available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Review

Epigenetic therapies in haematological malignancies: Searching for true targets

Lucia Altucci[a], *Saverio Minucci*[b,c,*]

[a]*Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli, Vico L. De Crecchio 7, 80138 Napoli, Italy*
[b]*European Institute of Oncology, Via Adamello 16, 20100 Milan, Italy*
[c]*Department of Biomolecular Sciences and Biotechnologies, University of Milan, Via Celoria 26, 20100 Milan, Italy*

ARTICLE INFO

Article history:
Received 24 February 2009
Accepted 3 March 2009
Available online 5 April 2005

Keywords:
Epigenetic therapy
Acute myeloid leukaemia
Lymphoma
Histone deacetylase
DNA methyltransferase
Histone methylase and demethylase
Targeted therapy

ABSTRACT

Epigenetic alterations complement genetic mutations as a molecular mechanism leading to cell transformation, and maintenance of the cancer phenotype. Of note, they are reversible by pharmacological manipulation of the enzymes responsible for chromatin modification: indeed, epigenetic drugs (histone deacetylase inhibitors and DNA demethylating agents) are currently on the market, inducing proliferative arrest and death of tumor cells. These drugs, however, have been effective only in a few tumor types: the lack of consistent clinical results is mainly due to their use in a poorly targeted approach, since the epigenetic alterations present in cancer cells are mostly unknown. In a few cases (notably, leukemias expressing RAR and MLL fusion proteins), the molecular mechanisms underlying tumor-selective and tumor-specific epigenetic alterations have started to be deciphered. These studies are revealing a dazzling complexity in the mechanisms leading to alterations of the epigenome, and the need of combination therapies targeting different chromatin modifiers to reach an effective reversion of epigenetic alterations.

1. Introduction

The concept of epigenetics includes the heritable changes that do not involve an alteration of the genome at the level of DNA sequences.[1,2] Recent progresses have highlighted the key role of epigenetic mechanisms in ensuring the appropriate control of biological processes, such as imprinting, X chromosome inactivation, or the establishment and maintenance of cell identity. The functional significance of this epigenetic control becomes apparent in its deregulated state: alterations of both genetic and epigenetic mechanisms are responsible for the establishment and progression of cancer, as well as other diseases (for recent reviews, see Refs. [3,4]). In addition to genetic alterations, aberrant epigenetic regulation, such as silencing of tumour suppressors, is used by cancer cells to escape control mechanisms.[5] Thus, compounds able to influence the epigenetic status of a cell have promise for cancer treatment: several epigenetic enzymes have been targeted with small molecules leading to the development of 'epigenetic therapies'. Here, starting from the clinical results obtained so far, we discuss (focusing on specific examples) the principles that should underlie their use, based on the mechanistical basis of the involvement of epigenetic alterations in cancer.

* *Corresponding author:* Address: European Institute of Oncology, Via Adamello 16, 20100 Milan, Italy. Tel.: +39 02 57489832; fax: +39 02 94375090.
E-mail address: saverio.minucci@ifom-ieo-campus.it (S. Minucci).
0959-8049/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2009.03.001

2. Epigenetic therapies: Poorly targeted therapies

The concept of targeted therapies has evolved to a dogma of inescapable success of pharmacological intervention against key molecular players in the pathogenesis of diseases.[6] In part, this view derives from the striking results of initial clinical applications: in cancer, Glivec has revolutionised the treatment of chronic myeloid leukaemia (CML) patients, targeting the enzymatic activity of the fusion protein (bcr-abl) which is the molecular trigger of the disease.[7]

Indeed, molecularly targeted agents appear to lead to reduced attrition rates in drug development, if compared to drugs lacking a well-defined, disease specific mechanism of action: this may be particularly relevant in clinical areas such as oncology, where for decades the only available therapies were focused on agents that – although efficacious – had a narrow therapeutic index since they were targeting basic biological functions common to both normal and tumour cells.[8]

Cancer cells show global changes in chromatin structure (DNA methylation and histone post-translational modifications) that lead to stable alterations in gene expression and, potentially, other nuclear functions (such as DNA replication and repair). Interestingly, unlike genetic lesions, those alterations are potentially reversible since the underlying DNA sequence is unchanged: this fundamental difference between genetic and epigenetic alterations makes the epigenome much more amenable to the development of therapeutic strategies. Indeed, the demonstration that small molecules with the capacity to interfere with enzymatic activities acting in chromatin modification have antitumour potential has led to the development of a series of drug discovery programmes in epigenetics, which has culminated in the approval by regulatory authorities of a small number of drugs for use in selected forms of cancer.[9]

Although these results have been widely propagandised as an additional example of a successful targeted approach, in our view this is not entirely true. It will be rather hard, in fact, to find well-defined correspondences between the case of Glivec in CML and the use of epigenetic drugs in cancer. In CML, a specific genetic alteration (the presence of the t(9:22) chromosomal translocation, leading to generation and expression of the bcr-abl fusion protein) is the initiating event leading to transformation of the normal target cell, which remains dependent on the continuous expression of the oncogene and its altered kinase activity for survival and transformed phenotype.[7] Inhibition of the bcr-abl associated kinase activity (by Glivec or other inhibitors) therefore leads to the interruption of critical pathways for the tumour cells and to induction of cell death (Fig. 1A). Currently, drugs interfering with epigenetic enzymes (such as DNA methyltransferases and histone deacetylases, the most advanced targets in the epigenetic arena) have been used in the vast majority of cases rather differently, and less specifically (detailed in the following sections of this review): we surmise, therefore, that one of the major goals of both basic and applied research in this area should be the search for a set of epigenetic alterations in tumour cells that lead to a transcriptional (and potentially related to other nuclear functions) landscape critical for cell survival, and that – when interfered by epigenetically targeted drugs – causes cell death (Fig. 1B). Indeed, the lack of a targeted approach could be – in our opinion – an important factor in the relatively weak anti-tumour effect of epigenetic drugs on the majority of cancer patients.

