Acetylation and histone deacetylase inhibitors in cancer

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1. The diverse field of epigenetics

In recent years, the role of epigenetics in the development and treatment of cancer has gained interest and the effects of internal and external factors on the epigenetic profile are under investigation. The term “epigenetics” refers to modifications that influence phenotype without altering genotype. Epigenetic changes are potentially reversible but generally stably maintained during the cell cycle. Since Feinberg et al. described differences in DNA methylation in human cancer in 1983 [66], several mechanisms of epigenetic control have been identified such as DNA methylation and histone modifications including acetylation, methylation and phosphorylation. Recently Seligson et al. reported that specific histone modification patterns are predictive of the risk of prostate cancer recurrence [183] and Fraga et al. showed that a profound disruption in histone modification patterns is a common feature of cancer [70]. Epigenetic abnormalities can be acquired during fetal development and during the course of a life contributing to common cancer risk in adults [65]. Recently Fraga et al. reported that, although monozygotic twins are epigenetically identical during the first years of life, the patterns of global and locus specific epigenetic modifications and gene expression patterns in monozygotic twin pairs diverge as they become older [70]. These differences could be explained by the influence of external factors such as smoking, physical activity and diet [17,61] as well as by accumulation of small defects in epigenetic information, that could be considered an “epigenetic drift” associated with the aging process [13].

In 1974, Kornberg suggested that the structural organization of chromatin is based on a repeating unit of about 200 base pairs and eight histone molecules [116]. A year later Baldwin proposed that this histone octamer serves as a protein spool around which DNA is wrapped [7]. In the same year Oudet et al. provided the first electron microscopic images of eukaryotic genome proving the existence of the repeating uniformly sized particles in DNA [151]. Referring to their nuclear origin and their resemblance to “nu bodies” [148], Oudet et al. named these spherical particles nucleosomes. Since then several others have tried to elucidate the structure of the histone complex, but it took until 1991 to solve the definitive X-ray structure of the octameric histone core of the nucleosome [2]. A chronologic review of the discoveries that led to the establishment of the nucleosome as a repeating unit in DNA was published in 2003 by Olins and Olins [149].

The nucleosome core consists of 147 base pairs [57] of double-stranded DNA wrapped 1.65 times around an octamer of two copies each of histone 2A (H2A), 2B (H2B), 3 (H3) and 4 (H4) protein (Fig. 1). The atomic structures of the dimerized H2A [106] and H3 and H4 [117] are shown in Fig. 2. There are >120 direct atomic interactions of the nucleosome core with the DNA backbone at 14 super helix locations [126]. The repeating nucleosome core particles assemble into higher order helices, which are stabilized by a linker histone H1 to further condense the chromatin and together make up the nucleosome. About 25% of nucleosome core histone is comprised of amino-terminal tails that protrude the enveloping DNA double helix [206].
Fig. 1. Schematic structure of histones in nucleosomes. (a) The core proteins of nucleosomes are designated H2A (Histone 2A), H2B (Histone 2B), H3 (histone 3) and H4 (histone 4). Each histone is present in two copies, so the DNA (black) wraps around an octamer of histones called the core nucleosome. (b) The amino terminal tails of core histones, lysines (K) in the amino-terminal tails of histones H2A, H2B, H3 and H4 are potential acetylation/deacetylation sites for histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation neutralizes the charge on lysines. A, acetyl; C, carboxyl terminus; E, glutamic acid; M, methyl; N, amino terminus; P, phosphate; S, serine; Ub, ubiquitin. (Reproduced with permission from Nature Reviews Cancer (Nat. Rev. Cancer 1(3) (2001), 194–202), copyright (2001) Macmillan Magazines Ltd.)

Although they are unstructured on a single nucleosome level, these N-terminal tails are thought to mediate interactions with other nucleosomes and chromatin proteins affecting higher order chromatin structure. The highly efficient way of DNA packaging by nucleosome formation compresses the DNA ~10,000 times forcing chromatin in a repressive state and inaccessible for nuclear processes like transcription. When Oudet et al. provided the first images of repeating uniformly sized particles in DNA in 1975, they hypothesized that their observations might have a function in genomic expression. Consistent with these predictions Boeger et al. showed in 2003 that nucleosomes can unfold completely at transcriptionally active promoters [18]. An overview on the dynamic structure and function of chromatin has been published by Hansen in 2002 [89].

2. The histone code

In 1993, Turner presented the first evidence that post-translational modification of histone tails was functionally significant [191]. In the years that followed, increasing experimental data provided support for the hypothesis that distinct patterns of covalent histone marks make up a histone ‘language’. Encoded on histone tail domains and read by other proteins or proteins modules, these modifications are thought to determine the transcriptional state of genes. Post-translational modifications important in the development and progression of cancer include acetylation, methylation, phosphorylation and ubiquitination. Figure 1 gives a schematic overview of the core nucleosome and important modification sites on amino terminal tails.

In 2000, Strahl and Allis referred for the first time to this language as the ‘histone code’ defining it as “multiple histone modifications acting in a combinatorial or sequential fashion on one or multiple histone tails, specifying unique downstream functions” [187]. An important concept in this hypothesis is that the histone code uses combinations of modifications on each histone and that modifications on different histone tails may be interdependent. It is therefore essential that histone modifications are site-specific and that these modifications cause site specific chromatin modification [187]. Recently two other hypotheses have been added to explain the important function histone modifications serve. The ‘modification cassettes model’ proposes that numerous residues in linear strings of densely modifiable sites can have a large array of different biological readouts by forming cassettes. In the second model neighboring modifications act together as ‘binary switches’ [68].
3. Nucleosome dynamics and epigenetic modifications

Although DNA packaging is necessary to store the ~2 meters of DNA that make up the human genome in the nucleus, the complex structure of nucleosomes and many interactions with the DNA backbone impairs accessibility to chromatin. Access to chromatin is essential for proteins that regulate biological processes like transcription, DNA repair and replication to exert their function. To counterbalance the repressive nature of chromatin, a sophisticated mechanism has evolved that regulates chromatin accessibility.

3.1. Chromatin remodeling complexes and chromatin modification complexes

Unlike assumed previously, nucleosomes have emerged to be highly specialized, serving many functions in the regulation of individual genes and chromosome regions. Although all nucleosomes contain a histone octamer around which DNA is wrapped, chromatin remodeling and chromatin modifying complexes allow nucleosomes to have a specialized and dynamic composition [31].

N-terminal histone tails, which protrude from the surface of the chromatin polymer, possess many post-translational modification sites. These modification sites can be recognized by ATP-dependent nucleosome-remodeling complexes that possess chromatin-binding domains that recognize specific modifications patterns (see below). ATP-dependent chromatin-remodeling complexes regulate chromatin accessibility using ATP hydrolysis to weaken histone-DNA contacts. At least three ways of creating access to nucleosomal DNA by chromatin remodeling complexes have been identified [110]: octamer sliding, DNA looping and histone substitution [64,102,136]. By weakening DNA con-
tacts these complexes are able to expose DNA to proteins that for instance regulate transcription to reach their target sites.

An overview of recent literature on chromatin remodeling complexes is provided by Cosgrove et al. [55] and Cairns [31].

The ε-amino groups evolutionarily conserved on lysine residues of histones tails can be post-translationally modified by chromatin-modifying enzymes causing acetylation, methylation and ubiquitination. Such seemingly small modifications determine not only the structural organization of chromatin, by virtue of their ionic charges, but also attract other proteins that possess modification-specific chromatin-binding domains (see below) to sites in the chromatin where transcription may be regulated [205].

3.2. Chromatin-binding domains

As described, both chromatin remodeling and chromatin modification make use of chromatin-binding domains, called protein motifs, which recognize specific modifications caused by chromatin modifying complexes on histone tails and non-histone proteins. Several chromatin-binding domains can be identified. Below three domains important in the context of the histone code hypothesis will be discussed: bromodomains, chromodomains and SANT domains.

3.2.1. Bromodomains

Bromodomains form an extensive family of small protein domains that preferentially bind acetylated peptides irrespective of the protein to which it belongs [58]. Bromodomains are widely distributed among different enzymes such as chromatin remodeling enzymes that utilize ATP to modify chromatin structure, but also in subunits of the chromatin remodeling complexes that do not have a catalytic function. In that case bromodomains mainly help to recognize previously modified chromatin and stabilize the complex [103]. Bromodomains have also been found in enzymes that cause methylation and acetylation. Interestingly, since histone acetyl transferases (HATs) acetylate a wide variety of target proteins (see below), the presence of a bromodomain on most HATs suggests self perpetuation through a positive feedback loop [58].

3.2.2. Chromodomains

As with bromodomains, chromodomains bind to their target protein independent of the protein to which they belong. Although their distribution among enzymes is more restricted than with bromodomains, chromodomains have been found in ATP-dependent chromatin remodeling factors, histone acetyltransferases and methyltransferases too. Chromodomains have been shown to recognize methyl-lysines [10], DNA and RNA and point to an involvement in protein–protein interactions [21]. However their exact function in the context of gene expression is not yet fully understood.

