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Histone deacetylase inhibitors for cancer therapy: An evolutionarily ancient resistance response may explain their limited success

John A. Halsall and Bryan M. Turner*

Histone deacetylase inhibitors (HDACi) are in clinical trials against a variety of cancers. Despite early successes, results against the more common solid tumors have been mixed. How is it that so many cancers, and most normal cells, tolerate the disruption caused by HDACi-induced protein hyperacetylation? And why are a few cancers so sensitive? Here we discuss recent results showing that human cells mount a coordinated transcriptional response to HDACi that mitigates their toxic effects. We present a hypothetical signaling system that could trigger and mediate this response. To account for the existence of such a response, we note that HDACi of various chemical types are made by a variety of organisms to kill or suppress competitors. We suggest that the resistance response in human cells is a necessary evolutionary consequence of exposure to environmental HDACi. We speculate that cancers sensitive to HDACi are those in which the resistance response has been compromised by mutation. Identifying such mutations will allow targeting of HDACi therapy to potentially susceptible cancers.

Keywords:
- cancer
- chromatin
- deacetylase
- epigenetic drugs
- evolution
- histone modification

Introduction

It is now almost 40 years since the first demonstration that treatment of cells in tissue culture with salts of butyric acid (a short chain fatty acid, SCFA) caused increased levels of histone acetylation [1]. This was accompanied by slowed cell cycle progression [2] but no dramatic, overall change in RNA synthesis [3]. It was shown that hyperacetylation resulted from inhibition of deacetylase activity [4]. These early experiments demonstrated the rapid turnover of histone acetate groups, and provided an invaluable method for production of acetylated histones for structural and functional analyses. The observation was particularly exciting in view of the possible link between histone acetylation and transcription [5], and that low concentrations of butyrate could induce differentiation of an erythroleukemia cell line into non-dividing, hemoglobin synthesizing cells [6].

These experiments all predated elucidation of the role of histones in DNA packaging, identification of enzymes responsible for turnover of histone acetates, formulation of the histone code hypothesis and realization of the extraordinary complexity of the processes by which histone post-translational modifications regulate chromatin function (reviewed in [7, 8]). But despite the vastly increased amount of information at our disposal, and increasingly sophisticated technologies by which to generate even more, fundamental questions posed nearly 40 years ago about the functional role of histone acetylation, remain unanswered [9]. The problem is particularly acute in view of the increasing clinical use of histone deacetylase inhibitors (HDACi), both simple SCFA salts such as sodium valproate, and more complex reagents.

In this article, we ask how it is that most normal cells are able to tolerate the extreme hyperacetylation of histones and other proteins induced by HDACi and why just a few types of cancer are so sensitive. If histone acetylation really is...
Histone deacetylase inhibitors are effective in treating certain cancers

In 2006, the US Food and Drug Administration (FDA) approved the use of a histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA, marketed by Merck as Zolinza), for the treatment of cutaneous T-cell lymphoma (CTCL). The drug is remarkably effective against this rare cancer, as is the structurally unrelated inhibitor depsipeptide, a bicyclic peptide marketed as Romidepsin. More recently, two further hydroxamic acid HDACs have also been granted FDA approval, Belinostat against peripheral T-cell lymphoma and Panobinostat, against multiple myeloma. These successes show that HDACi, as a class of drugs, can be effective chemotherapeutic agents [10, 11].

Recently, there has been a proliferation of trials testing HDACi against a variety of cancers, often in combination with other drugs, such as aza-cytidine (a DNA methyltransferase inhibitor) and Bortezomib (a proteasome inhibitor) [10, 12]. Inhibitors for which completed trials have been reported, and the cancers against which they have been tested, are listed in Tables 1 and 2. Although hematological malignancies remain the major clinical targets for HDACi, several have now been trialed against more common solid tumors, including lung, breast and prostate cancer (Tables 1 and 2). Results have been mixed; the variability of response, both between and within cancer types is striking, and remains unexplained [13].

Understanding how HDACi affect different cell types is complicated by the fact that protein deacetylation is carried out by a family of enzymes, within which individual members differ in their sensitivities to different HDAC inhibitors. Fortunately, a substantial body of research since purification and characterization of the first HDAC in 1996 [14] has provided valuable insights into the HDAC family and the factors that regulate its activities.