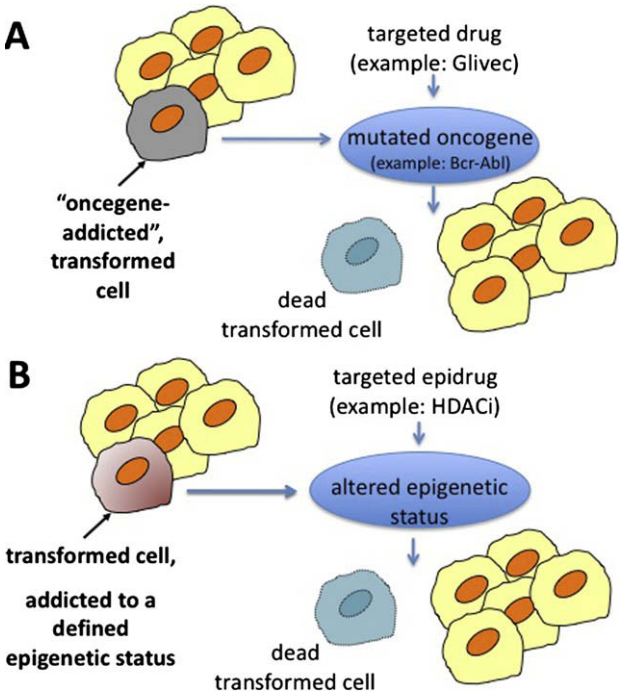

Fig. 1 – Targeted therapy as a paradigm for epigenetic therapy ('Epi-cures'). (A) General rationale for the use of molecularly targeted agents in cancer (example: Glivec in leukaemias). (B) Epigenetic therapies must identify tumour-specific epigenetic alterations, which lead to the use of the appropriate epigenetic drugs in a targeted way.

Here, we will try to show (taking as a paradigm haematological malignancies, and focusing when necessary on selected subtypes of leukaemias) that there are different mechanisms acting in different tumour types to impose an altered epigenetic landscape, and we will review the effect of epigenetic therapies in these same tumour types: the results so far add to the complexity of the picture, revealing that – at least in the current mode of treatment, and with the currently available inhibitors – epigenetic therapies may have non-epigenetic effects, and may act on both targeted epigenetic alterations and on intrinsically generic epigenetic features of the tumour cells (in some cases apparently with relevant antitumour effects).

3. Epigenetic drugs in clinical trials for haematological malignancies

Notwithstanding the caveats expressed above, the interest in epigenetic therapy is fully justified by the fact that in several clinical contexts they have shown a varying level of efficacy. Here, we summarise the most relevant clinical results obtained using epigenetic drugs.

3.1. HDAC inhibitors

Four major classes of histone deacetylases have currently been identified, three of which (I, II and IV) are considered here since they share a common catalytic site, and can be targeted by similar molecules. HDAC inhibitors are a diverse group of compounds able to induce histone hyperacetylation and changes in a large set of proteins.[10]

Several HDAC inhibitors (HDACis) exhibit impressive antitumour activity potentiated by little toxicity in *in vitro*, *ex vivo* and *in vivo* models and are now involved in clinical trials as monotherapies as well as in combination with other drugs.[11] Several classes of HDACis have been identified including short-fatty acids (such as butyric acid), hydroxamic acids (such as suberoylanilide hydroxamic acid, SAHA and trichostatin A, TSA), cyclic tetrapeptides and benzamides (such as MS-275). While it is well established that an important component of the action of HDACis is the induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, many sets of data point to the exciting potential of HDACis with selective anti-cancer activity but indicate the involvement of different molecular pathways. It has become quite clear that modulation of gene expression by HDAC inhibitors is not sufficient to describe their effects. The extensive research involving HDAC inhibitors has shown that they have effects in addition to the modulation of gene expression through chromatin remodelling. Therefore, focusing on a unique target is likely to be inadequate in explaining the actions of HDAC inhibitors. Moreover, HDAC inhibitors can induce acetylation of protein targets, including transcription factors and heat shock proteins, which may contribute to apoptosis. The effects of HDAC inhibitors on the extrinsic (TRAIL, Fas and death receptors)[12,13] and intrinsic (mitochondrial) death signalling pathway[14] have been extensively studied. The reported changes differed according to the tumour and cell type. Moreover, in leukaemia cells, HDAC inhibitor induced apoptosis was caspase dependent[12,13], whereas in myeloma cells it was independent of caspases.[15] Other possible mechanisms for HDAC inhibitor apoptosis, such as nuclear factor-kB activation, generation of ceramide, and modulation of heat shock proteins, have also been described.[16]