3.2.3. SANT domains

Unlike bromodomains and chromodomains, SANT domains primarily mediate interactions between remodeling complexes and unmodified chromatin substrates through the recruitment of chromatin modifying enzymes and by mediating interactions between histones and enzymes. SANT domains have been shown to be present in several components of complexes containing Histone Deacetylase (HDAC) or HAT activity; although no HAT or HDAC enzyme itself has been found to possess a SANT domain to date [58]. In ATP-dependent chromatin remodeling complexes, SANT domains are broadly present. By direct binding, ATP-dependent chromatin remodeling complexes cause conformational change of histone tails thereby promoting binding of modifying enzymes and subsequent catalytic processes. As is suggested by Yu et al., the interaction of SANT domains with unacetylated histone tails could block the binding of HATs, thereby maintaining a deacetylated state [214]. A review on the unique function of SANT-domains has been written by Boyer et al. in 2004 [22].

A more extensive review on the diversity of proteins containing protein motifs and their function has been published by de la Cruz et al. in 2005 [58].

4. Acetylation

As briefly discussed above, modification of histone tails is an important feature in the regulation of gene expression. Limited by the 20 encoded amino acids available, modification of proteins extends the range of molecular structures and functions possible. Many proteins are post- or co-translationally acetylated. Out of the more than 200 covalent modifications that have been reported, acetylation is the most common modification of eukaryotic proteins affecting many protein functions such as transcription, nuclear import, microtubule function, hormone responses, peptide-receptor recognition, DNA binding and protein–protein interactions. Modification of proteins not only changes its
molecular structure, but also provides binding sites for modification recognizing protein motifs such as bromodomains. The acetylation status of histones is regulated by the antagonistic actions of HATs and HDACs. Both HATs and HDACs function as part of multiprotein complexes with other proteins [81,177], or self-associate with each other [84,197].

4.1. HDACs

HDACs may be classified into four subfamilies, i.e., class I, class II, class III and the HDAC11-related enzymes (Table 1) [82]. The class I HDACs possess a molecular weight of 22 to 55 kDa and share homologous catalytic sites. The class II HDACs range from 120 to 135 kDa and, unlike the class I HDACs, exhibit greater diversity within the class. HDAC6 and −10 for example are characterized by duplicated HDAC catalytic domains while the other members of the same class display only one catalytic domain [197]. Class I HDACs are located exclusively in the nucleus with the exception of HDAC8 which has recently been shown to be predominantly expressed in the cytosol [201]. Class II HDACs shuttle between the nucleus and cytoplasm depending upon perceived cellular signals. Class III HDACs are known as the Sir2-like HDACs due to their homology to yeast Sir2 proteins. While class I and II are Zn-dependent HDACs, the class III HDACs are Zn-independent and NAD-dependent. In addition, the HDAC11-related enzymes, which share the features of class I and II HDACs but may have a distinct physiological role, could potentially constitute a fourth class of HDACs.

Despite the existence of several HDAC subfamilies, the different HDACs are by no means redundant. Robyr et al. generated ‘acetylation maps’ by inactivating six different HDACs, demonstrating that only a small degree of functional overlap was present among the different HDACs [171]. HDAC5 and 9 are for example involved in stress response of the heart [41], HDAC2 in apoptosis [220], HDAC1 in modulating the cell cycle and HDAC8 in smooth muscle contractility [200]. HDAC activity also differs between different types of tissues [48,109]. Screening of HDAC expression in human prostate cancer for instance revealed distinct class I HDAC profiles between stromal and epithelial cells [201] and in vitro experiments with prostate cancer cell lines showed a marked increase in HDAC levels for most HDACs compared to normal prostate tissue [99]. Furthermore, different isoforms of HDACs may have distinct localization and functions [152]. Even during embryonic development the levels of HDACs are continuously changing [129]. Targeted disruption of both HDAC1 alleles results in severe proliferation defects and retardation in development, leading to embryonic lethality [121]. Certain disease states can be characterized by loss or gain of specific or generalized HDAC activity, for example, reduced protein expression of HDAC1 and −2 proteins and decreased enzymatic HDAC activity is observed in asthma patients [97], while class II HDACs suppress cardiac hypertrophy [130] and higher expression of HDAC2 and −9 has been reported in many colon cancer cell lines compared with the primary cell from corresponding normal tissue [152,220].

Malignant diseases are exemplified by aberrant transcriptional regulation which may be triggered by increased recruitment of HDACs to the site of transcriptional initiation. In acute promyelocytic leukemia (APL) for example, it has been shown that the PLZF/RARα fusion protein causes oligomerization of RAR, imposing an altered interaction with transcriptional co-regulators that recruit HDACs [133]. The resulting continuous activity of the HDAC-complex at the promoters of target genes of the PLZF/RARα fusion protein leads to a repression of these genes and is the determinant of resistance to retinoic acid (RA) treatment [83]. Co-treatment with HDACIs relieves this transcriptional repression and leads to differentiation of myeloid cells in vitro and induced a clinical response in an RA-resistant APL patient [203]. Oligomerization and altered recruitment of HDACs are also responsible for malignant transformation by the non-APL Acute Myeloid Leukemia (AML) fusion protein AML1-ETO [127,202], which blocks RA signaling too [134], suggesting that interference with the RA pathway by HDAC recruitment may be a common theme in AMLs [134]. Thus, as malignant cells express

| Table 1 | Classification of HDAC subfamilies |
|---------|-------------------------------|
| Class I | Class II | Class III | Class IV |
| HDAC1   | HDAC4   | SIRT1     | HDAC11   |
| HDAC2   | HDAC5   | SIRT2     |          |
| HDAC3   | HDAC6   | SIRT3     |          |
| HDAC8   | HDAC7   | SIRT4     |          |
| HDAC9   | HDAC10  | SIRT5     |          |
|         | SIRT6   | SIRT7     |          |

The classification is based on the homology of human HDACs to yeast HDACs, their sub-cellular expression and enzymatic activity. HDAC – histone deacetylase, SIRT – silent information regulator.
increased HDAC activity, irrespective of the trigger of such activity, it is thought that inhibitors of HDACs return this transient aberrant transcription in malignant cells to the transcription status of their normal cell counterpart, while leaving the surrounding normal cells unaltered. With this premise, HDAC inhibitors (HDACIs) were investigated for activity and found to be relatively non-toxic to normal cells in vitro and in vivo despite the accumulation of acetylation in normal cells as well as tumor cells [96]. Successful preclinical studies and development of animal models has resulted in a number of HDACIs being evaluated in human clinical trials (see below).

4.2. HATs

Although hyperacetylation has been mainly associated with transcriptional activation, the effects of acetylation depend on the setting and the genes involved. As is shown for instance by Guidez et al. [86] acetylation of lysine residues can also lead to transcriptional repression. Acetylation of internal lysine residues is facilitated by the action of HATs. By definition HATs acetylate histone tails by transferring acetyl groups from acetyl coenzyme A (acetyl-CoA) onto the ε-amino group of conserved lysine residues. However, HATs are also able to acetylate non-histone proteins and are therefore, sometimes referred to as FATs (factor acetyltransferases). Post-translational acetylation of lysine residues occurs in histones, high mobility group (HMG) proteins, transcription factors, nuclear receptors and α-tubulin [159]. Important in the context of cancer is that post-translational acetylation of the ε-amino group of lysine residues is known to be reversible, making it an attractive therapeutic target. HATs are composed of several domains making them substrate- and site-specific under distinct physiological situations. Three super families of HATs can be identified based on their composition: GNAT (Gcn5-related N-acetyl transferases) [142], MYST (named after its founding members MOZ, Ybf2/Sas3, Sas2 and Tip60) [20] and p300/CBP (CREB-binding protein) [8,147].

4.2.1. GNAT super family

The GNAT super family acetylates a distinct subset of genes. All GNAT enzymes contain four functional domains (Fig. 3): an amino terminal, a catalytic motif domain, Ada2p interaction domain and a carboxy-terminal bromodomain. The amino-terminal is variable in length and is thought to facilitate recognition of nucleosomal substrates [207]. The catalytic motif domain actually contains up to four conserved motifs labeled A–D of which motif A, the highly conserved acetyl-CoA binding site, is common to HATs from all super families and essential for HAT activity [35] (Fig. 3). The Ada2p interaction domain enables GNATs to acetylate physiologically relevant nucleosomal substrates in vivo. Ada2 proteins interact with DNA-bound transcriptional activators [35]. The C-terminal bromodomain binds to acetylated lysine residues facilitating protein–protein interactions. Secondly, the bromodomain is able to affect HAT activity by autoacetylation causing the HAT to fold into an inactive state [182].

4.2.2. MYST super family

Besides an amino terminal tail, an acetyltransferase domain A and a C-terminal domain, MYST super family HATs sometimes contain zinc-finger domains and a chromodomain. The function of the chromodomain in the context of HATs is unclear. Likely, chromodomains serve as chromatin targeting modules for the MYST family similar to the bromodomains of the GNAT family (Fig. 3).

