Epigenetic Modifications as Therapeutic Targets

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Abstract

Epigenetic modifications work with genetic mechanisms to determine transcriptional activity and, while somatically heritable they are also reversible, making them good therapeutic candidates. Epigenetic changes can precede disease pathology and thus are diagnostic indicators for risk, and can act as prognostic indicators for disease progression. Histone deacetylase inhibitors and DNA methylation inhibitors have been FDA approved for several years and are clinically successful. More recently, histone methylation and microRNAs have also gained attention as potential therapeutic targets. The presence of multiple epigenetic aberrations within a diseased tissue and the abilities of cells to develop resistance suggest that combination therapies may be most beneficial. This review will focus on recent examples of using epigenetic modifications to evaluate disease risk, progression and clinical response and will describe the latest clinical advances in epigenetic therapies concentrating on treatments which combine epigenetic therapeutics with each other and with cytotoxic agents to increase clinical response.

Epigenetics is defined as heritable changes in gene expression that do not result from an alteration in the DNA sequence itself. DNA methylation, histone variants and modifications, and nucleosome positioning work together to determine the epigenetic landscape of a cell. DNA methylation occurs when a methyl group is added to the 5′ position of the cytosine ring of CpG dinucleotides. Histones can be covalently modified by the addition of a variety of modifications (methyl, acetyl, phospho, ubiqityl, or sumo groups) and whether the modification has a facilitory or inhibitory effect on transcription depends on which residue is modified and the type of modification. Nucleosomes consist of DNA wrapped around a core of 2 copies of each H2A, H2B, H3 and H4 histone proteins, thus integrating DNA methylation and histone modifications. Variants of core histone proteins, such as H3.3 and H2A.Z, also occur at specific genomic loci to alter the stability of nucleosome occupancy. The localization of nucleosomes within genomic regulatory regions plays an important role in creating permissible or refractory environments for transcription. These different aspects of epigenetic regulation work in concert to determine the epigenetic state of a cell and hence, its transcription profile.

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Epigenetic Disease Mechanisms

Epigenetic aberrations have been well established in cancer[1, 2] and occur in several other diseases including diabetes[3], lupus[4], asthma[5] and a variety of neurological disorders[2, 6, 7,8] (Table 1 and references within). In cancer, there is a global loss of DNA methylation (hypomethylation), particularly in gene bodies and intergenic regions, including repetitive elements, leading to genomic instability. This global hypomethylation is accompanied by increased *de novo* methylation (hypermethylation) of many promoters of tumor suppressor and other genes that are contained within CpG islands, resulting in permanent gene silencing (Figure 1). In addition to changes in DNA methylation, there is a global loss of H4K16 acetylation and H4K20 tri-methylation, as well as increased expression of BMI1, a component of the polycomb repressive complex 1 (PRC1), and EZH2, a component of PRC2, which act to inhibit gene expression[1, 9]. Interestingly, recent evidence has demonstrated that genes that are targets of the PRC in embryonic stem cells are more likely than others to become methylated in cancer, potentially linking different epigenetic silencing mechanisms[10-12].

Epigenetic modifications can be used to stratify disease[13] and predict clinical outcome[14, 15]. H3 acetylation and H3K9 di-methylation can discriminate between prostate cancer (PCA) and non-malignant prostate tissue and H3K4 tri-methylation can be a significant predictor of PSA recurrence[16]. EZH2 expression is an independent prognostic marker that correlates with the aggressiveness of prostate, breast and endometrial cancers[17]. Expression of the DNA repair gene O(6)-methylguanine-DNA methyltransferase (MGMT) antagonizes chemotherapy and radiation treatment[18] thus, silencing of MGMT by hypermethylation correlates with positive treatment response. Furthermore, epigenetic alterations can also precede tumor formation and thus are potential diagnostic indicators of disease risk[19]. For example, H. pylori bacterial infection is associated with DNA hypermethylation of specific genes, which are often methylated in cancer[20]. Thus, reversal of epigenetic alterations that occur as a result of an acute illness may prevent the progression to a more chronic disease state.