Despite efforts still needing to be made in the understanding of the anticancer mechanism(s) of HDAC inhibitors, many clinical trials at present are actually based on their use against cancer. SAHA (vorinostat, Zolinza®; Merck) was approved by the US FDA in October 2006 for the treatment of cutaneous manifestations in patients with cutaneous T-cell lymphoma (CTCL) who have progressive, persistent or recurrent disease on or following two systemic therapies. Thereby, SAHA is the first of a new class of anticancer agents (<http://www.fda.gov/cder/foi/label/2006/021991lbl> 2006). Apart from CTCL, vorinostat alone or more frequently in combination, is being used in clinical trials for many solid and haematological cancers, including pancreatic, breast, several types of leukaemia and lymphoma, thyroid, multiple myeloma, mesothelioma, NSCLC and colon cancer.[9–11] Moreover, vorinostat is also being applied in trials for pre-neoplastic manifestations such as MDS or myeloproliferative disorders. Apart from SAHA, a number of hydroxamates are being used in phase I–III clinical trials as anticancer agents including the indolylethylamino-methylcinnamyl hydroxyamides LAQ-824 and LBH-589 (panobinostat).[17,18] However, whereas the development of LAQ-824 was terminated due to the possibility of toxicity problems, panobinostat is currently involved in a phase II/III clinical trial for chronic myeloid leukaemia (CML), refractory CTCL and multiple myeloma (MM). Panobinostat has been reported as being highly active against HDAC class I and IIa (IC_{50}s = 14 to 3 nM), but less potent against HDAC6 and HDAC8.[19] Belinostat (formerly PXD-101) is another cinnamyl hydroxamate which inhibits both class I and class II HDACs in the submicromolar range. Belinostat is being used in phase II clinical trials for haematological malignancies as well as for many solid tumours.[20,21] The cyclic peptide romidepsin (formerly known as FK-228) is involved in clinical trials for CML, AML and CTCL.[22–24] The benzamide MS-275 has been shown to induce apoptosis of acute myeloid, B-chronic lymphocytic leukaemia cells, Jurkat lymphoblastic T cells and prostate cancer cells.[25,13,26] This HDACi is used in clinical trials in combination with other anti-tumour agents.[27,25] MGCD-0103 is a more recent benzamide developed by MethylGene. MGCD-0103 behaves as a HDAC1/2-selective HDACi[19] and is currently being used in phase II clinical trials for the treatment of haematological malignancies and in phase I/II trials for solid tumours.[28]

Although it is tempting to speculate that HDACis kill cells by causing cell death or differentiation, their pleiotropic actions suggest that this is an oversimplification. For example, attention has been recently focused on the ability of HDAC inhibitors to act as 'protein acetylases' altering the tubulin deacetylases (such as HDAC6), and interfering with the function of chaperone proteins.[29] The diverse mechanism(s) of HDACi action suggest that the simple acetylation of histones might not be the optimal tool to determine their activity, given that histone acetylation might be necessary, but not sufficient, for response. An improved understanding of the mechanism of action of HDAC inhibitors will be crucial for the rational design of future combination trials. A good deal of research interest concerns the issue of whether HDAC inhibitors will work more effectively when used in combination with existing anti-tumour drugs. Apparently, the synergistic effects generated from the combined use of HDACis and chemotherapy will be one of the hot issues in cancer therapy research.[30] Indeed, there is currently considerable interest in the development of regimens combining HDACis with other targeted agents that can enhance HDACi lethality, such as cyclin-dependent kinase inhibitors, Hsp90 antagonists, proteasome inhibitors and tyrosine kinase inhibitors.[31–33] Whether this approach will lead to improved antitumour action and selectivity is likely to be answered in the next few years.

3.2. DNA demethylating agents

The term DNA demethylating agents refers to compounds able to induce transient DNA hypomethylation. DNA methylation is the addition of a methyl group to a CpG site.[34] These sites bunch together in areas known as 'CpG islands' frequently in the proximity of gene promoters. DNA methylation, both aberrant and physiologic, can result in gene

silencing and in the corresponding gene inactivation, due to either mutations or deletions, of tumour suppressor genes.[4]

Actually, two hypomethylating agents are approved and widely used: 5-azacitidine[35] and 5-aza-20-deoxycitidine (decitabine).[36] These two drugs have significant activity in patients with higher risk myelodysplastic syndromes (MDS). Decitabine is a nucleoside analogue that indirectly inhibits DNMTs, thus resulting in global hypomethylation.[37] The finding of aberrant DNA methylation as a critical event in cancer, and the reversion by hypomethylating agents, has led to an increasing interest in performing clinical trials with these compounds in MDS and acute myelogenous leukaemia (AML). Some concerns regarding the use of DNMT inhibitors have been raised as a result of the finding of genome instability in mice with reduced DNA methylation levels due to prolonged demethylating treatment,[38] even if those results are not directly comparable to the transient DNMT inhibition applied in cancer patients. Initially, decitabine was used in several clinical trials in AMLs and higher risk MDS and some chronic myelogenous leukaemias (CML). In the initial studies, decitabine resulted in better responses compared with supportive care. Although the results of these initial studies are of great relevance, alternative schedules that did not require hospital admission have been tested.[39] The main toxicities were myelosuppression and its complications. In all cases the treatment with decitabine was associated with improved survival despite the fact that complete remissions were lower when compared with chemotherapy. This is probably due to the low induction of mortality observed. This notion represents a change in practice, as we may agree to a lower response rate if mortality is significantly lower and translates into better survival. 5-Azacitidine is the other nucleoside analogue, structurally related to decitabine. In contrast to decitabine, 5-azacitidine is a ribose structure that is incorporated into RNA and requires the activity of ribonucleotide reductase (RNR) to be incorporated into DNA and to exert its hypomethylating effect. Recent studies have reported a significant effect on survival in patients that received 5-azacitidine versus other treatments.[40] Interestingly, patients with alterations of chromosome 7 derived the most benefit: clearly, these evidences will need further study.