4.2.3. p300/CBP super family

Contrary to the GNAT super family, the p300/CBP super family generally acts as a global transcriptional
activator. p300 and CBP are two of the most widely studied HATs in the context of transcriptional regulation. They are largely interchangeable in function and are therefore, often referred to as p300/CBP. As co-activators for transcription, p300/CBP is particularly recruited to promoters by DNA-bound transcription factors that need p300/CBP to function in transcriptional activation. p300/CBP contain at least two independent regions for interaction with multiple transcription factors such as c-Jun [9] and nuclear hormone receptors [101]. Furthermore p300/CBP contains a bromodomain, three Zinc finger regions (cys, ZZ and TAZ domains) and a HAT domain containing a p300/CBP specific E motif (Fig. 3). The distribution of p300/CBP among the different target proteins might provide a mechanism for integrating several signaling and transcription response pathways since p300/CBP is present in limited amounts in the cell [101]. Research on HATs to date is basic and preclinical with limited, if any, clinical candidates. We preferred to include HATs because of their potential as therapeutic targets. A more detailed review on histone acetyltransferases is provided by Roth et al. [177].

5. Histone deacetylase inhibitors

The important role of acetylation and deacetylation in transcriptional regulation makes HATs and HDACs a promising target for anticancer therapies. Although the field of epigenetics is in its infancy, discoveries and translation to the clinic have moved at a remarkable speed. Drugs targeting epigenetic changes are in phase I trials and are moving to phase II trials for several types of histone deacetylase inhibitors (HDACIs). DNA methyl transferase inhibitors were recently approved by the US FDA for their clinical benefit in patients with myelodysplasia.

The microbial toxin Trichostatin A (TSA) was an early compound that was identified to possess HDAC inhibitory activity. After establishing its efficacy as an HDACI, TSA was used to model other HDACI such as vorinostat (also known as suberoylanilide hydroxamic acid, SAHA) and m-carboxycinnamic acid bis-hydroxamide (CBHA; Memorial Sloan-Kettering Cancer Center) [170], which in turn served as a template for PXD-101 (Prolifix Ltd/CuraGen Corp) and LAQ-824 (Novartis AG). Another natural compound to be discovered as a non-competitive inhibitor of HDAC was sodium butyrate (SB) [36]. Like other HDACIs of the same structural type, the clinical relevance of SB was limited by its short half-life and low potency. However, several improved derivatives of SB were later characterized and screened, including the drugs phenyl butyrate, phenyl acetate (which is the β-oxidation product of phenyl butyrate in vivo), valproic acid (VPA), and AN-9 (pivaloyloxymethyl butyrate, Titan Pharmaceuticals Inc), the prodrug form of butanoic acid [146].

The rate of development of newer HDACIs has greatly increased following the elucidation of the HDACI–HDAC interaction [67]. The ability of HDACIs to chelate zinc bound to the active site of the HDAC appeared to be crucial to maximum inhibitory activity, as exemplified by the general structure illustrated in Fig. 4, which is based on vorinostat. This explained why HDACIs such as the aliphatic acids, which lack a zinc binding moiety, required higher concentrations (mM) of drug for activity, compared with the nanomolar quantities of drugs of the hydroxamic acid type. As a result of this research, a novel class of HDACIs with a Zn$^{2+}$-chelating motif-tethered to short-chain fatty acids has been synthesized [125]. Cyclic peptides, such as FK-228 (National Cancer Institute/Gloucester Pharmaceuticals Inc), which do not possess the characteristic structure outlined above, are also active at HDAC inhibition at nanomolar concentrations, and research conducted by Furumai et al. helped to decipher the mechanism of action of such compounds [73]. Cellular reducing activity converts FK-228 to its active, reduced form (redFK), which possesses a functional sulfhydryl group capable of interacting with the zinc in the active-site pocket of HDACs. Because FK-228 is more stable than redFK in medium and serum, FK-228 may be the natural prodrug which is activated after incorporation in to the cells. More recent developments of HDACIs belong to the class of electrophilic ketones and include various trifluoromethyl ketones and α-ketoamides which are active at micromolar concentrations [71,195]. Special attention should also be given to the emergence of...
HDAC-specific HDACIs such as Tubacin, an HDAC6-specific HDACI [88] and MGCD0103 an HDAC1-3 specific HDACI [137].

5.1. Mechanisms of action

By changing the acetylation status of histone tails HDACIs modify the histone code leading to changes in gene expression [187] (see above). Furthermore, by accumulating acetylated histones, HDACIs relax chromatin structure by virtue of their ionic charges thereby enhancing the accessibility of the transcription machinery to DNA leading to increased transcription [205]. The accumulation of acetylated histones may also affect cell cycle progression. By inhibition of chromatin separation, the ability of tumor cells to undergo mitosis is altered [50]. Additionally, HDACIs specifically affect the cell cycle of tumor cells by targeting cells with deficient cell cycle checkpoint controls causing apoptosis [204]. Generation of reactive oxygen species also plays a role in HDACI-induced cell death. In a study published in 2005, Rueffli et al. showed that vorinostat-induced Bid cleavage and disruption of the mitochondrial membrane lead to cell death in the absence of activation of key caspases [179]. Recent work by Xu et al., suggests that anti-tumor effects of vorinostat in treated cancer cells is achieved in part by induction of polyploidy followed by senescence [208].

6. The cellular and biological effects of HDAC inhibitors

HDACIs demonstrate pleiotropic effects on cells in culture and in vivo. Their effect is dependent on several factors including cell type. Recently reported frameshift mutations in HDAC2 in colon, gastric and endometrial tumors have shown to diminish the effect of treatment with some, but not all, HDACIs using colorectal cell lines in vivo [172]. Other factors that are important for the response to HDACI include dose and duration of treatment and whether HDACIs are given in combination with other agents.

6.1. Activity in vitro of HDACIs

TSA was evaluated for its biological activity in a variety of mammalian cell lines and was demonstrated to prolong the half-life of acetylated histones in mouse mammary gland tumor cells [211]. Pulse-chase experiments revealed that histone hyperacetylation induced by TSA was not due to increased acetylation but was a result of decreased deacetylation of histones. Many mammalian cell lines have since been investigated for their sensitivity to various HDACIs. Examples include differentiation by TSA in breast cancer cell lines [198], apoptosis in endometrial cancer cell lines [188] and hepatoma cells [45], and apoptosis of neuroblastoma cell lines by CBHA either administered alone or in combination with retinoic acid [52]. Without testing its ability to act as an HDAC inhibitor, Carducci et al., demonstrated the pro-apoptotic effect of PB on prostate cancer cell lines [40]. Later, Butler et al., demonstrated the anti-proliferative effect of vorinostat on prostate cancer cell lines and simultaneously demonstrated the HDAC inhibitory activity by detecting presence of acetylated histones [28].

6.1.1. Cell cycle effects

HDACIs arrest growth in different phases of the cell cycle in different cell lines [163]. Most HDACIs, with the notable exception of tubacin, exert a dose-dependent effect on growth arrest in G1 [88]. The arrest is mediated by the p53-independent induction of p21CIP1/WAF1 [169,199], loss of activity of cyclin-dependent kinase (Cdk) [181] and transcriptional inactivation of the enzymes CTP synthase and thymidylate synthetase, which are responsible for DNA synthesis in the S phase [220]. G2 arrest has been detected in comparatively fewer cell lines and requires a higher dose of HDACIs compared with G1 arrest [27,181]. However, higher doses of HDACIs also induce apoptosis of cancer cells (as discussed below). Research by Qiu et al. and Warrener et al. has emphasized this dilemma by highlighting the role of checkpoints in cell cycle progression [163,204]. Cells exhibiting an intact G2 checkpoint were growth arrested on treatment with HDACIs while cells possessing a defective G2 checkpoint undergo apoptosis within hours of mitotic exit [204] (Table 2).

6.1.2. Induction of apoptosis

Treatment of cells with HDACIs can induce or repress genes to cause cell cycle arrest in different cancer cell types. HDACIs can also increase expression of pro-apoptotic genes such as Bak, Bax, CD95, CD95 ligand, gelsolin, p53, GADD45, DRAK1, Apaf-1, DFF45, caspase-9, caspase-8, caspase-3, Bim, Bid and Bad, and decrease expression of anti-apoptotic genes including Bcl-2, Bcl-xL, c-FLIP, survivin, XIAP, Mcl-1 and NFkB [3,44,62,91,92,98,135,217]. Apoptosis, mediated by varied pathways, was induced in many cell lines treated with HDACIs as a function
Table 2

| Effects            | Process                  | Pathway                                      | References |
|--------------------|--------------------------|----------------------------------------------|------------|
| Cell cycle arrest  | G1-arrest                | p53-independent p21 induction                | [169,199]  |
|                    |                          | Loss of Cdk activity                         | [181]      |
| Apoptosis          | Defective G2/M checkpoint| Loss of the G2/M checkpoint results in catastrophic mitosis | [163,204]  |
|                    | Mitochondrial injury     | Induction of expression of pro-apoptotic genes | [199]      |
|                    | p21-deficiency           | Induction of p21 inhibits apoptosis by inducing G1 cell cycle arrest | [27]       |
|                    |                          | Conversely p21-deficient cell lines do not arrest cells in G1. p21-deficiency can therefore cause catastrophic mitosis in cells with a defective G2/M checkpoint. |           |
| Generation of ROS  |                          | Activation of caspase cascade                | [174,179]  |

HDAC – histone deacetylase, Cdk – cyclin-dependent kinase, ROS – reactive oxygen species.

of dose and duration of incubation. In U937 human leukemia cells, vorinostat induced differentiation at low doses (sub-micromolar) but at higher doses the drug caused apoptosis by triggering mitochondrial injury [199]. The apoptotic effect of HDACIs is also cell line-specific, as demonstrated by the analysis of a large panel of HDACI-sensitive cell lines, in which selected cell lines required up to 10-fold lower concentrations of HDACIs in order to achieve apoptosis [27]. Cancer cells were revealed to be 10-fold more sensitive to hydroxamates than normal fibroblasts, possibly due to the loss of the G2 checkpoint, a feature exploited by HDACIs to selectively kill proliferating and non-proliferating tumor cells but not normal cells [26] (Table 2).

