inhibitors specific for HDAC4 and HDAC6 [73–76]. They are derivatives of classical class I HDACs inhibitors aroyl-pyrrolyl–hydroxyamides (APHAs), showing selectivity towards class IIa HDACs. The modified linker region provides this selectivity. Compared to the original class I inhibitors, they exhibit a decreased cytotoxic effect [73]. Despite this fact, MC1568 and MC1575 show some cytostatic effects in melanoma cells [76] and in ER + breast cancer cells [74]. The anti-proliferative effect is provoked by a block in the G1 phase of the cell cycle, through the induction of the Cdk inhibitor p21/Cip1/Waf1 [74]. MC1568 efficacy

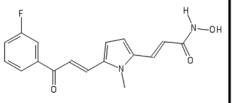
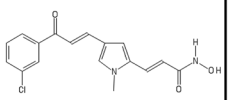
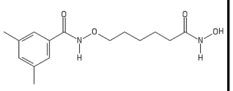
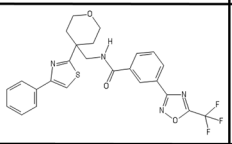
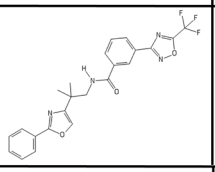
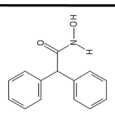
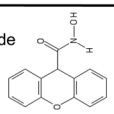
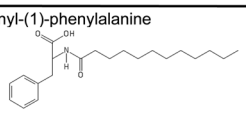
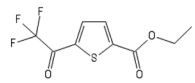
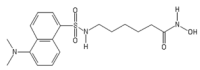
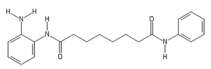
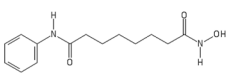
Name	Structure	IC50 (μM) CLASS I HDAC1	HDAC2	HDAC3	HDAC8	CLASS IIa HDAC4	HDAC5	HDAC7	HDAC9	CLASS IIb HDAC6	HDAC10	CLASS IV HDAC11	Substrate	Re.
MC1568		38.72	NA	NA	NA	0.22	0.22	0.22	0.22	0.22	NA	NA	Acetyl.pep	[73] [75]
MC1575		>20	>20	>20	>20	5	NA	NA	NA	NA	NA	NA	Class I Acetyl-H3 Class IIa Trifluoroacetyl Lys	[74]
LMK235		0.320	0.881	NA	1.278	0.0119	0.00422	NA	NA	0.0557	NA	0.852	Class I ac-H3 Class IIa Boc-Lys trifluoro-acetyl-AMC Class IIb and IV ac-p53	[71]
TMP269		>100	>100	>100	4.2	0.157	0.097	0.043	0.023	8,2	>100	>100	Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9	[26]
TMP195		>100	>100	>100	11.7	0.111	0.106	0.046	0.009	47,8	>100	>100	Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9	[26]
N-hydroxy-2,2 -diphenylacetamide		>10	6.06	NA	66	0.75	0.14	0.39	NA	>10	NA	NA	Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(e-trifluor methylacetyl-AMC HDAC6/8 ^{3}H-histone H4 peptide	[80]
N-hydroxy-9H -xanthene-9-carboxamide		NA	NA	NA	NA	0.25	0.11	0.05	NA	NA	NA	NA	Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC HDAC6/8 ^{3}H-histone H4 peptide	[80]
N-lauronyl-(1)-phenylalanine		>100	NA	NA	NA	NA	NA	21	NA	>100	NA	NA	Boc-Lys-(Ac)-AMC	[81]
Ethyl 5-(trifluoracetyl) thiopene-2-carboxylate		5.7	NA	3.5	NA	0.32	NA	NA	NA	0.55	NA	NA	Fluor de Lys HDAC1,3 Trfluoroacetamide -Lys HDAC4,6	[67]
Compound 2		0.95	1.38	1.12	3.98	0.33	0.40	2.56	NA	0.13	0.42	0.48	Fluor de Lys	[85]
BML-210		37.06	22.76	5.09	>300	NA	NA	>300	NA	>300	>300	NA	Competition binding	[118]
SAHA		0.22	0.56	1.79	2.74	>10	1.3	>10	>10	0.027	0.11	0.082	Class I, IIb, IV Fluor de Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC	[80] [85]

Fig. 3 Structures and summary of the available literature data on the IC50 for the proposed class IIa inhibitors

in cancer cells finds rationality in the capability of up-regulating the tumor suppressor Brahma, repressed by HDAC9 [77]. Curiously, MC1568 has been reported stabilizing the HDAC4-MEF2D complex in differentiated C2C12 myoblasts, thus impairing instead of favoring myogenesis [78].