With the growing development of technologies to analyze the epigenome, a new field: pharmaco-epigenomics - is emerging, where epigenetic profiles can be used to identify molecular pathways for cancer drug sensitivity[21] and be used in determining the best therapeutic approach. In non-small-cell lung cancer, an unmethylated IGFBP3 promoter is indicative of responsiveness to cisplatin based chemotherapy[22]. A polymorphism in the CYP2C19*2 variant of cytochrome P450 requires higher valproic acid (VPA) doses to achieve target plasma concentration levels[23]. Furthermore, monitoring epigenetic changes can be used to measure treatment efficacy and disease progression. Methylation of PITX2 can be used to predict outcome of early breast cancer patients following adjuvant tamoxifen therapy[24]. Patients with *p16* hypermethylation had lower bladder cancer recurrence rates after IL-2 treatment compared to patients with no *p16* hypermethylation after IL-2 treatment[25]. Since epigenetic mechanisms determine which genes and hence signaling pathways can be activated, the presence of distinct modifications on specific genes and subsets of genes can aid at several steps in determining and monitoring optimal therapeutic approaches.

Epigenetic modifications are somatically heritable yet also reversible, making them good candidates for potential drug treatments. Multiple epigenetic abnormalities are typically present in diseased tissue leading to an altered epigenetic landscape. Cancer cells may become 'addicted' to the aberrantly developed epigenetic landscape and, consequently, be more sensitive than normal cells to epigenetic therapy, in a process similar to an inverted oncogene addiction. One example of oncogene addiction is MET, a tyrosine kinase that acts as a receptor for Hepatocyte Growth Factor (HGF) and functions in normal cells to control tissue homeostasis[26]. MET can be aberrantly activated in cancer, by ligand dependent mechanisms or by overexpression[26]. Interestingly, while MET plays roles in both normal and cancer cells, the latter are more sensitive to MET inhibition due to increase reliance on MET signaling[26]. Thus, cancer cells become dependent (and consequently addicted) to increased activity of a few highly important oncogenes. It is possible that cancer cells undergo a parallel process by which they become dependent (and consequently addicted) to aberrant silencing/inactivation of a few highly important tumor suppressor genes. Since it is well known that several tumor suppressor genes are silenced in cancer by epigenetic mechanisms[1] it is possible that cancer cells become addicted to their aberrant epigenetic landscape and, consequently, more sensitive to the epigenetic therapy than normal cells.

DNA Methylation

In cancer there is global hypomethylation accompanied by hypermethylation of a subset of gene promoters contained within CpG islands leading to gene silencing (Figure 1)[1]. This hypermethylation has recently been described to also extend past the boundaries of CpG islands into "DNA shores" [27]. DNA methyltransferases 3A/B are responsible for *de novo* DNA methylation patterns, which are then copied to daughter cells during S-phase by DNMT1. DNA methylation inhibitors have been well characterized and tested in clinical trials[28]. 5- Azacytidine (5-Aza-CR; Vidaza) is a nucleoside analog that incorporates into RNA and DNA and is FDA approved to treat high risk myelodysplastic syndromes (MDS) patients and successful clinical results have recently been reported (Table 2)[29]. 5-Aza-2-deoxycytidine (5-Aza-CdR; Decitabine) is the deoxy derivative of 5-Aza-CR and is incorporated only into DNA. At low doses both azanucleosides act by sequestering DNMT enzymes after incorporation into DNA thus leading to global demethylation as cells divide, while at higher doses they induce cytotoxicity. Potential new drugs that can be used in the clinic include zebularine, a cytidine analog that acts similarly to 5-Aza-CR, but has lower toxicity, increased stability and specificity[30] and S110, a decitabine derivative that has increased stability and activity, which has shown promise in pre-clinical studies (Figure 2)[31]. In addition to inhibiting DNA methyltransferase activity, azanucleosides also act through unspecific mechanisms, which likely contribute to their clinical effectiveness. Promoter DNA methylation can be used to molecularly classify[21], [32],[33] predict progression of cancer[34], [35] and direct therapeutic approach[36],[37]. For example, DNA methylation of specific promoters may identify a sub-population of colorectal cancer that is responsive to 5-fluorouracil[36]. Furthermore, reversing MLH1 silencing by DNA methylation inhibitors restores sensitivity to cisplatin[38] suggesting that inclusion of DNA methylation inhibitors may increase the effectiveness of conventional chemotherapy. Successful conventional chemotherapy depends on activation of pro-apoptotic genes that respond to cytotoxic agents