Recently, apoptosis has been reported to be induced by generation of reactive oxygen species (ROS) by various HDACIs (reviewed in reference [164]). ROS production leads to activation of the caspase cascade and degradation of critical proteins such as p21CIP1/WAF1, p27kip1, Bcl-2 and pRb. Proof of this was established by administration of the free radical scavenger L-N-acetylcysteine, which blocked MS-275-mediated mitochondrial injury and apoptosis [174]. In acute T-cell leukemic cell lines, vorinostat induced a cell-death pathway acting via cleavage of Bid and production of ROS [179]. Conversely, SB, which induces apoptosis independently of ROS generation or activation of the extrinsic pathway, prompted degradation of anti-apoptotic proteins Bcl-2 and p21CIP1/WAF1 in human leukemia cell lines [176]. Induction of p21CIP1/WAF1 was also observed in breast cancer cell lines co-treated with SB and tumor necrosis factor (TNF)-α, TNF-related apoptosis inducing ligand (TRAIL) or anti-FAS agonist antibody [49] (Table 2).

6.1.3. Gene expression affected by HDAC inhibition

Gene expression profiles of T24 bladder and MDA breast carcinoma cells treated with TSA or other HDACIs were studied to define a common set of genes that are induced or repressed by HDAC inhibition [78]. Examples of those genes induced and repressed are provided in Table 3.

6.1.4. Acetylation of non-histone proteins

Phylogenetic studies have proven that the evolution of HDACs predates the evolution of histones, indicating that there are non-histone substrates of HDACs [82]. p53 represents one example of a non-histone protein that is maintained in the acetylated state by HDACIs [192,216]. Other examples include the transcriptional repressor Bcl-6 [14], the 90-kDa heat shock protein (Hsp90) [215] and tubulin [88]. Hyper-acetylation of Ku70 protein on treatment with HDACIs results in release of Bax, which may provide one of the underlying mechanisms behind apoptosis [53].

HDACIs demonstrate pleiotropic effects by altering transcriptional status and preventing/inhibiting proliferation of tumor cells. Studies have exhibited synergism in gene re-expression and anti-proliferative effects when HDACIs are combined with other agents. The pleiotropy arises from the variety of histone deacetylase substrates, many of which are still unknown. The name ‘histone’ deacetylase inhibitors may be misleading, as many ‘non-histone’ substrates have been identified for HDACs.

6.2. Activity in vivo

The antitumor activity of HDACIs was demonstrated in several in vivo models of cancer including human xenografts. Significant reduction in tumor volume of breast cancer xenografts and lung metastasis was observed in animals treated with valproic acid [80]. MS-275, pyroxamide (Aton Pharma/National Cancer Institute (NCI)) and vorinostat have also exhibited antitumor activity in many cancer cell lines in
Table 3
Genes induced or repressed by HDACIs

| Induced genes                                      | Repressed genes                                      |
|---------------------------------------------------|------------------------------------------------------|
| p21CIP1/WAF1 [169], and p27KIP1 [94]              | Thymidylate synthetase [78]                          |
| Retinoic acid receptor β (RAR β) [156]            | CTP synthetase [78]                                  |
| Estrogen receptor [209]                           | Genes controlled by signal transducer and activator of transcription-5 (STAT5) [167] |
| TRAIL cell surface death receptors:               | Bcr-Abl fusion gene [144,145]                        |
| TRAIL-R1/DR4 and TRAIL-R2/DR5 [217]               | VEGF receptors VEGFR-1 and VEGFR-2, and neuropilin-1 [60,162] |
| Semaphorin III, a vascular endothelial cell growth factor (VEGF) competitor [60] | Inactivation of the heat shock protein-90 (Hsp90) molecular chaperone leading to mutant and wild-type androgen receptor depletion. As well as other Hsp90 client proteins HER-2 (ErbB2), Akt/PKB, and Raf-1 [43] |
| α-Fucosidase [78]                                | Lipopolysaccharide (LPS) induced inflammatory cytokines TNF-α, interleukin (IL)-1β, IL-6 and interferon (IFN)-γ [124] |
| Histone H2B [78]                                 | Androgen receptor (AR) and prostate-specific antigen (PSA) [43] |
| α-Tubulin [78]                                    |                                                      |
| Glutaredoxin [78]                                 |                                                      |
| Metallothionein 1L [78]                           |                                                      |

vivo [28,54]. In addition to their role in direct growth arrest, death and differentiation of tumor cells in vivo, HDACIs act as chemopreventive agents to inhibit tumor growth by preventing neovascularization of the tumor and thus exerting an anti-angiogenesis function. TSA, vorinostat, FK-228 and LAQ-824 have all demonstrated an anti-angiogenic effect in vivo [60,120,162], and recently, the chemopreventive sulforaphane has also indicated HDACi activity [141].

6.2.1. Histone acetylation

With the exception of the small molecule tubacin, all known HDACIs maintain histones in the acetylated state [88]. For example, hyperacetylated histone H4 was detected in peripheral blood mononuclear cells (PBMCs) at 1 h and 2 h after a single injection of PXD-101 in tumor-bearing mice [158]. In another example, xenografts from mice treated with pyroxamide displayed increased levels of histone acetylation and increased expression of the cell cycle regulator p21CIP1/WAF1 compared with tumors from vehicle-treated control animals [29].

7. SIRT inhibitors

The above discussed HDAC inhibitors affect the activity of class I and II HDACs. Translational research on these compounds has resulted in phase 1 and 2 clinical trials testing its efficacy in cancer patients. Although it is known that class III HDACs such as Silent Information Regulator (SIRT) 1 have cancer rel-
evance, SIRT1 regulates gene silencing [85], p53 function [122,196] and plays a critical role in stress signaling [25], SIRT has been primarily appreciated for its role in the biology of aging. A few recent findings might shift the focus a little more in the direction of neoplasms. Ford et al. [69] reported in 2005 that inhibition of SIRT1 by siRNA in cancer cells causes growth arrest and/or apoptosis in absence of stress. Non-cancerous cells are shown to be refractory to SIRT1 silencing and the effect of SIRT1 inhibition is therefore highly cancer-specific. A paper published in March 2006 by Pruitt et al. [161] showed furthermore that pharmacologic, dominant negative and siRNA-mediated inhibition of SIRT1 in colon and breast cancer cell lines reactivates epigenetically silenced tumor suppressor genes (TSG) such as mismatch-repair gene MLH1 and cell-cell adhesion associated protein E-cadherin. Previously described synergistic reactivation of epigenetically silenced TSG using HDACIs have been shown to depend on pre-treatment with methyltransferase inhibitors to (partially) demethylate the promoter regions [32]. Surprisingly, the process of reactivation using SIRT-inhibitors takes place without the loss of promoter DNA hypermethylation. The recent findings suggest new directions for targeting reversal of gene silencing by epigenetic pathways and possible therapeutic approaches.

8. HAT regulation: The other side of acetylation

Over the past years substantial progress has been made in the field of HDACIs, but less research has
been done on the young field of HATs. Several papers however, have established a direct relationship between HAT activity and the development or progression of disease [154]. As mentioned earlier, since CBP and p300 are available in limited concentrations in the cell, competition for them between different transcription factors can facilitate integration of several signaling and transcription response pathways.

8.1. Gain of p300/CBP and HAT inhibitors

As a result of fusion to other proteins HATs can become oncogenic. These gain-of-function mutations presumably increase proliferation by inappropriately enhancing activation of certain transcription pathways. MOZ (monocytic leukemia zinc finger), a putative HAT, has been described in acute myeloid leukemia (AML), to be fused with at least two different gene products: CBP (CREB binding protein) [20] and TIF2, a nuclear receptor coactivator [37], causing gain-of-function. Furthermore, the fusion of MLL (a homeotic regulator, mixed lineage leukemia) with CBP in patients with therapy-related AML, myelodysplastic syndrome and CML and the fusion of MLL with p300 in patients with AML, suggests an important (oncogenic) role for these gain of function mutations. Initially HAT inhibitors were synthesized as mechanistic tool for researching idea to use a HAT activator to study its effect on HAT activators, especially in the context of cancer agents. A review on the implications of small molecule activators and inhibitors of HATs in chromatin therapy has been written in 2004 by Varier et al. [193].