From a mechanistic point of view, the most widely studied combination includes hypomethylating drugs and HDAC inhibitors.[41] Several studies have been reported with the combination of 5-azacitidine or decitabine with valproic acid (VPA).[42] These studies with VPA have been mainly phase I or II in AMLs and higher risk MDS. The relative weakness of VPA as a HDACi supports the notion that replacing VPA by more active HDAC inhibitors may result in better clinical combinations. Such studies are ongoing, using either 5-azacitidine or decitabine with several HDAC inhibitors. One such study is the combination of 5-azacitidine and MGCD0103, a class 1 HDAC inhibitor with activity in AML and MDS.[43,44]

Other nucleoside analogues, such as zebularine, have been developed to overcome the instability of decitabine and 5-azacitidine.[45] Unfortunately, zebularine has limited oral bioavailability in a pharmacokinetic study, and it is not clear whether active concentrations can be clinically achieved with oral administration.[46] Recently, additional compounds, such as the local anaesthetic procaine (and its derivative procainamide) and the main polyphenol compound in green tea, EGCG ((–)-epigallocatechin-3-gallate), have been shown to inhibit DNMTs and some of them are currently in phase I trials. Moreover, psammaplins have shown both DNMT and HDAC activity inhibition which might enable a combinatorial inhibition by using a single drug.[47] Finally, RG108 is the first rationally designed DNMT1 inhibitor with demethylating activity both in *in vivo* and *in vitro* models.[48] Unfortunately, its activity seems too weak to have any potential in clinical use.

4. Epigenetic alterations in haematological malignancies

Conceptually, there are two main classes of epigenetic alterations that are potentially found in tumour cells: those due to the direct action of the triggering transforming event, and those subsequent to the transformation process itself. Here, we mainly take into consideration the epigenetic alterations found in two subtypes of AML, caused by two distinct classes of fusion protein: RAR- and MLL-fusion proteins, respectively, since we have a relatively clear picture of their mechanism of action.

4.1. *Acute promyelocytic leukaemia (APL)*

Retinoic acid receptors (RARs) are the main effectors of the signalling network instigated by retinoic acid (RA), and are important modulators of several cell processes, including haematopoietic differentiation.[49] RARs are transcription factors that bind specific response elements and repress transcription of target genes in the absence of a ligand, and activate transcription when they become bound by RA: the ligand triggers a conformational change in the receptor, leading to a switch in the association with transcriptional cofactors: repressive (such as HDACs) in the unbound state, co-activating in the presence of RA.[50] APL is caused in 100% of cases by RAR translocations, yielding different RAR fusion proteins (in >90% cases, the fusion partner is *PML*, and the fusion protein is PML-RAR).[50] PML-RAR binds DNA with an altered specificity, expanding the repertoire of potential target genes for the fusion protein.[51] Additionally, physiological concentrations of RA are not able to induce the switch in the association with transcriptional coregulatory complexes, and PML-RAR maintains a repressive chromatin structure at target genes in the presence of RA.[52] There is a complex network of PML-RAR associated chromatin remodelling complexes: class I HDACs as part of Sin3 and NURD complexes, DNA methyl-transferases and factors able to bind methylated DNA (MBDs), and histone methyltransferases (see below).[53–56] In several model systems (mainly at the cellular level), recruitment of those activities has been shown to be required for the altered biological properties of the fusion protein.[53–56] These studies have led to the proposal of a general model where the effect of the fusion protein is to 'freeze' the chromatin structure of target genes in a strongly repressive conformation, resembling heterochromatin.[10] This structure is refractory to physiological stimuli that occur during haematopoietic differentiation and that require activation of PML-RAR target genes, thus resulting in a block of differentiation.[10]

Unfortunately, however, several bits of information are missing: for example, only a few studies are starting to address, systematically, chromatin changes induced by PML-RAR expression at target genes: most of the studies mentioned above make use of only one (the RARß promoter) or a few RAR target genes as exemplifying the entire picture, which is certainly not the case. Reassuringly, recent analyses confirm the ability of the fusion protein to recruit HDACs (HDAC1 in REF 57) and cause histone deacetylation and histone methylation (histone H3K9 trimethylation) of target genes.[57] One important element to be considered is that the use of artificial cell models, although of clear help in the design of models and hypothetical mechanisms of action, is not sufficient to fully validate the proposed mechanism of action: mechanistical studies have to be performed in 'real', patient-derived cells, and – whenever possible – *in vivo* (through the use of animal models).

4.2. *MLL-fusion proteins*

MLL is the mammalian homologue of the *Drosophila* Trithorax. As in flies, it exerts multiple roles during development, including haematopoietic development.[58] *MLL* is a large protein with several functional domains required for association with DNA, chromatin and other protein–protein interactions. Among these domains, a SET domain has shown histone methyltransferase activity with a specificity for the K4 residue of histone H3.[59,60] H3K4 methylation is associated with an active transcriptional state, and consistently *MLL* has been shown to be involved in the transcriptional activation of *HOX* genes and other targets.[61] Chromosomal translocations at the *MLL* locus have been found in several forms of leukaemia, and are extremely frequent in paediatric leukaemias.[62] Strikingly, the corresponding fusion proteins (generated by the fusion of the MLL N-terminal portion with >50 different fusion partner genes) invariably lose the SET domain, and are devoid of intrinsic histone methyltransferase activity.[62]

At a first glance, therefore, the situation appears to be similar to APL: while in this latter disease a bivalent transcriptional regulator is frozen in a repressive state through the fusion with *PML*, in the case of *MLL* fusions, a transcriptional activator, losing a key activating function, is potentially turned out into an inactive protein that could behave as a repressor factor.