– LMK235 (N-((6-(hydroxyamino)-6-oxoh exyl)oxy)-3,5-dimethylbenzamide) is a hybrid between two

classes of class I HDACis: the hydroxamic acids and the benzamides (Fig. 3) [71]. The specificity towards HDAC4 and HDAC5 is conveyed by the hydrophobic dimethyl substituted phenyl ring, which acts as a CAP group, matching class IIa active site better than class I [71]. This modification makes the molecule less toxic and more suitable for the treatment of some malignances, when compared to class I HDACis. Furthermore, LMK235 is able to re-sensitize cancer cells to cisplatin, better than SAHA [71].

- TMP269 and 195 (Figs. 3, 5) are two recently developed class IIa HDACis in which the classical hydroxamic Zn^{2+} binding domain is substituted by a trifluoromethyloxadiazolyl group (TFMO) [26] that highly resembles the trifluoromethylketone (TFMK) adopted by Bottomley and colleagues in their biochemical study of the ZBD [31]. The ring structure of the TFMO group increases its stability with respect to the highly unstable TFMK series of compounds [79]. Moreover, this TFMO moiety, differently from hydroxamate, acts as a non-chelating metal binding group, which interacts with the "catalytic" Zn^{2+}, through weak electrostatic interactions. As a consequence, the TFMO series has fewer off-targets compared to hydroxamates. Augmented selectivity is indirectly proved by gene expression profile studies in (PHA)-activated human peripheral blood mononuclear cells (PBMC) (Fig. 5). In these cells SAHA modulates the expression of 4,556 genes, whereas TMP195 regulates only 76 genes [26]. Curiously this finding is in accordance to what was observed in fibroblasts, where HDAC4 directly modulate only 76 genes [38]. To better characterize the transcriptome profile induced by their TFMO series of compounds, Lobera and colleagues purified T cells (CD3+), B cells (CD19+) and monocytes (CD14+) from the PHA-stimulated PBMC population and separately treated the three subpopulations with TMP195. T and B cells turned out to be very low sensitive to TMP195 (17 and 36 genes regulated, respectively); on the contrary the effect of the compound on monocytes was impressive (587 genes) and was not due to an increase in the expression of class IIa HDACs in these cells compared to the other two cell types. In particular the inhibitor interfered with monocytes to macrophages M-CSF (macrophage colony-stimulating factor)-induced differentiation. These findings candidate class IIa HDACs as druggable targets for immunological diseases [18, 71].
- *N*-hydroxy-2,2-diphenylacetamide and *N*-hydroxy-9H-xanthene-9-carboxamide (respectively, compound 6 and 13 in the original manuscript) are two diphenylmethylene hydroxamic acids characterized by Besterman group as class IIa HDACs specific inhibitors active in the μM range [80]. Both molecules exhibit a certain degree of symmetry and the second compound could be considered as the rigidification of the diphenyl moiety of the first (Fig. 3). This modification increases the specificity of the molecule towards HDAC7 [80].
- *N*-lauroyl-(l)-phenylalanine is a class IIa HDACi active in the μM range (Fig. 3) [81]. It was identified during a screening of a commercial available library of compounds. The specificity was scored not merely by classical measurements of HDAC activity but also through a fluorescence assay, which exploits the competition between a fluorescent substrate and the putative inhibitor for each purified HDAC [81]. This molecule shows anti-tumoral properties against ER+ breast cancer cells and can influence the expression of some MEF2-target genes (Fig. 5) [37].
- Ethyl 5-(trifluoroacetyl)thiophene-2-carboxylate [67] is the founder of a class of compounds, the trifluoroacetylthiophenes, that targets class II HDACs (class IIa and HDAC6) with some specificity. It was identified during a screening of a commercially available library of compounds using both the wt and the GOF mutant of HDAC4 as targets. It is a tripartite molecule characterized by: (i) a trifluoromethyl ketone group that chelates the active site zinc in a bidentate manner, (ii) the central thiophene ring that fits perfectly to class IIa active site and (iii) the amide group that interacts with the surrounding residues. The chemistry and the trifunctional nature of this compound justify its specificity.
- Tasquinimod (Fig. 4) is a promising drug for the treatment of advanced castration resistant prostate cancers [82, 83]. It acts by perturbing the tumor microenvironment. Differently from the aforementioned molecules it was not rationally designed or screened to target HDACs. Nevertheless, this carboxamide is able to enter the ZBD of HDAC4, keeping it in the inactive form and thus reorganizing the HDAC4 catalytic site. Tasquinimod-induced structural changes are causative of N-CoR/SMRT/HDAC3 displacement [43]. This finding is surprisingly considering the pronounced steric hindrance of the molecule, which is profoundly different from all SAHA derivatives. However, by virtue of its selective targeting of the "structural" Zn^{2+}", Tasquinimod molecular backbone could substitute SAHA as starting model for the development of specific inhibitors. From a molecular point of view the inactivation of HDAC4 prevents HIF-1α deacetylation, thus inducing its destabilization. Clinically, in hypoxic conditions the activation of HIF-1α transcriptional program stimulates the differentiation of tumor infiltrating myeloid derived suppressor cells into tumor-associated macrophage,