leading to cell death. DNA methylation of these pro-apoptotic genes can block cell death from occurring leading to chemotherapeutic resistance, thus reactivation of epigenetically silenced apoptotic genes can increase efficacy of chemotherapy. For example, APAF1 is silenced in metastatic melanoma cells and treatment with 5-Aza-CdR restores expression and chemosensivity[37]. Conversely, methylation induced silencing of DNA repair genes can be detrimental by leading to microsatellite instability[39] or beneficial by preventing the repair of genes targeted by chemotherapy causing cells to undergo apoptosis rather than repair[40]. Methylation induced silencing of cancer-testis antigens, such as NY-ESO-1, can protect cancer cells from being recognized by T cells. Treating cancer cells with demethylating agents can induce the expression of these antigens, allowing a reaction by engineered lymphocytes[41] suggesting that epigenetic therapy can be combined with immunotherapy for better results (Figure 1).

Despite the clinical successes achieved with DNA methylation inhibitors there is still room for improvement. Currently available DNA methylation inhibitors block DNA methylation at the enzymatic level leading to global DNA methylation inhibition. This is therapeutically beneficial as tumor suppressor genes are hypermethylated in cancer, however global hypomethylation may lead to activation of oncogenes and/or increased genomic instability. DNA hypomethylation can activate promoters within repetitive elements, for example LINE-1 hypomethylation can activate an alternative transcript of the MET oncogene in bladder cancer[42], suggesting a risk of global DNA demethylation agents. Developing DNA methylation inhibitors that targeted specific genes or groups of genes would overcome this limitation. Furthermore, DNA methylation inhibitors act during S-phase of the cell cycle, thus they preferentially affect rapidly growing cells. This is advantageous when treating rapidly dividing cancer cells but may be less clinically useful in treating diseases that are not characterized by rapid cell cycling. Furthermore, upon azanucleoside withdrawal the DNA methylation levels return to pre-treatment levels[6] demonstrating a continual need for DNA methyltransferase inhibition. Thus, while DNA methylation inhibitors are clinically successful, their lack of specificity, cell cycle dependency and need for continual administration leave room for the development of better therapies.

Histone Modifications

While DNA methylation is considered to be a very stable epigenetic modification, histone modifications are comparably more labile with their levels being maintained by a balance between histone modifying enzymes, which add or remove specific modifications. Aberrant histone modification levels result from an imbalance in these modifying enzymes in diseased tissue, thus correcting the increased or decreased level of a particular enzyme will restore the natural balance in the affected cells. In cancer, both histone methyltransferase and histone demethylase enzymes are misregulated and there is a high expression level of histone deacetylases (HDAC) and a global reduction in histone acetylation levels[1, 9, 43, 44, 45]. HDAC inhibitors have long been studied in the clinical setting as potential therapies (Figure 2) and recent clinical trials have been extensively reviewed elsewhere (Table 2)[46]. Histone deacetylase inhibitors (HDACi) can affect acetylation of non-histone proteins, as well as histones, potentially leading to more global effects[46]. Furthermore, non-isoform selective HDACi only target approximately 10% of all acetylation sites [47], thus more work

is necessary to understand the underlying basis for target specification of global and isoform specific HDACi. Currently, significant effort is underway to find new molecules that are able to selectively inhibit specific HDACs[48, 49] and thus avoid the side effects that occur with a global HDACi[50]. To date, specific inhibitors of HDAC6 (class II) and HDAC8 (class I) have been developed[48, 49]. The development of specific HDACi combined with a better understanding of the pathophysiology of diseases associated with alterations in HDACs will allow more rational therapy and, potentially, reduce side effects. For example, the HDAC inhibitor PCI-34051, which is derived from a low molecular weight hydroxamic acid scaffold, was recently shown to selectively inhibit HDAC8 and induce apoptosis specifically in T-cell lymphomas and not other tumor or normal cells, showing HDC8 plays an important role in the pathophysiology of this disease and suggesting the therapy with HDAC8 specific inhibitor may lead to less side effects[49]. While the identification of additional specific HDAC inhibitors will increase specificity and the possibility of personalized treatments, it may also potentially limit the likelihood of success in combinatorial therapy.