Actually, however, the situation is more complex. In fact, MLL-fusion proteins retain the capacity to activate transcription of target genes: this ability is mainly due to the recruitment of other chromatin remodelling abilities. Several MLL-fusion partners (and corresponding fusion proteins) are able to engage the histone methyltransferase DOT1: this enzyme is responsible for H3K79 methylation, coupled to transcriptional activation.[63] Strikingly, the H3K79 histone methylation pattern in MLL-AF4 expressing cells is dramatically divergent from normal cells, and also from other acute leukaemias of the same subtype, consistent with a specific gene expression pattern: 1000 promoter regions (including *HoxA* genes) show an altered pattern of H3K79 histone methylation (with large domains of 5–100 Kb showing aberrant methylation) in MLL-AF4 leukaemias and enhanced gene expression.[64,65] Among those MLL-AF4 gene targets, several haematopoietic developmental regulators appear to be expressed aberrantly in leukaemic cells, justifying their altered biological state.[65]

Other MLL-fusion partners (such as *EEN*) show the ability to interact with different chromatin remodelling complexes. Thanks to this interaction triggered by EEN, MLL-EEN can recruit CBP (histone acetylase) and PRMT1 (H4R3 histone methylase), triggering distinct chromatin modifications at target genes (*Hox* genes), consistent with their transcriptional activation. The ability to recruit these complexes is critical for MLL-EEN transforming capacity.[66]

4.3. *The rest of the picture: An unresolved epigenetic puzzle*

Besides these relatively well characterised examples, other leukaemias have shown a clear link between the leukaemogenic, transforming factor and its ability to induce epigenetic alterations. In a different form of AML, the fusion protein AML1-ETO is also able to recruit transcriptional co-repressor complexes (HDACs and DNMTs), induce a repressive chromatin structure (deacetylated histones and methylated DNA), and repress target gene transcription.[52] Interestingly, AML1-ETO has also been reported to silence RA target genes, establishing transcriptional repression of the RA signalling pathway as a common event in AMLs (directly mediated by an altered RAR in APL cells, and indirectly in other cases: see Ref. [67]).

Aberrant recruitment of chromatin remodelling complexes by transcription factors is not limited to AMLs: in non-Hodgkin lymphomas, we observe overexpression of the oncogene Bcl6, a member of a family of transcriptional repressors containing a POZ domain, that is able to recruit various corepressor complexes with HDAC activity.[68–70]

Strikingly, recurrent chromosomal translocations in leukaemias involve direct histone acetylating enzymes.[71] Both CBP/p300, and members of the MYST family of acetyltransferases are found in these rare rearrangements: although conservation of an aberrant enzymatic activity by the corresponding fusion proteins seem to be relevant for leukaemogenesis, only a few studies have addressed the mechanistical basis of the deregulated activity of the fusion proteins and looked at their transcriptional targets.[71]

These additional examples help to emphasise two important points: (i) deregulation of epigenetic mechanisms is a frequent tumourigenic event in haematological malignancies and (ii) we have only just started to scratch the surface in determining how this deregulation takes place.

In fact, a unique biological output (cell transformation) can be the consequence of paradoxically opposite epigenetic alterations. In the case of MLL-fusion proteins, for example, the epigenetic alteration leads to transcriptional activation of targets, while RAR fusion proteins repress transcription.[72] How can these opposing effects be reconciled?

One simple explanation would be that, irrespective of the primary genetic lesion and direct epigenetic alterations induced by the product of the genetic alteration, the ultimate epigenetic consequences (and deriving altered gene expression programmes) would be identical, or at least highly similar. In other terms, MLL-fusion proteins would activate primary targets, which in turn would lead to a transcriptional

profile resembling the pattern induced by RAR fusion proteins (irrespective of the mechanism of action of the fusion proteins). Unfortunately, this does not appear to be the case: gene expression profiles in different AML subtypes are quite distinct.[73] Indeed, the chromatin organisation of MLL-X leukaemias seems to be distinct from any other leukaemias, even those belonging to the same immuno-cytological phenotype.[64]

Perhaps the identity of the target cell (the cell where transformation takes place, and gives rise to the leukaemic clone) could play an important role in the definition of the epigenetic determinants required for transformation. In the case of MLL-fusion proteins, although a definitive conclusion has not been reached, it appears that both haematopoietic stem cells and progenitor cells can be targets for transformation,[74] while for APL the target cell has not been described as of yet.[75] Interestingly, APL blasts are arrested at a later stage during haematopoietic differentiation, and it is not excluded, therefore, that the target cell could be different: in this case, a different set of epigenetic alterations would be required to maintain cells 'frozen' at different stages during differentiation, and it is conceivable that different fusion proteins may achieve – in the context of a common transformed background – specific phenotypes, depending on the set of epigenetic alterations that they are able to induce.

5. Putting it all together: The epigenetic state of leukaemic cells and the response to epigenetic drugs

This short overview is sufficient to reinforce the conclusion that we are still far – in the field of epigenetics – from the application of the concept of 'targeted therapy', at least in the sense derived from the equation '*Bcr-Abl expression in CML = successful treatment with Glivec*'. In fact, it is very difficult to find convincing links between the pleiotropic effects shown by epigenetic drugs, the multiple pathways hit following treatment, and a direct effect on the epigenetic alterations induced by the transforming event (see also below). APL is a striking example of this discrepancy: in spite of the multiple epigenetic alterations instigated by the fusion protein, HDAC inhibitors induce apoptosis of leukaemic cells mainly by reactivation of the death receptor pathway, which is silenced in tumour cells through PML-RAR independent mechanisms.[12,13] If this is true for haematological malignancies (that have a better definition of epigenetic alterations in tumour cells), the situation is surely worse for solid tumours.

From one point of view, however, this consideration can be reassuring for those critics not satisfied with the clinical results obtained so far with epigenetic drugs: administration of a 'targeted' drug in an 'untargeted' way is a sure path to underestimation of its potential. So in the end, for now, we are using epigenetic drugs with schedules of treatment and modalities that resemble much more the use of standard chemotherapeutic drugs than Glivec. We could, therefore, be far away from the optimal clinical results that could be achieved with epigenetic therapy.