HDACi generally inhibit several members of the HDAC family

In view of the high degree of conservation of the HDAC catalytic site between all but the class III (NAD dependent) HDACs, it is not surprising that most inhibitors target more than one family member. The specificities of the
inhibitors currently in trials are shown in Table 1. This broad specificity makes it difficult to interpret the results of inhibitor treatment in terms of precise biochemical mechanisms. Attempts have been made, with some success, to synthesize derivatives specific for individual HDACs and one such (a benzamide, 4SC-202) [26, 27] is in a phase I clinical trial (Table 1). However, the functional redundancy of HDACs, particularly class I enzymes [20, 21], may reduce the clinical effectiveness of enzyme-specific inhibitors. It is also worth noting that the specificities of individual HDACs have been determined by using pure, recombinant enzymes against artificial substrates. The catalytic activity of recombinant HDACs is extremely low and unlikely to reflect the in vivo situation where the enzyme itself can be part of different multi-protein complexes and exposed to the various factors (such as inositol phosphates) that modify its activity. An indirect, mass-spectrometry-based assay has been devised to examine the in vivo sensitivities of the different HDAC complexes to common inhibitors [28]. Though technically challenging, such approaches may provide a deeper understanding of HDAC inhibitor sensitivities.

**Why most cells are tolerant of HDACi-induced hyperacetylation**

A recent paper from the authors’ laboratory [29], addresses the question of why cells are usually so tolerant of
Table 2. Abbreviations of cancer types used in Table 1 and the number of HDACi compounds which have completed trials or are approved for each cancer

| Abbreviation | Cancer                          | n  | Abbreviation | Cancer                          | n  |
|--------------|--------------------------------|----|--------------|--------------------------------|----|
| AHM          | Advanced hematological malignancies | 3  | MCL          | Mantle cell lymphoma            | 1  |
| ALL          | Acute lymphoblastic leukemia     | 3  | Mes          | Mesothelioma                    | 3  |
| AML          | Acute myeloid leukemia           | 7  | MM           | Malignant melanoma              | 2  |
| APL          | Acute promyelocytic lymphoma     | 1  | MuMy         | Multiple myeloma                | 7  |
| Br           | Breast                          | 6  | NBM          | Neuroblastoma                   | 1  |
| CLL          | Chronic lymphocytic leukemia     | 1  | NHL          | Non-hodgkin’s lymphoma          | 5  |
| CML          | Chronic myeloid leukemia         | 1  | NSCLC        | Non-small cell lung cancer      | 10 |
| Col          | Colorectal                      | 5  | Ov           | Ovarian                        | 3  |
| CTCL         | Cutaneous T-cell lymphoma        | 6  | Pan          | Pancreatic                      | 3  |
| DLBCL        | Diffuse large B-cell lymphoma    | 1  | PCV          | Polycythemia vera               | 1  |
| Gas          | Gastric cancer                  | 1  | Pr           | Prostate                        | 4  |
| GBM          | Glioblastoma multiforma          | 1  | PTCL         | Peripheral T-cell lymphoma      | 3  |
| Gli          | Glioma                          | 1  | RCC          | Renal cell carcinoma            | 2  |
| HCC          | Hepatocellular carcinoma        | 1  | Sar          | Sarcoma                        | 5  |
| HL           | Hodgkins lymphoma               | 3  | SCLC         | Small cell lung carcinoma       | 1  |
| HNC          | Head and neck cancer            | 2  | SLL          | Small lymphocytic lymphoma      | 1  |
| Liv          | Liver                           | 2  | TCL          | T-cell lymphoma                 | 1  |
| Lung         | Lung                            | 1  | Thy          | Thyroid                        | 2  |
| Lym          | Lymphoma                        | 6  |              |                                |    |

HDACi-induced protein hyperacetylation. How do they deal with the disruption of fundamental cell functions that would be expected to ensue? To address this, attempts were made to identify the earliest transcriptional and epigenomic responses to HDACi. Human lymphoblastoid cells were exposed to two chemically different HDACi (SAHA and sodium valproate) for 30, 60, and 120 minutes. At this early stage, and for both inhibitors, cells were found to undergo a progressive and coordinated change in expression of a small and distinctive set of genes. Growth-promoting cytokines were down-regulated, presaging a later slowing of cell growth, while there was a rapid up-regulation of genes encoding DNA binding proteins and transcription factors. But the most striking response was the consistent and severe down-regulation of genes encoding components of the six enzyme complexes responsible for protein acetylation, the lysine acetyltransferases, KATs [17].