Histone methyltransferase and demethylase enzymes are relatively more specific than HDACs in that they target a limited number of residues[51] however, like HDACs, lysine and arginine methyltransferase enzymes methylate non-histone proteins as well as histone proteins[52, 53]. A great deal of effort is underway to find drugs able to revert specific histone methylation marks or to target histone methyltransferase or histone demethylases. In this regard, a new class of oligoamine analogs was recently found that act as potent inhibitors of Lysine specific demethylase 1 (LSD1) (Figure 2). LSD1 targets the activating H3K4 mono and di-methylation mark but can also target the repressive H3K9me2 mark when complexed with the androgen receptor[43, 54]. Treatment of colon cancer cells with LSD1 inhibitors (e.g. SL11144) resulted in increased H3K4 methylation, decreased H3K9me2, and re-expression of the SFRP2 gene[55], demonstrating context specificity of LSD1 and its inhibitors. LSD1 inhibition in neuroblastoma, resulted in decreased proliferation *in vitro* and reduced xenograft growth[56]. Interestingly, LSD1 can also demethylate DNMT1 resulting in destabilization and loss of global DNA methylation maintenance[57]. The ability of LSD1 to affect both histone and DNA methylation, make it a promising target for epigenetic therapy.

The repression mediated by the H3K27 tri-methylation mark, occurs through the actions of two multi-subunit complexes, PRC1 and PRC2, H3K27me3 is deposited by EZH2 and then recognized and bound by PRC1, which can further recruit additional proteins to establish a repressed chromatin configuration[1]. Gene promoters which are marked by PRC2 (i.e. polycomb target genes) in embryonic stem cells have recently been shown to be far more likely than other genes to become methylated in cancer[10-12]. Similarly, polycomb targets in normal prostate cells also become methylated in prostate cancer[58]. Thus alterations in chromatin structure do not always coincide with changes in gene expression, rather DNA methylation replacement of polycomb repressive marks acts to "lock in" an inactive chromatin state through a process called "epigenetic switching"[58]. The mechanism underlying the predisposition of polycomb targets for DNA methylation is not fully understood, however some links have recently been uncovered. CBX7, a component of the PRC1 complex, can directly interact with DNMT1 and 3B at polycomb target genes[59].

While there is significant promise for drugs that target histone methylation enzymes, more work is necessary to determine their specificities and stability and there are currently no such drugs undergoing clinical trials. To date, the S-adenosylhomocysteine hydrolase inhibitor, 3-deazaneplanocin A (DZNep) has the most promising pre-clinical results of drugs, which target histone methylating enzymes (Figure 2). DZNep depletes cellular levels of PRC2 components (EZH2, EED and SUZ12) and consequently reduces H3K27me3 levels and induces apoptosis in breast cancer but not normal cells[60]. The effect of DZNep is similar to those observed when EZH2 is depleted by RNAi, suggesting that this drug may be more effective in cancers like prostate and breast, which rely on abnormally high EZH2 expression levels[61]. On the other hand, a subsequent study showed that DZNep also decreased H4K20me3 demonstrating that DZNep lacks specificity and acts more as a global histone methylation inhibitor, suggesting the need for further development of histone methylation inhibitors[62].

EZH2 activity can also be regulated by signaling cascades. For example, AKT phosphorylates EZH2 at serine 21, suppressing its methyltransferase activity and, consequently, reducing H3K27me3[63]. H3K27me3 levels can be restored using the PI3K/AKT inhibitor LY294002, opening a new therapeutic opportunity to repair epigenetic alterations by targeting upstream signaling pathways. Furthermore, in prostate cancer, the oncogenic ETS transcription factor ERG can bind to the *EZH2* promoter and induce over-expression. Thus, the pharmacological disruption of ERG activity could reduce EZH2 over-expression observed in cancer[64]. EZH2 is a particularly important example because it is frequently over-expressed and aberrantly targeted to genes in cancer[58], a process termed PRC reprogramming (Figure 1).