We must consider the additional possibility that we are not optimising towards epigenetic effects, but rather towards non-epigenetic effects of epigenetic drugs.[10] As an example, the oncogene bcl6 is acetylated following HDACi treatment, leading to its inactivation and cell cycle arrest/apoptosis in lymphoma cells.[76] Similarly, in APL cells, PML-RAR also exerts non-epigenetic effects, stimulating deacetylation and degradation of p53: HDACis – when cells are exposed to genotoxic agents, able to trigger p53 dependent pathways – lead to stabilisation of p53 in APL cells and its activation.[77] These are just a few examples: it is clear that HDAC inhibition leads to acetylation not only of histones, but of several other proteins that are normally acetylated/deacetylated as a mechanism to modulate their function.[10] The identification of the cellular acetylome has just started: a few proteomic studies have been published, revealing the first list of potential candidate targets for the non-epigenetic effects of HDACis.[78] The extravasation of epigenetic drugs into non-epigenetic areas is not limited to HDACs: histone methylase and demethylases have also been shown to be able to modify non-histone substrates, and therefore pharmacological modulation of their activities is also bound to have non-epigenetic effects.[79]

Can we be truly '*epigenetic*' in our modality of using epigenetic drugs? Undoubtedly, and unless selective modulators of histone modifications will be identified in the future, we are bound to have a mix of epigenetic and non-epigenetic effects using almost all of the currently available drugs, since they target enzymes acting on histone and non-histone substrates. One potential exception is the use of DNA demethylating agents, since their main action (at least for decitabine) is at the level of DNA. This can be an important property that could be exploited for the development of bona fide epigenetic therapies (see below).

In any case, however, it is imperative that a major effort be devoted to the systematic characterisation of epigenetic alterations in cancer cells, and to the identification of the targets of leukaemia and lymphoma associated oncogenes. It is only with a better understanding of these events that we will be better able to design appropriate strategies and identify key biomarkers of epigenetic treatment.[80] Technologies are just emerging that will facilitate this endeavour in the years to come: they are mainly based on high throughput sequencing approaches and can map genome-wide specific histone post-translational marks, or general features of chromatin.[81,82] In our view, it is unlikely that those epigenetic alterations will be due to the deregulated activity of one single enzyme: indeed, chromatin is continuously modified by the combinatorial work of several enzymatic activities, and, therefore, epigenetic alterations will most likely result in a spectrum of alterations due to different enzymes (although we cannot exclude that single histone post-translational modifications may be the dominant alteration – as is becoming apparent for MLL-fusion proteins). If this is the case, then the most effective – and perhaps the most unique – epigenetic therapies will be the combined treatment with more than one drug, and it is likely that DNA demethylating agents will be a constant and critical component of these epigenetic cocktails. Data discussed above are already showing that the clinical results of the first combination trials are more promising than the single agent studies, although they continue to be performed under suboptimal conditions. We envision, therefore, that a more detailed knowledge of epigenetic alterations

in tumour cells will lead to the identification of optimally suited combination therapies, able to fully exploit the potential of this approach. More study is certainly required in order to find the true and prominent location of epigenetic therapy in the map of anticancer strategies.

Conflict of interest statement

S.M. has stocks in Genextra Spa, a biopharmaceutical company that is currently developing HDAC inhibitors for cancer therapy.

Acknowledgements

We are indebted to Pier Giuseppe Pelicci, Eric So, Ciro Mercurio and Angela Nebbioso for stimulating discussions. Studies in the author's laboratories have been supported by the Associazione Italiana per la ricerca contro il cancro (AIRC), EU grants (Epitron LSHC-CT2005-518417; Apo-sys HEALTH-F4-2007-200767; CancerDip HEALTH-F2-2007-200620), La Regione Campania Legge 5/2002 annualità 2005, il Ministero italiano dell'Istruzione, Università e Ricerca (MIUR-PRIN 2006, 2007). LA dedicates this work to Lucia Latela, who died fighting against cancer.