Lys–CoA, a conjugate of the amino acid lysine and coenzyme A, specifically blocks the HAT activity of p300. Although Lys–CoA has been intensively used for transcription studies during in vitro and in vivo studies, the use of microinjection or cell permeabilizing agents is necessary since Lys–CoA is not easily taken up by cells in cell culture conditions. The same holds true for H3-CoA-20, a p300/CREB binding protein-associated factor (PCAF)-specific inhibitor of the same class [123], and anacardic acid (AA) the first naturally occurring HAT inhibitor. AA was isolated from cashew nut shell liquid which inhibits HAT activity from both p300 and PCAF very effectively [6]. Their inability to penetrate the cell membrane makes them unsuitable for future use in animal models and humans. Recognizing the importance of HAT inhibitors in a clinical setting the first cell permeable HAT inhibitor garcinol was reported by Balasubramanyam et al. in 2004. Garcinol is a polyisoprenylated benzophenone derived from Garcinia indica fruit rind and shows to be a potent inhibitor of both p300 and PCAF HATS in vitro and in vivo. Treatment of HeLa cells with garcinol was shown to inhibit activated histone acetylation, induce apoptosis and down-regulate gene expression of proto-oncogenes [5]. However, the effect of garcinol in normal (untransformed) cells and other cancer types remains to be elucidated.

8.2. Loss of p300/CBP and HAT activators

In addition to functioning as oncogenes p300 as well as CBP have been shown to be potent tumor suppressor genes. Mice heterozygous for loss of CBP have been shown to develop tumors. Consistent with these data Rubenstein–Taybi syndrome patients lacking one functional allele of CBP show a predisposition to cancer [131]. Interestingly mice heterozygous for loss of p300 have not been shown to develop tumors. Despite this lack of direct evidence for p300 acting as a tumor suppressor gene, heterozygosity studies show p300 involvement in a number of different cancer types in humans. Analysis of colorectal, gastric, and epithelial cancer samples for instance show missense mutations as well as deletion mutations in the p300 gene [140] and it has been found that colorectal tumors as well as 80% of glioblastoma is associated with a loss of heterozygosity of the p300 gene. Gayther et al. identified in 10/193 tumor samples and cancer cell lines (breast, colorectal, ovarian, lung, pancreatic cancer and glioma) truncation mutations, insertions and missense mutations of p300 with or without inactivation or deletion of the second allele. Although their study indicates that p300 mutations are relatively rare, they do support the idea that loss of p300 activity contributes to tumor development since the nature of the p300 mutations suggests that most of the mutations would clearly lead to a loss of function [75]. In that perspective, it would be an interesting idea to use a HAT activator to study its effect on p300 function. Little has been published however on HAT activators, especially in the context of cancer. By using HAT inhibitor AA, Balasubramanyam et al. synthesized the first small molecule HAT activator called CTPB (N-(4-chloro-3-trifluoromethyl phenyl)-2-ethoxy-6-pentadecyl-benzamide). Just like AA however, cells are poorly permeable to CTPB [5]. It remains to be seen in in vitro and in vivo studies whether HAT activators can serve as potential anti-cancer agents. A review on the implications of small molecule activators and inhibitors of HATs in chromatin therapy has been written in 2004 by Varier et al. [193].
9. HDACIs in clinical trials

Over the last five years more than 20 HDACIs have been investigated in cancer clinical trials, either alone or in combination with other agents. A brief summary of some selected clinical trials is presented in Table 4. This includes the early clinical trials which paved the way for more HDACIs to be evaluated, as well as the most recent trials showing promise.

9.1. Clinical toxicity and antitumor activity

Dose-limiting clinical toxicities and reported antitumor responses have been noted in Phase I and II clinical trials for the limited number of structurally varied HDACIs that have entered clinical testing. Short chain fatty acids such as phenylbutyrate show a dose-limiting toxicity (DLT) of somnolence and confusion when administered using prolonged intravenous infusion. This neurotoxicity has not been reported for the benzamide or hydroxamate HDACIs or for the carboxylate prodrug AN-9. Despite thrombocytopenia being a DLT for both CI-994 and depsipeptide, evidence for antitumor clinical activity upon oral daily dosing of CI-994 has been noted in patients with several epithelial types of advanced solid malignancies (including non-small cell lung cancer (NSCLC), renal cell carcinoma, and bladder cancer). Likewise, two Phase I trials of depsipeptide have suggested that patients with T cell leukemia or lymphoma, as well as other occasional cases of refractory malignancies, may achieve clinical benefit from this HDACI. Unfortunately, depsipeptide has been reported to be associated with a significant incidence of cardiac dysrhythmias and nonspecific electrocardiogram (EKG) abnormalities. In some patients, the hydroxamates, LAQ824 and LBH-589 too have demonstrated some EKG changes. Fatigue was commonly observed with vorinostat treatment but was not dose-limiting and was similar to that previously reported for depsipeptide. Importantly, many patients with solid cancers showed some degree of clinical improvement.

9.2. Pharmacokinetics and pharmacodynamics

Owing to the reversible nature of epigenetic modifications, and assuming these to be the key determinants of tumor growth, inhibition of intracellular HDAC activity or demethylation commonly will require continuous drug exposure to achieve maximal tumor cytostasis or apoptosis and clinical response. Rapid clearance, a high degree of protein binding, rapid metabolism, or rapid inactivation of reactive functional groups (i.e., epoxy groups) are factors that can adversely affect HDACI bioavailability and antitumor activity. Most HDACIs are rapidly metabolized in rodents and dogs. With a few exceptions, for example, LBH-589A (Novartis AG; \( t_{1/2} = 15 \) to 20 h) [11] and MS-275 \( (t_{1/2} = 100 \) h) [79,180], reported half-lives for HDACIs are a maximum of 1 h in humans. This short half-life poses a significant limitation to the design of both in vivo studies and clinical trials with HDACIs. In particular, butyrate and phenylbutyrate degraded rapidly after intravenous administration, requiring doses \( \geq 400 \) mg/kg/day to be administered as a continuous i.v. infusion for 120 h (repeated every 21 days) in certain clinical trials [39,203]. Therefore, most phase I trials continue to focus on the pharmacokinetics of different HDACIs. In case of drugs with a shorter half-life, continuous dosing may be required. However, continuous dosing is not always desirable. Therefore, drugs available as oral formulations like valproic acid and vorinostat, are more potentially attractive candidates. In the case of valproic acid, the already well-investigated neuropsychiatric drug, indicated availability of a pharmacokinetic profile with a \( T_{1/2} \) of \( \sim 14 \) h [194] and at therapeutically tolerated doses, effective plasma concentrations are in the achievable 0.5–0.75 mM range. The drug is conveniently bioavailable in an oral formulation. Trials on prostate cancer and other malignancies with this drug have been guided by this pre-existing knowledge.