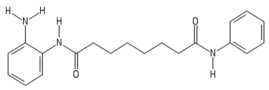
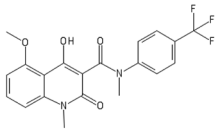
Name	Structure	DISPLACEMENT FROM PROTEIN BINDING IC50 (μM) HDAC4 - MEF2	HDAC4 - N-CoR	Assay	Re.
BML210		5	NA	Two-Hybrid in HeLa cells	[95]
Tasquinimod		NA	<1	Co-Ip in 293 cells	[43]

Fig. 4 Structure and binding interference properties of BML210 and Tasquinimod, two compounds capable of altering interaction of class IIa HDACs with their partners

which secrete pro-angiogenic factors [84]. Authors, therefore, proposed Tasquinimod as an anti-angiogenetic drug, which anti-cancer efficacy is being evaluated in pre-clinical models [43].

These last three molecules are considered unconventional inhibitors because, even though characterized by a tripartite motif, they are not SAHA derivatives.

Targeting the nuclear-cytoplasmic shuttling

In 2011, Brown group made the first attempt of blocking class II HDACs in the cytoplasm [85]. Starting from the structure of SAHA, they generated a couple of molecules by substituting the amino-phenyl group with a fluorescent dansyl group. This modification increases the specificity for class II HDACs in spite of a loss of reactivity against class I HDACs. If used in the μM range, the most effective molecule of the series, named compound 2 (Fig. 3), increases the fraction of cytoplasmic HDAC4 in prostate cancer cells PC3. The authors suggested that since the inhibitor accumulates in the cytoplasm, it binds HDAC4, thus impeding the interaction with importin-1α. As a consequence, the inhibitor increases the fraction of cells in the G1 phase of the cell cycle, the levels of p21/Cip1/Waf1, of acetylated H3 and tubulin. The increase of tubulin acetylation is probably due to the inhibition of HDAC6 [86] and seems to be unrelated to the suppression of class IIa [85].

It must be underlined that the IC50 values of these new inhibitors have been estimated by measuring the enzymatic activities of HDACs purified from mammalian cells, using the Fluor–de-Lys substrate [85]. Therefore, in the case of class IIa HDACs, it must be intended as indirect, deriving principally from the associated class I HDACs.

The strategy of interfering with class IIa HDACs nuclear accumulation could be attractive in oncology, as increasing evidences demonstrate that nuclear resident class IIa can display oncogenic functions [37, 38], but it might also present some drawbacks. First of all, class IIa HDACs possess also cytoplasmic functions [reviewed in 44], which could be amplified after inhibition of their nuclear import. Moreover, the cytoplasmic accumulation of class IIa HDACs is sometimes an indirect still uncertain effect of class I inhibition. For example the class I/II inhibitor LBH589, which is a SAHA derivate, confines HDAC4 in the cytoplasm in irradiated non-small cell lung cancer cells [87]. Considering all these drawbacks, the nucleus/cytoplasmic shuttling of class IIa HDACs seems to be the less druggable feature of these proteins.