The rapid depletion of KAT complex components inevitably diminishes the aberrant acetylation of histones and other proteins when the deacetylating enzymes are blocked. KAT and HDAC complexes have both been shown to target the acetylated promoters of active genes [30], so KAT depletion would be expected to counter the effects of HDACi at those loci where histone acetylation is associated with gene expression. We propose that this transcription-based response allows the cell to reduce, and eventually reverse, protein hyperacetylation at critical regulatory regions, thereby reducing the epigenetic disruption caused by HDACi.

It is notable that, even at the earliest time points, there was no consistent association between increased histone acetylation at transcription start sites and increased gene expression. This indicates that histone hyperacetylation, at least at the three lysines studied so far, does not drive increased gene expression in this system, in agreement with previous studies [31–34]. However, it is striking that the genes that change expression in response to HDACi, whether up- or down-regulated, are packaged in highly acetylated chromatin [29]. It seems that histone acetylation helps provide a chromatin context within which genes are able to change their transcriptional state in response to HDACi, and perhaps other regulatory signals.

Surprisingly, the most substantial change in histone modification we observed was an increase in the levels of the Polycomb-associated silencing mark H3K27me3, specifically at transcription start sites and mostly at genes whose expression did not change [29]. The change in H3K27me3 levels at TSS was shown to be required for the short term, HDACi-induced changes in transcription at some loci. A chemical inhibitor of the enzyme responsible for H3K27 methylation, EZH2, prevented the up- or down-regulation of selected groups of genes [29]. How the changes in H3K27me3 are triggered, and how they influence the response to HDACi, are questions that require further investigation.

Does a reversibly acetylated non-histone protein control the response to HDACi?

The lack of association between changes in histone acetylation and transcription over the early stages of HDACi treatment, suggests that histones themselves are not the primary driver of early transcriptional change. What is? In Fig. 1, we present a model in which the acetylated and non-acetylated isoforms of a master regulator protein enhance the expression of different sets of genes. The relative expression levels of these genes are dependent on the balance between acetylated and non-acetylated isoforms. HDACi cause the balance to shift dramatically toward the acetylated form, resulting in up-regulation of one set of genes and down-regulation of the other (Fig. 1). The response is self-limiting in that the universal down-regulation of KAT complex components by HDACi will diminish acetylation of the primary sensor, thereby increasing the non-
acetylated isoform and restoring transcription of KAT complex components (Fig. 1). As things stand, the model is entirely hypothetical. Experiments are needed to identify the primary sensor protein, the KAT and HDAC involved in its acetylation, the steps in the signal cascades that link the sensor to specific sets of genes and the chromatin-binding proteins that finally mediate the observed changes in gene expression. Depending on the number of steps in the signal cascades, it is likely that multiple gene regulatory proteins are involved for both down- and up-regulated genes (Fig. 1). Some proteins may be used for both sets of genes. It is important that both signal cascades are responsible for supporting transcription of their respective sets of target genes. Thus, the down-regulation of KAT genes and others in response to HDACi, is due to diminution of this positive transcriptional control, rather than active down-regulation.

The components of the signal cascades triggered by the primary sensor, and the chemical changes involved, remain to be determined, but could involve phosphorylation, mediated by kinases and phosphatases as in many other signaling pathways, or other reversible modifications, including protein acetylation. In the latter case, the involvement of Class III deacetylases would protect the cascade from disruption by HDACi, to which these enzymes are resistant [16]. It has become clear that reversible lysine acetylation is a key regulator of enzyme function and metabolism in both eukaryotic cells and bacteria [35–37].

HDACi are natural products that can kill or manipulate competing organisms

It is, at first sight, surprising that human cells have the ability to mount such a carefully calibrated response to various inhibitors of a specific enzyme family. However, the fact that so many HDACi are natural products, suggests an evolutionary rationale through which these findings are readily explained.

HDACi of various chemical shapes and sizes are secreted by a wide range of organisms, often to kill, or at least suppress the growth of, competing life forms, (reviewed by Salvador and Luesch [38]). Some examples are presented in Table 3. Short chain fatty acids, all broad spectrum HDAC inhibitors, are prolific by-products of bacterial metabolism [39, 40]. Other, chemically more complex, HDACi seem to be specifically synthesized. The first hydroxamic acid-based HDAC inhibitor to be identified, Trichostatin A (TSA), is secreted by selected species of bacteria and is an antifungal antibiotic [41, 42].

Three types of bacterial HDACi based on a cyclic depsipeptide chemical backbone have been identified, FK228/Romidepsin [43], spiruchoatin [44] and largazole [45, 46]. The organisms they act against remain to be identified, though fungi are likely targets (Table 3).