9.3. Biomarkers of evaluation

Because of the differentiating properties of epigenetic modifiers, the conventional marker of evaluation, PSA, is often upregulated even when tumor burden is reduced [39]. Hence, PSA cannot be used as a marker of disease prognosis when using differentiating agents. Till date, acetylation of histones in PBMCs is relied upon as a marker of exposure to HDACI, in both blood and solid cancers. As markers of effect in blood cancers, acetylation of the p21 gene and upregulation of the protein is a vital endpoint of drug activity, but not of antitumor response. In order to avoid biopsies, and in the absence of validated biomarkers of effect in surrogate tissue, the inability to evaluate acetylated histones in solid tumors, has led to difficulty in trial design. However, many novel designs of evaluating patient and tumor response to HDACI are currently underway. These designs include treating patient populations...
| Class          | HDACI       | Structure | Cancer          | Schedule                                        | Outcome                      | Adverse events                                                                 | Reference |
|---------------|------------|-----------|-----------------|------------------------------------------------|-------------------------------|-------------------------------------------------------------------------------|-----------|
| Cyclic peptides | Depsipeptide | ![Depsipeptide](image) | Hematological malignancies (Phase I) | 13 mg/m² i.v. on days 1, 8, and 15 every 4 weeks | CR, PR: none Evidence of antitumor activity and histone acetylation increases of at least 100%. Increase in p21 promoter H4 acetylation, p21 protein and 1D10 antigen expression. | Progressive fatigue, nausea, and other constitutional symptoms prevented repeated dosing. Neither life-threatening toxicities nor cardiac toxicities were noted. | [30]      |
|               |            |           | CTCL + PTCL (Phase II) | CR: 21% (3/14 CTCL) PR: 29% (4/14 CTCL) PR: 24% (4/17 PTCL) | | Fatigue, N/V, granulocytopenia, hypocalcemia, neutropenia, thrombocytopenia. Cardiac: non-specific ST-T wave changes, but no change in cardiac function. | [155]     |
| Butyric acid  | AN-9       | ![AN-9](image) | NSCLC (Phase II) | 2.34 g/m²/day i.v. in 6 h infusion on 3 consecutive days every 3 weeks | PR: 6.43% (3/47) SD > 12 weeks: 30% (14/47) | Fatigue, nausea, dysgeusia, dyspnea (G1-2: 9%, G4: 4%), chest pain (G1-2: 6%, G3-4: 4%) | [105,168] |
| Aliphatic acid | Phenylacetate | ![Phenylacetate](image) | Solid tumors (Phase I) | Single i.v. bolus followed by 14-day continuous i.v. infusion (maintaining blood concentration at 200–300 µg/ml) | SD > 9 months: 17% (1/6 glioblastoma) SD > 2 months: 33% (3/9 HRPC) Nonlinear pharmacokinetics, induction of drug clearance | Confusion, lethargy, emesis DLT: reversible CNS depression | [189]     |
|               |            |           | Malignant glioblastoma (Phase II) | 400 mg/kg/d continuous i.v. infusion 2 weeks/4 weeks | CR: none PR: 7.5% (3/40) SD: 17.5% (7/40) >50% reduction of tumor: 7.5% (3/40) treatment failure < 2 months: 75% (30/40) | Infection, malaise, fatigue, lethargy, reversible disorientation, somnolence, weakness, N/V, edema, granulocytopenia. | [42]      |
| Class       | HDACI        | Structure | Cancer (trial phase) | Schedule | Outcome                          | Adverse events                                                                 | Reference |
|-------------|--------------|-----------|----------------------|----------|----------------------------------|-------------------------------------------------------------------------------|-----------|
| Phenyl butyrate | solid tumors (Phase I) | 150–515 mg/kg/day as 120 h i.v. infusion every 21 days | CR: none SD: 8% (2/24, 19 PCa) SD: 8% (2/24, 19 PCa) Significant bone pain reduction in HRPC Recommended phase II dose: 410 mg/kg/day for 120 h | DLT: neurocortical (excessive somnolence, confusion), hypokalemia, hyponatremia, hyperuricemia (2 patients, 515 and 345 mg/kg/day) MTD: 410 mg/kg/day for 5 days Other toxicities: fatigue, nausea | [39]     |
| Valproic acid | solid tumors (Phase I) | 9–45 g/day p.o. in 3 doses/day | PR, CR: none SD > 6 months: 25% (7/28, 12 PCa) Recommended phase II dose: 27 g/day | dyspepsia (G1-2), edema (G1-4), fatigue, neurocortical toxicity, N/V DLT: N/V, hypocalcemia at 36 g/day (2/7) MTD: 27 g/day | [77]     |
| Hydroxamate vorinostat | advanced cancer (Phase I) | 30–120 mg/kg/day as 2 × 1 h i.v. infusion for 5 days every 2 weeks | PBC: hyperacetylation observed in majority of patients Recommended phase II dose: 60 mg/kg | neurological toxicity (G3-4, 9/26, dose 75-, 90- and 120-mg/kg) no hemalogical toxicity >G3-4 MTD | [4,153] |
|              | solid tumor hematological malignancies (Phase I) | 200 mg qd, 400 mg qd, 600 mg qd, or 400 mg bid per day p.o. | CR: 1/73 PR: 4% (3/73 + 2 unconfirmed) linear pharmacokinetics from 200 to 600 mg, Mean $T_{1/2}$: 91–127 min dose-dependent accumulation of acetylated histones from 200–600 mg | MTD: 400 mg qd and 200 mg bid for continuous daily dosing and 300 mg bid for 3 consecutive days per week dosing. DLT: anorexia, dehydration, diarrhea, and fatigue | [107]    |
| Class | HDACI | Structure | Cancer Schedule | Outcome | Adverse events | Reference |
|-------|-------|-----------|----------------|---------|----------------|-----------|
| Solid tumors + hematological malignancies + refractory lymphomas (Phase I) | LAQ-824 | ![LAQ-824](image) | 75–900 mg/m²/day in 2 h i.v. infusion 3–5 days/week for 1–3 weeks | Objective tumor regression with clinical improvement in tumor related symptoms: 4 patients | Fatigue (G1-3), anorexia (G1-2), vomiting (G1-2), diarrhea (G1-3), constipation (G1-4), hypokalemia, non-specific EKG changes, increased creatinine, dysgeusia MTD: 300 mg/m²/day × 5 days for 3 weeks (2/5 hematological), 900 mg/m²/day × 5 days for 3 weeks (1/6) DLT: leucopenia (G3-4), thrombocytopenia (G3-4), acute respiratory distress, hypotension (G3) | [108] |
| advanced CTCL (Phase II) | | | 400 mg daily until disease progression or intolerable toxicity | Objective Response Rate was 29.5% (18 PR including 1 with later CR) | AE ≥ Grade 3 included fatigue (5%), pulmonary embolism (5%), nausea (4%) and thrombocytopenia (4%). Twenty-five pts discontinued due to progressive disease. Causes of the 3 deaths on study were: unknown (d 2), ischemic stroke (d 227) and disease progression (d 52) | [150] |
| LAQ-824 | | | Dose-escalating i.v. infusion on days 1–3 for 21 days | Increase and maintenance of acetylation for 24 h, inhibition of Hsp90 activity with increased expression of Hsp70 and decreased downstream target c-Raf | Not reported | [118] |
| Class       | HDACI | Structure | Cancer (trial phase) | Schedule                                                                 | Outcome                                                                 | Adverse events                                                                                           | Reference |
|------------|-------|-----------|----------------------|--------------------------------------------------------------------------|--------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|-----------|
|            |       |           | advanced solid tumors + hematological malignancies (Phase I) | 6–200 mg/m²/day as 3 h i.v. infusion on days 1–3 for 21 days             | PK analysis showed dose proportionality, $T_{1/2}$: 6–26 h                  | QTcF day 1: no change QTcF day 3: dose-related increases of $<20$ msec QTcF $>60$ msec: 10% (8/77) at 36–200 mg/m². QTc $>500$ msec: 1/77 (200 mg/m²) Frequent non-specific T wave flattening Small increase in troponin without increase in CK MB (2/77) | [178]     |
|            | PDX101| ![Structure](image1.png) | advanced solid tumors (Phase I) | 150, 300, 600, 900 and 1200 mg/m² as 30 min. i.v. infusion 5 days every 3 weeks Ongoing Schedule: 1000 mg/m² | SD $>2$ cycles: 7/21 linear kinetics displayed $T_{1/2}$: 0.5–1 h PMN: Dose dependent histone H4 hyperacetylation, increases in p21, p19, and Apaf-1 expression | DLT: 14% (3/21, all 1200 mg/m²), fatigue (G3), reversible atrial fibrillation (G3), diarrhea (G3) and lethargy. All other AE were $\leq$ G2. No hematological toxicity was observed | [186]     |
|            | LBH-589| solid tumors (Phase I) | 1.2–7.2 mg/m²/day as 30 min. i.v. infusion day 1–3 and 8–10 every 3 weeks (arm 1) or day 1–3 and 15–17 every 4 weeks (arm 2) | SD: 46% (6/13) Rapid onset (1 h) of prolonged acetylation (up to 7 days) in some patients. $T_{1/2}$: 15 to 20 h. | Neutropenia (G3, 1/13), hypoglycemia (G3, 1/13), thrombocytopenia (G2, 2/13), anemia (G2, 2/13) DLT: prolonged thrombocytopenia (G2, 7.2 mg/m²/day in arm 1) | [11]      |
| Benzamides | MS-275| ![Structure](image2.png) | solid tumors + lymphomas (Phase I) | $>2$ mg/m²/day (dose escalation) p.o. 4/10 weeks or 4/6 weeks | $T_{1/2}$: 39–80 h Linear PK suggested PBMC: increased histone H3 acetylation was apparent at all dose levels | MTD: 10 mg/m² DLT: N/V, anorexia, and fatigue | [25]      |
Table 4
(Continued)

| Class                  | HDACI Structure | Cancer (trial phase) | Schedule | Outcome | Adverse events | Reference |
|------------------------|-----------------|----------------------|----------|----------|----------------|-----------|
| solid tumors + lymphomas (Phase I) |                 | 2–6 mg/m²/2 weeks p.o. or 2 mg/m² p.o. twice weekly for 2/3 weeks or 4 mg/m²/week p.o. for 3 weeks | $T_{1/2}$: 100 h  $T_{max}$: 0.5–2.0 h  PBMC: increased histone H3 acetylation.  HDACI observed.  PR: 6% (1/17)  SD: 17% (3/17) | Hyophosphatemia, asthenia, nausea, anorexia.  MS-275 p.o. on the daily schedule was intolerable at a dose and schedule explored.  AE: hyophosphatemia, asthenia, nausea and anorexia (all G1–3) | [79,180] |
| Metastatic melanoma (Phase II) |                 | 3 mg on day 1 + 15 or 7 mg on day 1 + 8 + 15 of a 4 week cycle | SD for 8–48 weeks: 29% of 3 mg group and 21% of 7 mg group Stabilization of certain metastatic lesions No tumor response was noted | Nausea (32%, G1–G2), hyophosphatemia (29%, G1–G3), diarrhea (18%, G1–G2) | [90] |
| MGCDO103 advanced solid tumors (Phase I) |                 | 12.5, 20, 27, 36 and 45 mg/m²/day 3 days/week, 2 out of 3 weeks | SD (>2 cycles) seen in renal cell cancer (2 patients, 4 and 6 cycles) and colorectal cancer (1 patient, 4 cycles) Dose-dependent $T_{1/2}$: 7.7–11.3 h ($\pm$0.6–1.6) Maximal HDAC inhibition in patients co-administered an acidic beverage which reduces previous PK variability. | AEs: grade 1–3 fatigue (91%), grade 1–2 nausea (70%) and vomiting (48%), anorexia (26%), constipation (39%) and dehydration (14%). MTD has not yet been reached | [38,184] |
| advanced solid tumors + NHL (Phase I) |                 | 12.5–27 mg/m²/day p.o. | SD > 6 cycles: 1/27 to date (thymic carcinoma) HDACI correlates positively with histone acetylation in PBC lasting >24 h post-dosing. $T_{1/2}$: 8.8 h | Fatigue (20/27, 5/20 G3), nausea 13 pts (13/27, 1/13 G3), anorexia (8/27), vomiting (6/27) and diarrhea (5/27) | [76,100] |