Class IIa HDACs N-terminus, which allows their interaction with some partners, such as MEF2 family of TFs

As discussed above, class IIa HDACs' N-terminal region (Fig. 1) mediates the interaction with multiple partners and contains a glutamine-rich domain (with the exception of HDAC7) that allows homo- and heterodimerization among the different class IIa members [12, 88]. The best-characterized class IIa transcriptional partners are the MEF2s proteins [49, 89]. Several of the biological functions attributed to class IIa HDACs are the results of the MEF2s transcriptional repression [27, 37, 38]. The phenotype of the single knock-out of class IIa HDACs could be explained as the effect of MEF2 over-activation in bone (HDAC4), heart (HDAC5/9) and cardiovascular system (HDAC7), in relation to the district in which the single HDACs are more abundant [90–92]. Hence, the design of an inhibitor that displaces class IIa HDACs from MEF2s could be a good approach to selectively interfere with this specific repressive exploit. A limitation to this strategy concerns the promiscuity of the class IIa HDACs sequence required for this interaction (aa 166-184 in HDAC4). In fact, this stretch of amino acids is also involved in the interaction with additional partners, among which, the nucleoporin Nup155 [93] and the demethylase JARID1B [94] (Fig. 1). An alternative plan to influence the MEF2-

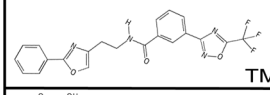
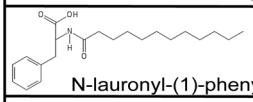
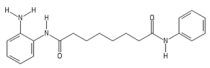
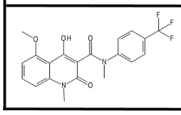
Compound	Cells	Treatment	Up-reg. MEF2-targets	Experiment	Re.
 TMP195	PBMCs PHA-stimulated	3μM 60 hrs	*ASB2,CCL1,ATP1B2*	DNA Microarray	[26]
 N-lauronyl-(1)-phenylalanine	MCF7	100μM 48 hrs	*KLF2, RHOB, NR4A1 KLF3, MARK1, GADD45γ*	qRT PCR	[37]
 BML-210	SK-UT-1 SK-LMA-1 DMR	10μM 36 hrs	*KLF2, RHOB, NR4A1*	qRT PCR	[38]
 Tasquinimod	LNCap	50μM 24 hrs	*IRS1, DTNA, ARRDC3 LHX4, KCNG1, ISL2*	DNA Microarray	[82]

Fig. 5 Summary of the available literature data on the effect of class IIa HDACs inhibitors on MEF2s-dependent transcription. MEF2s are the foremost characterized transcriptional partners of class IIa HDACs. Hence, an effect of these inhibitors on the expression of MEF2s target genes is an important read-out of their activity

HDAC axis could be targeting the region of MEF2s that interacts with class IIa HDACs. Using this approach, BML-210 (Figs. 3, 4, 5), a weak class I HDAC benzamide inhibitor, was found to interact through its aminophenyl-group with the hydrophobic residues of MEF2s (aa 66-69) thus displacing class IIa HDACs [95]. Using the crystal structure of the HDAC9–MEF2B complex as a guide [96], authors generated a panel of more powerful BML-210 derivatives. In the next future it will be important to further improve the specificity of these compounds to exclude residual targeting of class I HDACs.

Conclusions and perspectives

The identification of molecules that could reset the transcriptional profile in neoplastic cells has raised many hopes for new anti-cancer therapies [97]. Unfortunately today this goal has been only partially reached. Nevertheless an epigenetic therapy against cancer is still subject of intense research. A new impetus in this field was given by the discovery of the demethylases [98, 99] and the synthesis of their specific inhibitors [100]. A more niche-research concerns class IIa HDACs and their selective inhibitors, which are hypothesized to be less powerful than pan-HDACis but more specific. However, these studies are still in their infancy and the applicability of class IIa HDACis in clinic requires still intense laboratory characterization. Additional experiments and data are mandatory to characterize and understand the contribution of these molecules to epigenetic changes in vivo. Up to now, information about the impact of class IIa HDACis on RNA non-coding world and the role of class IIa HDACs in stemness maintenance are very limited [101]. In parallel the efforts trying to design, isolate and characterize new compounds, acting as epigenetic regulators must persist. In addition, a robust in vitro pre-clinical characterization of molecules already available is needed to define: their molecular mechanism of action, their ideal context of utilization and off-targets effects. All these efforts are justified by the benefits that drug-induced genetic reprogramming could exert on different diseases.

Certainly anti-cancer therapy is the first and most important scope. Nevertheless, the involvement of class IIa HDACs in the regulation of Glut4 [102–105], of the NF-kB pathway [106, 107] and of many neuronal activities [108–111] could stimulate studies about the employment of class IIa HDACis for the treatment of diseases other than cancer, such as diabetes [112], neurodegenerative disorders [113, 114] and inflammatory diseases [26, 115–118]. There are opportunities out there; we just have to find out what is the best compound for each specific application.

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