G9a and GLP (G9a like protein) are histone methyl-transferases that catalyze H3K9 di-methylation and are often over-expressed in tumors[65]. Knockdown of G9a in prostate cancer cells demonstrates a critical role for this protein in regulating centrosome duplication and chromatin structure, suggesting that G9a is important to perpetuate the malignant phenotype and, thus, a putative target in cancer therapy[66]. As a result there is significant interest in developing G9a/GLP inhibitors and thus far the most efficient inhibitor is BIX-01294 (adiazepin-quinazolin-amine derivative), which is able to transiently reduce bulk H3K9me2 levels in several cell lines[67]. BIX-01294 binds to the SET domain of GLP in the same groove where the target lysine (H3K9) binds, preventing the binding of the peptide substrate and, consequently, the deposition of methylation marks at H3K9[68].

Several other histone methyltransferases and demethylases have also been associated with diseases and, thus, could be potential targets for epigenetic therapy. For instance, MMSET, a H4K20 methyltransferase, is overexpressed in myeloma cell lines and required for cell viability[69]. SMYD3, an H3K4 methyltransferase, is also highly expressed in cancer and seems to play a role in carcinogenesis as a coactivator of ERalpha[70]. GASC1, an H3K9 and H3K36 demethylase, is often amplified in cancer and its inhibition results in decreases cell proliferation[71].

Despite a lack of specificity, targeting histone modifications has been clinically successful. Hopefully the development of therapeutics that target specific histone modifying enzymes

will retain or increase their therapeutic success while decreasing side effects due to the lack of specificity. On the other hand targeting individual histone modifying enzymes may decrease clinical efficacy due to compensation by other histone modifying enzymes potentially leading to resistance. Designing personalized cocktails of inhibitors based on an individual's need may help overcome the potential problems of compensation and resistance.

microRNAs

miRNAs are able to induce heritable changes in gene expression without altering DNA sequence, and thus contribute to the epigenetic landscape. In addition miRNAs can both regulate, and be regulated by, additional epigenetic mechanisms. Expression of miRNAs is misregulated in several diseases including cancer[72] and neurodegeneration[73]. For example, miR-101 targets EZH2 for degradation and is down-regulated in several types of cancer, resulting in increased EZH2 expression and consequently H3K27me3 levels and decreased tumor suppressor gene expression[61, 74]. Re-expression of miR-101 leads to reduced H3K27me3 and inhibits colony formation and cancer cell proliferation[61, 74]. Expression of miR-143 in colorectal cancer cells[75] and the miR-29 family in lung cancer cells[76] reduced DNMT3A and DNMT3A and B levels, respectively, and resulted in decreased cell growth and colony formation. Treatment of cells with 5-Aza-CdR and 4-phenylbutyric acid resulted in miR-127 activation, which in turn downregulated the BCL6 oncogene in bladder cancer cells[77]. In fact, treatment with 5-Aza-CdR alone is sufficient to reactivate miR148a, miR34b/c and miR-9, a group of miRNAs with metastasis suppression activity[78]. In addition to inducing aberrantly repressed miRNAs using epigenetic drugs, replacement gene therapy may also be useful in re-establishing miRNA expression. Viral vectors generated by cloning individual or groups of human miRNAs has been successful in pre-clinical assays using a mouse model of hepatocellular carcinoma in which miR-26a expression by an adeno-associated virus (AAV) resulted in apoptosis and inhibition of cancer cell proliferation in the absence of toxicity[79]. microRNA gene therapy has an advantage, over the conventional RNAi, in that it is unlikely to generate a strong type I interferon response because double-strand RNA is not introduced to the cell and furthermore, the AAV vector is less immunogenic than previous generations of viral vectors used for gene transfer[80].

Abnormally high expression of miRNAs can be targeted using recently developed locked-nucleic-acid (LNA)-modified phosphorothioate oligonucleotide technology. Locked-nucleic-acid-modified oligonucleotides contain an extra bridge in their chemical composition leading to enhanced stability compared to their unmodified counterparts. miRNAs can be generated using these LNA modified phosphorothioate oligonucleotides to create LNA-antimiRs, which can be delivered systemically. In pre-clinical assays with primates, intravenous injections of LNA-antimiR complementary to the 5′ end of miR-122 antagonized liver specific expression of this miRNA without toxicity[81]. Based on these promising results, phase 1 trials are currently underway. LNA-antimiRs may be used to target aberrantly expressed miRNAs in other diseases, such as cancer. For example, miR-155 is up-regulated in lung adenocarcinoma compared to non-cancerous lung tissue and patients with higher miR-155 expression had lower survival rates than patients with lower miR-155 expression, suggesting that miR-155 is a promising target for LNA-antimiR therapy (Table1)[82]. Several other miRNAs are up-regulated in cancer and could,