AML – acute myeloid leukemia, CNS – central nerve system, CRi – complete remission, CTCL – cutaneous T-cell lymphoma, CR – complete response, DAC – 5-Aza-2′-deoxycytidine, DNMT – DNA methyl transferase, DLT – dose-limiting toxicity, ECG – electrocardiogram, ER – estrogen receptor, GSTPi – glutathione-S-transferase Pi, HDACI – histone deacetylase inhibition, HRPC – hormone refractory prostate cancer, HGB F – fetal hemoglobin, i.v. – intravenous, MDS – Myelodysplastic Syndrome, N.A. – non available, NHL – non-Hodgkin’s lymphoma, NSCLC – non-small-cell lung cancer, N/V – nausea/vomiting, PCa – prostate cancer, p.o. – per oral, PMN – peripheral mononuclear cells, POD – post-operative death, PR – partial response, PTCL – peripheral T-cell lymphoma, RR – relative risk, SD – stable disease.
Table 5
Preclinical combination of HDACIs with other agents

| HDAC inhibitor(s) | Combination agent(s) | Rationale/mechanism underlying synergism                                                                 | Reference |
|------------------|----------------------|---------------------------------------------------------------------------------------------------------|-----------|
| TSA, FK-228, Phenylbutyrate, vorinostat CBHA | Drugs or hormones that act on retinoic acid, estrogen receptors or other nuclear receptors | Aberrant fusion proteins bind to RARE (or other nuclear receptors) where HDAC-containing repressor complexes are recruited to silence expression of genes from these promoters. Ligands and HDACIs reactivate such silenced and repressed chromatin to cause expression of hormone-inducible genes to overcome retinoid resistance. | [51,52,56,59,132,156,190] |
| TSA, vorinostat, FK-228, Phenylbutyrate FR901228 | DNA methyl transferase inhibitors (e.g., DAC, decitabine) | Eliminated the dominant effect of hypermethylation of promoters. | [12,19,32,74,104,114,139,160,221] |
| 3-n-Butyrate, 5-Fluorouracil (5-FU) | | Enhanced apoptosis. | [24] |
| FK-228, Phenylbutyrate, vorinostat | Flavopiridol (NCl) | Flavopiridol, a synthetic Cdk inhibitor, interfered with expression of the cellular Cdk inhibitor p21CIP1/WAF1, to cause apoptosis as opposed to cell cycle arrest and differentiation induced by HDACIs. | [1,143,175] |
| vorinostat, Phenylbutyrate, Apicidin LAQ-824 | STI-571 | HDACIs caused apoptosis in Imantinib-resistant cells and enhanced apoptosis in Bcr-Abl expressing cells. | [112,144,145,212,213] |
| Sodium butyrate | Topoisomerase II inhibitors (e.g., etoposide, epirubicin) | HDACIs upregulated topoisomerase II expression, which in turn rendered cells sensitive to topoisomerase II inhibitors. | [119] |
| LAQ-824, LBH-589 | 17-AAG (Hsp90 antagonist) | Inhibition of chaperone association of Hsp90 with Flt-3 and Bcr-Abl, resulting in polyubiquitination and proteosomal degradation of Flt-3 and Bcr-Abl. Levels of Flt-3 and Bcr-Abl were greatly attenuated to result in enhanced apoptosis. | [15,165] |
| vorinostat, FK-228 | Standard chemotherapy agents (e.g., VP-16, ellipticine, doxorubicin, cisplatin, oxaliplatin) | Enhanced apoptosis. | [113] |
| TSA, vorinostat, MS-275, FK-228 | γ-Irradiation | γH2AX foci expression was prolonged and histones were inhibited from participating in DNA repair. vorinostat also caused differential expression of several oncoproteins and DNA damage repair proteins (epidermal growth factor receptor, AKT, DNA-PK, and Rad51) that affect susceptibility of cells to radiation induced damage response | [16,33,34,46,111,218] |
| vorinostat, Sodium butyrate | Bortezomib | Enhanced apoptosis. | [213] |
Table 5
(Continued)

| HDAC inhibitor(s) | Combination agent(s) | Rationale/mechanism underlying synergism | Reference |
|-------------------|----------------------|------------------------------------------|-----------|
| Sodium butyrate, TSA, vorinostat | Activators of extrinsic, receptor-mediated apoptotic pathway (TRAIL, TNF-α) | HDACIs sensitized cells to TRAIL by decreasing FLIP protein expression to cause cell death. | [93,95,173] |
| LAQ824 | Apo-2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) | Exposure to LAQ824 increased the mRNA and protein expressions of the death receptors DR5 and/or DR4, but reduced the mRNA and protein levels of cellular FLICE-inhibitory protein (c-FLIP). As compared with treatment with Apo-2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or LAQ824 alone, pretreatment with LAQ824 increased the assembly of Fas-associated death domain and caspase-8, but not of c-FLIP, into the Apo-2L/TRAIL-induced death-inducing signaling complex. This increased the processing of caspase-8 and Bcl-2 interacting domain (BID), augmented cytosolic accumulation of the prodeath molecules cytochrome-c, Smac and Omi, as well as led to increased activity of caspase-3 and apoptosis. Treatment with LAQ824 also down-regulated the levels of Bcl-2, Bcl-x(L), XIAP, and survivin. Partial inhibition of apoptosis due to LAQ824 or Apo-2L/TRAIL exerted by Bcl-2 overexpression was reversed by cotreatment with LAQ824 and Apo-2L/TRAIL. | [87] |
| vorinostat | Signal transduction modulators (e.g., STI-571) | Effectively induced apoptosis in Bcr/Abl+ cells that were STI-571-resistant and non-resistant. | [144,145,212] |
| vorinostat | ErbB signaling inhibitors (e.g., CI-1033 (Pfizer Inc)) | Dual targeted therapy abrogated EGFR and Akt signaling. | [47] |
| LAQ-824 | VEGFR tyrosine kinase inhibitor (PTK-787/ZK-222584) | Increased anti-angiogenic factors in tumor and surrounding endothelial cells, and inhibited tumor growth. | [162] |
| AQ-824 | Herceptin and/or Taxotere or Epothilone B against human breast cancer | Causes down regulation of the mRNA and protein expression of Her-2, which correlated with the attenuation of pAKT. Promoting the proteasomal degradation of Her-2 and sensitizes human breast cancer cells (with Her-2 amplification) to Herceptin and apoptosis due to tubulin polymerizing agents, Taxotere and Epothilone B. | [72] |

5AC – 5-Aza-cytidine, DAC – 5-Aza-2′-deoxycytidine, CNS – central nerve system, CTCL – cutaneous T-cell lymphoma, CR – complete response, MTD – maximum tolerated dose, DLT – dose-limiting toxicity, ECG – electrocardiogram, GSTP1 – glutathione-S-transferase Pi, HDACI – histone deacetylase inhibition, HRPC – hormone refractory prostate cancer, HGB F – fetal hemoglobin, N.A. – non-available, ND – not determined, NHL – non-Hodgkin’s lymphoma, NSCLC – non-small-cell lung cancer, N/V – nausea/vomiting, p.o. – per oral, PMN – peripheral mononuclear cells, POD – post-operative death, PR – partial response, PTCL – peripheral T-cell lymphoma, RR – relative risk, SD – stable disease.
### Table 6
Clinical Trials of HDACIs with other agents