REFERENCES

1. Altucci L, Stunnenberg HG. Time for epigenetics. *Int J Biochem Cell Biol* 2009;**41**:2–3.
2. Baylin SB, Schuebel KE. Genomic biology: the epigenomic era opens. *Nature* 2007;**448**:548–9.
3. Esteller M. Epigenetics in cancer. *N Engl J Med* 2008;**358**:1148–59.
4. Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;**128**:683–92.
5. Ballestar E, Esteller M. Epigenetic gene regulation in cancer. *Adv Genet* 2008;**61**:247–67.
6. Hait WN, Hambley TW. Targeted cancer therapeutics. *Cancer Res* 2009;**69**:1263–7 [discussion 1267].
7. Druker BJ. Translation of the Philadelphia chromosome into therapy for CML. *Blood* 2008;**112**:4808–17.
8. Walker. Do molecularly targeted agents in oncology have reduced attrition rates? *Nat Rev Drug Discov* 2009;**8**:15–7.
9. Mai A, Altucci L. Epi-drugs to fight cancer: from chemistry to cancer treatment, the road ahead. *Int J Biochem Cell Biol* 2009;**41**:199–213.
10. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer* 2006;**6**:38–51.
11. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* 2006;**5**:769–84.
12. Insinga A, Monestiroli S, Ronzoni S, et al. Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. *Nat Med* 2005;**11**:71–6.
13. Nebbioso A, Clarke N, Voltz E, et al. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. *Nat Med* 2005;**11**:77–84.
14. Lindemann RK, Newbold A, Whitecross KF, et al. Analysis of the apoptotic and therapeutic activities of histone deacetylase inhibitors by using a mouse model of B cell lymphoma. *Proc Natl Acad Sci USA* 2007;**104**:8071–6.
15. Fandy TE, Shankar S, Ross DD, Sausville E, Srivastava RK. Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma. *Neoplasia* 2005;**7**:646–57.
16. Rosato RR, Grant S. Histone deacetylase inhibitors: insights into mechanisms of lethality. *Expert Opin Ther Targets* 2005;**9**:809–24.
17. Arts J, Angibaud P, Marien A, et al. R306465 is a novel potent inhibitor of class I histone deacetylases with broad-spectrum antitumoral activity against solid and haematological malignancies. *Br J Cancer* 2007;**97**:1344–53.
18. Remiszewski SW, Sambucetti LC, Bair KW, et al. N-hydroxy-3-phenyl-2-propenamides as novel inhibitors of human histone deacetylase with in vivo antitumor activity: discovery of (2E)-N-hydroxy-3-[4-[[(2-hydroxyethyl)[2-(1H-indol-3-yl)ethyl]amino]methyl]phenyl]-2-propenamide (NVP-LAQ824). *J Med Chem* 2003;**46**:4609–24.
19. Khan N, Jeffers M, Kumar S, et al. Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem J* 2008;**409**:581–9.
20. Qian X, Ara G, Mills E, LaRochelle WJ, Lichenstein HS, Jeffers M. Activity of the histone deacetylase inhibitor belinostat (PXD101) in preclinical models of prostate cancer. *Int J Cancer* 2008;**122**:1400–10.
21. Steele NL, Plumb JA, Vidal L, et al. A phase 1 pharmacokinetic and pharmacodynamic study of the histone deacetylase inhibitor belinostat in patients with advanced solid tumors. *Clin Cancer Res* 2008;**14**:804–10.
22. Byrd JC, Marcucci G, Parthun MR, et al. A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. *Blood* 2005;**105**:959–67.
23. Piekarz RL, Robey R, Sandor V, et al. Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: a case report. *Blood* 2001;**98**:2865–8.
24. Piekarz RL, Frye AR, Wright JJ, et al. Cardiac studies in patients treated with depsipeptide, FK228, in a phase II trial for T-cell lymphoma. *Clin Cancer Res* 2006;**12**:3762–73.
25. Lucas DM, Davis ME, Parthun MR, et al. The histone deacetylase inhibitor MS-275 induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells. *Leukemia* 2004;**18**:1207–14.
26. Qian DZ, Wei YF, Wang X, Kato Y, Cheng L, Pili R. Antitumor activity of the histone deacetylase inhibitor MS-275 in prostate cancer models. *Prostate* 2007;**67**:1182–93.
27. Hess-Stumpp H, Bracker TU, Henderson D, Politz O. MS-275, a potent orally available inhibitor of histone deacetylases – the development of an anticancer agent. *Int J Biochem Cell Biol* 2007;**39**:1388–405.
28. Moradei OM, Mallais TC, Frechette S, et al. Novel aminophenyl benzamide-type histone deacetylase inhibitors with enhanced potency and selectivity. *J Med Chem* 2007;**50**:5543–6.
29. Bali P, Pranpat M, Bradner J, et al. Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. *J Biol Chem* 2005;**280**:26729–34.
30. Carew JS, Giles FJ, Nawrocki ST. Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. *Cancer Lett* 2008;**269**:7–17.
31. George P, Bali P, Annavarapu S, et al. Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML

cells with activating mutation of FLT-3. *Blood* 2005;**105**:1768–76.
32. Heider U, von Metzler I, Kaiser M, et al. Synergistic interaction of the histone deacetylase inhibitor SAHA with the proteasome inhibitor bortezomib in mantle cell lymphoma. *Eur J Haematol* 2008;**80**:133–42.
33. Nawrocki ST, Carew JS, Maclean KH, et al. Myc regulates aggresome formation, the induction of Noxa, and apoptosis in response to the combination of bortezomib and SAHA. *Blood* 2008;**112**:2917–26.
34. Robertson KD, Wolffe AP. DNA methylation in health and disease. *Nat Rev Genet* 2000;**1**:11–9.
35. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol* 2002;**20**:2429–40.
36. Kantarjian HM, O'Brien S, Cortes J, et al. Results of decitabine (5-aza-2′-deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. *Cancer* 2003;**98**:522–8.
37. Yang AS, Doshi KD, Choi SW, et al. DNA methylation changes after 5-aza-2′-deoxycytidine therapy in patients with leukemia. *Cancer Res* 2006;**66**:5495–503.
38. Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. *Science* 2003;**300**:489–92.
39. Kantarjian HM, O'Brien S, Huang X, et al. Survival advantage with decitabine versus intensive chemotherapy in patients with higher risk myelodysplastic syndrome: comparison with historical experience. *Cancer* 2007;**109**:1133–7.
40. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol* 2009;**10**:223–32.
41. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999;**21**:103–7.
42. Soriano AO, Yang H, Faderl S, et al. Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome. *Blood* 2007;**110**:2302–8.
43. Garcia-Manero G, Assouline S, Cortes J, et al. Phase 1 study of the oral isotype specific histone deacetylase inhibitor MGCD0103 in leukemia. *Blood* 2008;**112**:981–9.
44. Jain N, Rossi A, Garcia-Manero G. Epigenetic therapy of leukemia: an update. *Int J Biochem Cell Biol* 2009;**41**:72–80.
45. Zhou L, Cheng X, Connolly BA, Dickman MJ, Hurd PJ, Hornby DP. Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. *J Mol Biol* 2002;**321**:591–9.
46. Holleran JL, Parise RA, Joseph E, et al. Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine. *Clin Cancer Res* 2005;**11**:3862–8.
47. Pina IC, Gautschi JT, Wang GY, et al. Psammaplins from the sponge *Pseudoceratina purpurea*: inhibition of both histone deacetylase and DNA methyltransferase. *J Org Chem* 2003;**68**:3866–73.
48. Brueckner B, Boy RG, Siedlecki P, et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res* 2005;**65**:6305–11.
49. Collins SJ. Retinoic acid receptors, hematopoiesis and leukemogenesis. *Curr Opin Hematol* 2008;**15**:346–51.
50. Minucci S, Pelicci PG. Retinoid receptors in health and disease: co-regulators and the chromatin connection. *Semin Cell Dev Biol* 1999;**10**:215–25.
51. Kamashev D, Vitoux D, De Thé H. PML-RARA-RXR oligomers mediate retinoid and rexinoid/cAMP cross-talk in acute promyelocytic leukemia cell differentiation. *J Exp Med* 2004;**199**:1163–74.
52. Minucci S, Nervi C, Lo Coco F, Pelicci PG. Histone deacetylases: a common molecular target for differentiation treatment of acute myeloid leukemias? *Oncogene* 2001;**20**:3110–5.
53. Carbone R, Botrugno OA, Ronzoni S, et al. Recruitment of the histone methyltransferase SUV39H1 and its role in the oncogenic properties of the leukemia-associated PML-retinoic acid receptor fusion protein. *Mol Cell Biol* 2006;**26**:1288–96.
54. Di Croce L, Raker VA, Corsaro M, et al. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* 2002;**295**:1079–82.
55. Morey L, Brenner C, Fazi F, et al. MBD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks. *Mol Cell Biol* 2008;**28**:5912–23.
56. Villa R, Pasini D, Gutierrez A, et al. Role of the polycomb repressive complex 2 in acute promyelocytic leukemia. *Cancer Cell* 2007;**11**:513–25.
57. Hoemme C, Peerzada A, Behre G, et al. Chromatin modifications induced by PML-RARalpha repress critical targets in leukemogenesis as analyzed by ChIP-Chip. *Blood* 2008;**111**:2887–95.
58. Dou Y, Hess JL. Mechanisms of transcriptional regulation by MLL and its disruption in acute leukemia. *Int J Hematol* 2008;**87**:10–8.
59. Milne TA, Briggs SD, Brock HW, et al. MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell* 2002;**10**:1107–17.
60. Nakamura T, Mori T, Tada S, et al. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol Cell* 2002;**10**:1119–28.
61. Ernst P, Mabon M, Davidson AJ, Zon LI, Korsmeyer SJ. An Mll-dependent Hox program drives hematopoietic progenitor expansion. *Curr Biol* 2004;**14**:2063–9.
62. Zeisig BB, Cheung N, Yeung J, So CW. Reconstructing the disease model and epigenetic networks for MLL-AF4 leukemia. *Cancer Cell* 2008;**14**:345–7.
63. Okada Y, Feng Q, Lin Y, et al. HDOT1L links histone methylation to leukemogenesis. *Cell* 2005;**121**:167–78.
64. Krivtsov AV, Feng Z, Lemieux ME, et al. H3K79 methylation profiles define murine and human MLL-AF4 leukemias. *Cancer Cell* 2008;**14**:355–68.
65. Guenther MG, Lawton LN, Rozovskaia T, et al. Aberrant chromatin at genes encoding stem cell regulators in human mixed-lineage leukemia. *Genes Dev* 2008;**22**:3403–8.
66. Cheung N, Chan LC, Thompson A, Cleary ML, So CW. Protein arginine-methyltransferase-dependent oncogenesis. *Nat Cell Biol* 2007;**9**:1208–15.
67. Fazi F, Zardo G, Gelmetti V, et al. Heterochromatic gene repression of the retinoic acid pathway in acute myeloid leukemia. *Blood* 2007;**109**:4432–40.
68. Lemercier C, Brocard MP, Puvion-Dutilleul F, Kao HY, Albagli O, Khochbin S. Class II histone deacetylases are directly recruited by BCL6 transcriptional repressor. *J Biol Chem* 2002;**277**:22045–52.
69. David G, Alland L, Hong SH, Wong CW, DePinho RA, Dejean A. Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. *Oncogene* 1998;**16**:2549–56.
70. Dhordain P, Albagli O, Lin RJ, et al. Corepressor SMRT binds the BTB/POZ repressing domain of the LAZ3/BCL6 oncoprotein. *Proc Natl Acad Sci USA* 1997;**94**:10762–7.
71. Yang XJ. The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. *Nucl Acids Res* 2004;**32**:959–76.

72. So CW, Cleary ML. Dimerization: a versatile switch for oncogenesis. *Blood* 2004;**104**:919–22.
73. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 1999;**286**:531–7.
74. Krivtsov AV, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. *Nat Rev Cancer* 2007;**7**:823–33.
75. Puccetti E, Ruthardt M. Acute promyelocytic leukemia: PML/RARalpha and the leukemic stem cell. *Leukemia* 2004;**18**:1169–75.
76. Bereshchenko OR, Gu W, Dalla-Favera R. Acetylation inactivates the transcriptional repressor BCL6. *Nat Genet* 2002;**32**:606–13.
77. Insinga A, Monestiroli S, Ronzoni S, et al. Impairment of p53 acetylation, stability and function by an oncogenic transcription factor. *EMBO J* 2004;**23**:1144–54.
78. Kim SC, Sprung R, Chen Y, et al. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol Cell* 2006;**23**:607–18.
79. Huang J, Sengupta R, Espejo AB, et al. P53 is regulated by the lysine demethylase LSD1. *Nature* 2007;**449**:105–8.
80. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 2007;**8**:286–98.
81. Gargiulo G, Minucci S. Epigenomic profiling of cancer cells. *Int J Biochem Cell Biol* 2008;**41**:127–35.
82. Gargiulo G, Levy S, Bucci G, et al. NA-Seq: a discovery tool for the analysis of chromatin structure and dynamics during differentiation. *Dev Cell* 2009;**16**:466–81.