theoretically, be used as LNA-antimiR targets. For example, miR-21, is up-regulated in several types of cancer (lung, breast, colon, gastric and prostate carcinomas, endocrine pancreatic tumours, glioblastomas and cholangiocarcinomas) and targets the tumor suppressor PTEN (Table 1)[83]. Thus, miRNAs can alter the epigenetic machinery and also be regulated by epigenetic alterations, creating a highly controlled feedback mechanism, making it a suitable target for epigenetic therapy and possibly an epigenetic drug itself.

One unique advantage of targeting miRNAs is the ability of one miRNA to regulate several target genes and multiple cellular process. In that way, if the level of one or a few miRNAs has changed in a pathological state, several different pathways could be consequently altered. Rather than trying to identify and directly target the proteins in multiple pathways it would be more effective to restore the physiological level and functions of the misregulated miRNA(s). This clinical potential highlights the importance of better understanding miRNA profiles in healthy and diseased tissues in order to develop better therapeutic strategies. Furthermore, multiple miRNAs that target different steps of an over-active pathway can be combined to increase efficacy and allow for customization of therapies to individual patients. While the unique composition of miRNA based therapy provides many benefits, additional research is necessary to determine the best method of delivery and increase miRNA stability to ensure efficacy.

Combined Epigenetic Therapies

The presence of multiple epigenetic aberrations in a single tissue, the ability to develop resistance and the discovery that common sets of genes are regulated by distinct epigenetic mechanisms at different biological stages demonstrates the need and highlights the potential success of combinatorial therapy. Combining epigenetic therapies to increase efficacy has been done for over 25 years[84, 85] and has demonstrated both additive and synergistic effects depending on the targets[6, 44]. Extensive work has evaluated the clinical benefits of combined DNMT and HDAC inhibition and comprehensive reviews have been published elsewhere[1, 39, 46, 86, 87]. A recent phase II multicenter study examining the combination of 5-Aza-CR and the HDAC inhibitor VPA in patients with higher risk MDS found that therapeutic levels of VPA may increase 5-Aza-CR efficacy[23]. Sequential administration of DNMT and HDAC inhibitors demonstrated clinical efficacy in patients with hematologic malignancies[46, 86]. However, other studies found no correlation between baseline methylation levels or methylation reversal and positive clinical outcome in MDS and AML patients following 5-Aza-CR and entinostat treatment[88]. The mechanism behind the clinical efficacy of combined DNMT and HDAC remains controversial and additional studies investigating potential genetic or epigenetic determinants of responsiveness will be helpful. In addition to inducing apoptosis in cancer cells, another therapeutic approach is to induce differentiation of cancer cells. To this end, 5-Aza-CR and VPA treatment followed by all trans-retinoic acid in patients with acute myeloid leukemia or high-risk myelodysplastic syndrome resulted in global hypomethylation and histone acetylation and clinical response in nearly half of treated patients[89].

While targeting histone de-methylation enzymes is still in its infancy, early pre-clinical studies alone (described above) and combined with other epigenetic therapies are promising.

SFRP2 (a negative regulator of Wnt signaling) expression was re-established *in vitro* and *in vivo* in a human colon cancer model following LSD1 and DNMT inhibition resulting significant growth inhibition of established tumors[55]. Interestingly, in addition to histone residues, LSD1 can also demethylate DNMT1 providing the ability to target both histone methylation and DNA methylation using a single compound. Co-treatment with the HDAC inhibitor, Panobinostat, further enhanced DZNep's reduction of EZH2 levels leading to increased p16, p21, p27 and FBX032 expression and apoptosis in cultured AML cells and mouse models[90]. Thus, while clinical trials targeting histone methylation and demethylation enzymes are not currently underway it is an area of active investigation with significant promise.