Most fungal species seem to be resistant to HDACi, though mechanisms of resistance have only rarely been reported [47], and some even make their own. Examples include the linear polyketide depedecin [48] and the cyclic tetrapeptides HC-toxin [47, 49], and Trapoxin [50]. Another fungal HDACi, apicidin (a cyclic tetrapeptide made by Fusarium spp.) specifically kills some apicomplexan parasites (e.g. Plasmodium berghei) [51, 52]. In a survey of 52 Fusarium isolates, only one produced apicidin [53]. Some more complex, multicellular eukaryotes also secrete HDACi. Marine sponges synthesize HDACi based on either a cyclic tetrapeptide backbone (azumamides) [54] or bromotyrosine (Psammaplin A) [55–57], and may serve to deter micro-organisms sharing the same aquatic environment. The isothiocyanate-based HDACi sulforaphane, is produced by cruciferous plants such as broccoli [58] and first became of interest because of its ability to suppress the growth of transformed cells in tissue culture [58–60]. It remains to be shown how this inhibitor, and HDACi in general, might be involved in the cancer-preventing effects of cruciferous vegetables and other dietary components [60–63].

Competition for space and resources in the microbial world is often intense,
Hypotheses

and it is no surprise that an almost infinite variety of coping strategies have evolved. The energy and resources that some organisms devote to synthesizing sometimes chemically complex deacetylase inhibitors is presumably justified by the selective advantage they confer. The processes by which bacterial HDACi kill or deter competing organisms remain to be established, but it is likely to be important that fungi and protists (eukaryotes) have chromatin-based epigenetic control systems that bacteria (prokaryotes) lack.

Eukaryotic chromatin-based control systems provide a likely target for manipulation by prokaryotes

All life forms on earth consist of one or another of just two cell types, prokaryotic (bacteria and archaea) and eukaryotic (everything else, including all complex multicellular life forms). Though derived from a common ancestor [64], the two cell types are qualitatively different. Eukaryotes are classically distinguished by possession of a nuclear envelope, microtubules, and mitochondria (or equivalent) [65]. In addition, although prokaryotes have histone-like proteins that bind DNA [66], only eukaryotes package their DNA as chromatin, which invariably is, or once was [67], based on the canonical eight-histone nucleosome core particle [7, 68]. Thus, chromatin, nucleosomes and epigenetic control systems based on chromatin modifications, are uniquely eukaryotic. In this respect, they are likely targets for competing prokaryotes. We suggest that the HDACi secreted by some bacteria provide one example of this targeting.

Eukaryotes would be expected to have evolved responses to environmental HDACi, and we suggest that the transcription-based resistance mechanism present in human cells is an example of this [29]. As a competitive strategy, the secretion of HDACi will succeed only while the competing species are susceptible. Once they become resistant, new strategies are required. This may explain why most bacteria do not secrete, or are not known to secrete, HDACi, and why most eukaryotes seem resistant to their effects.

The resistance response to HDACi may be evolutionarily very ancient. It is estimated that the first prokaryotic life forms emerged about 3.5 billion years ago, 1 billion years or so after the planet was formed [69]. The emergence of the first eukaryotes is hard to establish, but is unlikely to be more than 2 billion years ago, perhaps much less [70]. The first eukaryotes emerged into ecosystems dominated by prokaryotes, who would have used all means at their disposal to see off the new competitors; targeting their evolving, but uniquely eukaryotic, chromatin, and epigenetic signaling networks would be a promising approach (Fig. 2).

Evolution generally proceeds by small steps, by selection of small mutational changes to existing systems. The development, by early eukaryotes, of defensive strategies to cope with prokaryotic HDACi, must have proceeded in the same way. In this respect, the simple model presented in Fig. 1 suggests a possible evolutionary pathway for the establishment, and progressive improvement, of a resistance response. All cells have systems by which they maintain metabolic homeostasis in the face of environmental change and their own growth and reproductive cycles. The signal cascades shown in Fig. 1 could be viewed as components of a homeostatic system for maintenance of levels of a key metabolite, acetylCoA. The intracellular concentration of acetylCoA has been shown to regulate levels of protein acetylation [36, 71], and the primary sensor postulated in Fig. 1 could have evolved from a sensor used to monitor the concentration of acetylCoA and regulate expression of genes encoding acCoA-producing or or acCoA-metabolising enzymes.