| HDACI       | Combining agent | Structure (trial phase) | Cancer Schedule | Outcome | Adverse events | Reference |
|-------------|-----------------|-------------------------|-----------------|---------|----------------|-----------|
| Valproic acid | Epirubicin      | Advanced solid tumors (Phase I) | VPA loading dose followed by 6 oral doses (q12h) prior to epirubicin in 3-week cycles | PR: 19% (7/37) SD/minor response: 43% (16/37) H3 and H4 acetylation and topo II expression correlated to VPA dose, plasma concentration and response | DLT: somnolence (1/42) and neutropenia (1/42) MTD: 160 mg/kg/d VPA combined with 100 mg/m²/d epirubicin | [138] |
| DAC         |                 | Advanced cancer (Phase I) | DAC: 20–47 mg/m²/day for 10 days every 28 days VPA: 11.9–25.7 mg/kg/day p.o. | SD for 6 months: 5% (1/22) SD for 4 months: 15% (3/22) Median LINE methylation decreased from 65% to 61% after 10 days treatment, but had returned back to baseline at the start of the next cycle | Drowsiness (5/22) Tremor (4/22) Hypomagnesemia (2/22) Anemia G2 (2/22) Neutropenia (2/22) DLT: neutropenic fever (1/22 at 37.5 mg/m²) | [23] |
| Decitabine  |                 | relapsed/refractory AML + untreated AML with age >60 (Phase I) | 15–20 mg/m²/day decitabine as 1 h i.v. infusion for 10 days every 28 days ± 15 mg/kg/day p.o. VPA on days 5–21 every 28 d | Decrease DNA methylation: 64% (9/14) Depletion of DNMT: 67% (4/6) >100% increase of p15 or ER expression: 77% (10/13) of which all had a clinical response <100% increase of p15 or ER expression: 23% (3/13) of which none had a clinical response decitabine Cmax: 93 ng/ml (n = 7) CR: 13% (2/15) CRi: 27% (4/15) 4/15 patients had clinical improvement | Not reported | [115] |
Table 6
(Continued)

| HDACI     | Combining agent                | Structure                      | Cancer                                                                 | Schedule                                                                 | Outcome                                                                 | Adverse events                                                                 | Reference |
|-----------|--------------------------------|-------------------------------|------------------------------------------------------------------------|--------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------|----------|
| DAC       | and ATRA                        | ![Structure](image1)          | High risk MDS + relapsed/refractory AML + untreated AML                | DAC: 75 mg/m2/day for 7 days ATRA: 45 mg/m2/day p.o. on days 3–7 VPA: 50, 62.5 or 75 mg/kg/day p.o. for 7 days | All patients received fixed doses of DAC and ATRA. The dose VPA varied between the patients. CR: 6% (1/16, 75 mg/kg VPA) Marrow blasts <5%: 13% (2/16) CRP (CR minus bone marrow response): 6% (1/16) Median LINE methylation decreased during treatment from 62% to 58%, but returned to baseline by day 0 of the next cycle | Confusion (G3): 1/6 in 50 mg/kg VPA treated patients, 0/6 in 62.5 mg/kg VPA treated patients and 2/6 in 75 mg/kg VPA treated patients. | [185]    |
| Vorinostat| 5-fluorouracil and leucovorin and oxaliplatin (FOLFOX) | ![Structure](image2)          | Advanced colorectal cancer                                            | vorinostat: 100–300 mg p.o. twice daily on day 1–7 every 2 weeks FOLFOX: 400 mg/m2 leucovorin + 85 mg/m2 oxiplatin over 2 h followed by 2500 mg/m2 5-FU over 24 h on days 4–5 every 2 weeks | SD for 5 months: 1/6 SD for 2 months: 3/6 2 liver patient biopsies showed major downregulation of thymidilate synthase after 3 days treatment with vorinostat | Neutropenia (1/8, G2), mucositis (1/8, G2), N/V (2/8, G2) | [63]     |
| MS-275    | 13-cis retinoic acid            | ![Structure](image3)          | Advanced solid tumors                                                | MS-275: 4–5 mg/m2 p.o. once per week CRA: 1 mg/kg/day p.o. twice daily for 3 weeks every 4 weeks | $T_1/2$ MS-275: 108.2 ± 47.9 h Clearance MS-275: 9.4 ± 6.0 L/h/m² Median CRA levels: 182.4 ng/ml 38% (5/13) remained on treatment for >4 months Histones isolated from PBMCs post-therapy showed transient but consistent acetylation Post-therapy liver lesion biopsies showed increased histone acetylation and decreased phosphorylated ERK and STAT3 protein expression | MTD: 5 mg/m² MS-275 + 1 mg/kg CRA (G3 hyponatremia, neutropenia, anemia) Fatigue (G1–2) | [157]    |

AML – acute myeloid leukemia, ATRA – all-trans retinoic acid, CRA – 13-cis retinoic acid, CR – complete response, DAC – 5-Aza-2′-deoxycytidine, DNMT – DNA methyl transferase, DLT – dose-limiting toxicity, ER – estrogen receptor, HDACI – histone deacetylase inhibitor, i.v. – intravenous, MDS – myelodysplastic syndrome, MTD – maximum tolerated dose, N/V – nausea/vomiting, p.o. – per orale, PBMC – peripheral mononuclear cells, PR – partial response, PTCL – SD stable disease, VPA – valproic acid.
with HDACIs in the window period before surgery (pre-prostatectomy for example) and evaluating biomarkers of drug activity in post-surgery samples. Similarly, in case of DNA Methyl Transferase Inhibitors (DNMTIs), the frequency of methylation markers, like GSTPi, RASSF1A, CDH1, EDNRB1, which are frequently methylated in prostate cancer [128,210], etc. may be evaluated. However, it is still not known if decreases in methylation of these genes or others have independent prognostic significance.

10. Combination studies with HDACIs

*In vitro*, HDACIs have been combined with an array of chemically and structurally diverse compounds. In some cases, the combination was supported by a rationale, and in others the mechanism underlying the synergistic activity of the combination was subsequently analyzed. A summary of selected preclinical combination studies is provided in Table 5. Many of these preclinical observations have now forwarded to phase I/II clinical trials. Table 6 provides an overview of clinical trials using HDACIs in combination with other agents.

11. Conclusion

In the clinic, HDACIs are competing with conventional chemotherapeutic drugs, and are favorable because of their low toxicities. Further analysis into the common pathways between conventional drugs and HDACIs, significance of individual HDACs and their isoforms, and other substrates of HDACs will reveal the mechanism behind the success of HDACIs in the clinic. HDACIs with a more favorable PK such as MS-275 and to a lower extent LBH-589 and LAQ-824 may be preferred than the aliphatic HDACIs, many of which have very short half lives. Ease of administration and type of toxicity are additional considerations that will influence the development of next generation HDACIs. Currently, studies on mechanism of action of HDACIs are ongoing and the next few years should see a continued increase in the number of HDACIs under investigation in clinical trials.

Glossary of abbreviations

| Abbreviation | Definition |
|--------------|------------|
| AA           | anacardic acid |
| Acetyl-CoA   | acetyl coenzyme A |
| AML          | acute myeloid leukemia |
| AN-9         | pivaloyloxyethyl butyrate |
| APL          | acute promyelocytic leukemia |
| ATP          | adenosine triphosphate |
| ATRA         | all-trans retinoic acid |
| CBHA         | m-carboxycinnamic acid bis-hydroxamide |
| CBP          | CREB-binding protein |
| Cdk          | cyclin-dependent kinase |
| CML          | chronic myeloid leukemia |
| CNS          | central nerve system |
| CR           | complete response |
| CRA          | 13-cis retinoic acid |
| CTCL         | cutaneous T-cell lymphoma |
| CTPB         | N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide |
| DAC          | 5-Aza-2'-deoxycytidine |
| DLT          | dose-limiting toxicity |
| DNA          | deoxyribonucleic acid |
| DNMT         | DNA methyl transferase |
| DNMTI        | DNA methyl transferase inhibitor |
| ER           | estrogen receptor |
| ECG/EKG      | electrocardiogram |
| FAT          | factor acetyl transferases |
| GNAT         | Gcn5-related N-acetyl transferases |
| GSTPi        | glutathione-S-transferase Pi |
| H2A          | histone 2A |
| H2B          | histone 2B |
| H3           | histone 3 |
| H4           | histone 4 |
| HAT          | histone acetyl transferase |
| HDAC         | histone deacetylase |
| HDACI        | histone deacetylase inhibitor |
| MDS          | myelodysplastic syndrome |
| HGB F        | fetal hemoglobin |
| HMG          | high mobility group |
| HRPC         | hormone refractory prostate cancer |
| Hsp90        | 90-kDa heat shock protein |
| i.v.         | intravenous |
| Lys-CoA       | conjugate of amino acid lysine and coenzyme A |
| MLL          | mixed lineage leukemia |
| MOZ          | monocytic leukemia zink finger |
| MTD          | maximum tolerated dose |
| MYST         | super family of HATs named after its founding members MOZ, Ybf2/Sas3, Sas2 and Tip60 |
| N.A.         | not available |
| NCI          | National Cancer Institute |
| NHL          | non-Hodgkin’s lymphoma |
| NSCLC        | non-small cell lung carcinoma |
| N/V          | nausea/vomiting |
PCa prostate cancer  
PCAF p300/CREB binding protein (CBP)-associated factor  
PBMC peripheral blood mononuclear cells  
PK pharmacokinetic  
PMN peripheral mononuclear cells  
p.o. per oral  
POD post operative death  
PR partial response  
PTCL peripheral T-cell lymphoma  
RA retinoic acid  
RNA ribonucleic acid  
ROS reactive oxygen species  
RR relative risk  
SAHA suberoylanilide hydroxamic acid  
SB sodium butyrate  
SD stable disease  
SIRT silent information regulator  
TNF α tumor necrosis factor α  
TRAIL TNF-related apoptosis inducing ligand  
TSA trichostatin A  
TSG tumor suppressor gene  
US FDA United States Federal Drug Administration  
VPA valproic acid

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