Epigenetic and Cytotoxic Therapies

Conventional chemotherapy can rapidly induce cell death in cancer cells. However, resistance to standard chemotherapy can be established through epigenetic and DNA repair mechanisms[22]. As a result, epigenetic therapeutics can be combined with more conventional therapies to induce responsiveness or overcome resistance to cytotoxic treatments. Preconditioning with epigenetic drugs could reverse the epigenetic alteration(s) that conferred resistance restoring chemotherapeutic sensitivity. For example, 5-Aza-CR treatment can reverse DNA methylation, thereby overcoming the gene silencing, which led to chemotherapeutic resistance[91]. On the other hand, methylation induced silencing of DNA repair genes (i.e. MGMT) correlates with a positive clinical response to chemotherapy. Thus, whether the combination of DNA methylation inhibitors and chemotherapy will be successful may depend on the epigenetic profile of an individual tumor. Clinical findings in patients with previously untreated non-small cell lung cancer treated with the HDAC inhibitor, vorinostat, in combination with carboplatin and paclitaxil were sufficiently promising to warrant a phase 2 study which also showed promising results (Table 2)[92, 93].

Conclusions/Perspective

Epigenetic therapy has been established as a successful treatment approach for a variety of malignancies. Inhibition of DNMTs and HDACs are two FDA approved cancer treatments. While the mechanism(s) behind the therapeutic benefit of DNMT and HDAC inhibition are not fully understood, ongoing and future studies that combine genomic sequencing and expression data may provide the keys to understanding the mechanism(s) underlying responsiveness. Furthermore, histones can be phosphorylated, ubiquitylated and sumoylated in addition to methylated and acetylated, however these modifications have been less well studied in the context of disease and may offer additional therapeutic targets.

One important challenge in epigenetic therapy is defining which genes are the drivers (i.e. group of genes necessary to be epigenetically silenced for disease to occur) and the passengers (i.e. group of genes that are epigenetically silenced due to aberrant activity of the epigenetic machinery, but are not necessary for disease to occur). Recent advances in high throughout technologies like genome-wide sequencing, combined with RNA profiling, chromatin immunoprecipitation (ChIP) or bisulfite conversion, have led to the generation of large amounts of data that can be integrated to form a comprehensive understanding of the

epigenetic alterations that are common and specific to various disease states. Assimilating these large datasets will likely assist in identifying epigenetic alterations that are causational and those that are merely correlative. Thus, in the future it may be possible for patients to be screened, using high throughout technologies, and classified by epigenetic alterations of the driver genes allowing the use of personalized targeted therapies.

Currently, epigenetic therapies are successfully used in the clinic to treat hematological malignancies, however little success has been achieved in treating solid cancers (Table 2)[94]-[97]. Initial clinical trials used treatment regimens later found to be less than optimal and resulted in few cases of positive clinical response. Administering more recently developed dosing and treatment schedules in addition to subtyping tumor types based on molecular signatures may increase the efficacy of epigenetic therapy for solid tumors. Furthermore, solid tumors are heterogeneous cell populations, often consisting of cells at a variety of stages of differentiation, thus determining which of these cells harbor epigenetic alterations and ensuring that therapeutic agents maintain stability and are able to penetrate the cellular mass and reach affected cells and will increase the likelihood of clinical success.

The recognition of epigenetics as a significant contributor to normal development and disease opens an expanding new avenue for drug discovery and therapeutics. Epigenetic therapies can be combined with conventional therapies to develop personalized treatments, used to convert unresponsive tumors and may allow for lower dosing which can limit side effects of treatment improving quality of life and treatment compliance.