It would be wrong to assign the prokaryote-eukaryote interactions proposed here to the evolutionary past. We live in a world in which prokaryotes are ubiquitous, and even, by some measures, the predominant life form [72]. The human body is host to a variety of bacterial species, some of which are closely involved in key physiological functions, and are disrupted in disease [73]. For example, colonic bacteria give rise to millimolar concentrations of

Table 3. Natural products that act as histone deacetylase inhibitors

| Inhibitor               | Chemical type        | Source organism       | Target organism       | References |
|-------------------------|----------------------|-----------------------|-----------------------|------------|
| Butyrate et al.         | Short chain fatty acid derivatives | Most bacteria | Eukaryotic cells | [4]        |
| Trichostatins           | Hydroxamic acid derivatives | Bact; *Streptomyces hygroscopicus* | Fungi (Trichophyton, Aspergillus) | [41, 42] |
| FK228 (Romidepsin)      | Cyclic depsipeptide   | Bact; *Chromobacterium violaeum* | Fungi? | [43] |
| Spiruchostatin          | Cyclic depsipeptide   | Bact; *Pseudomonas chlorophila* | Fungi? | [44] |
| Largazole               | Cyclic depsipeptide   | Marine cyanobacterium (*Symploca sp*) | unknown | [45, 46] |
| Depudecin               | Linear polyketide     | Fungus (*Alternaria brassicicola*) | unknown | [48] |
| HC toxin                | Cyclic tetrapeptide   | Plant fungus (*Cochliobolus carbonum*) | Green plants | [47, 49] |
| Trapoxins               | Cyclic tetrapeptide   | Fungus (*Helicoma ambiens*) | unknown | [50] |
| Apicidin                | Cyclic tetrapeptide   | Fungus (*Fusarium pallidoroseum*) | Apicomplexan parasites | [51–53] |
| Azumamides              | Cyclic tetrapeptide   | Marine sponge (*Myccale izunensis*) | unknown | [54] |
| Psammaplin A            | Linear bromotyrosine  | Marine sponge (*Psammaplysilla sp*) | unknown | [55, 56] |
| Sulforaphane            | Isothiocyanate        | Cruciferous plants (eg broccoli) | Pathogenic fungi? | [58, 59] |

The table shows the basic chemical structures of naturally occurring HDACi, organisms that make them and, where known, the organisms against which they act in vivo. Detailed chemical structures for the inhibitors listed can be found in the review by Salvador and Luesch [38].

Fig. 1 could have evolved from a sensor used to monitor the concentration of acetylCoA and regulate expression of genes encoding acCoA-producing or or acCoA-metabolising enzymes.
salts of various short chain fatty acids in the mammalian large intestine [74–77]. Cells of the colonic epithelium, including differentiating stem cells involved in replacement of the surface layer, must therefore accommodate inhibitory concentrations of bacterial HDACi. Given the subtlety with which prokaryotes can influence patterns of gene expression in neighbors through chemical signals [78], this is unlikely to be the only situation in which endogenous micro-organisms influence our epigenetic systems [73]. Nor is it likely that HDACs are the only targets.

**Conclusions**

This article began with a description of the potential value of HDACi as chemotherapeutic drugs, progressed through the possible influences of HDACi in the co-evolution of prokaryotic and eukaryotic micro-organisms, and has finished by highlighting the ongoing chemical interactions between complex eukaryotes (including ourselves) and the microbial world. This summary returns to where we started, by noting that the existence of a resistance response to HDACi, and its evolutionary provenance, has clinical implications. Certain cancers may be sensitive to HDACi because their resistance mechanism has been disrupted (Fig. 2), perhaps by mutation of one or more of its essential genes, or components of the (as yet hypothetical) primary sensor or signal cascade (Fig. 1). Identification of such mutations will help target individual cancers susceptible to HDACi. Similarly, combining HDACi with drugs that undermine the resistance mechanism specifically in cancer cells, may open the way to more successful treatment of currently refractory cancers through combination therapy. It will also be interesting to search for other pathways by which prokaryotic metabolites might deregulate epigenetic control systems peculiar to eukaryotes, and to ask by what means eukaryotes defend themselves against such manipulation. Understanding these interactions may be crucial in maximizing the clinical benefit of the many chemotherapeutic drugs based on natural products.

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Figure 2. How a resistance response to histone deacytase inhibitors (HDACi) might influence both evolution of eukaryotes and response of tumor cells to chemotherapy. We propose that eukaryotes have evolved a response that allows them to deal with the hyperacetylation caused by environmental (often bacterial) HDACi (upper part). Cancer cells in which this response remains intact can resist chemotherapeutic HDACi, whereas those in which the response has been compromised, either by mutation or through additional drug treatment, are killed, leading to a period of remission (lower part).

**Hypotheses**
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