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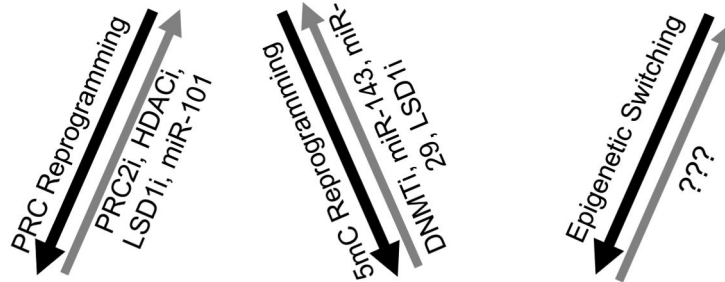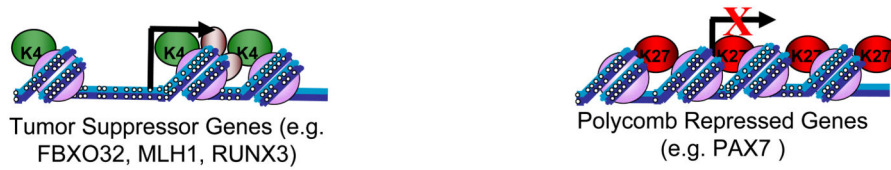
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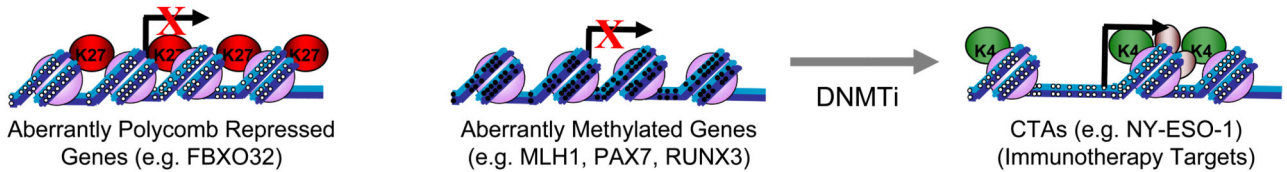
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Normal

Tumor Suppressor Genes (e.g. FBXO32, MLH1, RUNX3)

Polycomb Repressed Genes (e.g. PAX7)

PRC Reprogramming

PRC2i, HDACi, LSD1i, miR-101

5mC Reprogramming

miR-143, miR-29, LSD1i

DNMTi,

Epigenetic Switching

???

Cancer

Aberrantly Polycomb Repressed Genes (e.g. FBXO32)

Aberrantly Methylated Genes (e.g. MLH1, PAX7, RUNX3)

DNMTi

CTAs (e.g. NY-ESO-1) (Immunotherapy Targets)

⬤ : **Acetylation**   ⬤⬤ : **Tri-methylation**   ●: **Methylated CpG**   ○: **Unmethylated CpG**

Figure 1. Epigenetic Aberrations of CpG Island Promoters in Cancer and the Epigenetic Therapies That Target Them

Tumor suppressor genes (e.g. FBXO32, MLH1 & RUNX3) are expressed in normal cells and become silenced in cancer cells. This can occur by PRC reprogramming (e.g. FBXO32), where the polycomb group protein EZH2 catalyses the methylation of H3K27 or by 5mC reprogramming (e.g. MLH1, RUNX3) due to de-novo DNA methylation by DNMT3A and DNMT3B. Polycomb mediated repression can be targeted by inhibitors of PRC2, like DZNep and re-expression of these genes can be enhanced by HDAC and LSD1 inhibitors allowing acetylation of H3/4 and methylation of H3K4, respectively. Polycomb mediated repression can also be reversed by inducing miR-101 expression, which inhibits the expression and function of EZH2. 5mC reprogramming can be reversed, mainly by DNMT inhibitors, but also by re-expression of miR-143 and miR-29, two miRNAs that target de-novo DNMTs. LSD1 inhibitors may also reactivate tumor suppressor genes by inhibiting DNMT1 stabilization leading to loss of DNA methylation maintenance. Genes, which are polycomb repressed in normal cells (e.g. PAX7), can undergo epigenetic switching by gaining DNA methylation, thus losing their plasticity during transformation. Currently it is not known whether the treatment of cancer cells with DNMTi alone will reverse epigenetic switching to restore the polycomb repressed state or whether it will re-activate this set of genes. Cancer-Testis Antigens (CTAs, e.g. NY-ESO-1) can become silenced by DNA methylation in cancer. Treatment with DNMT inhibitors can induce CTA expression, allowing the immune system to recognize and kill the cancer cells. Black arrows represent
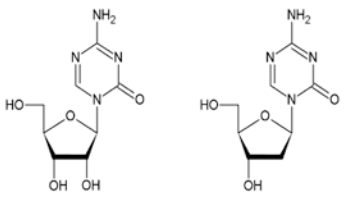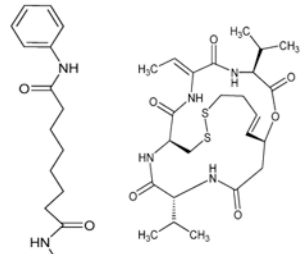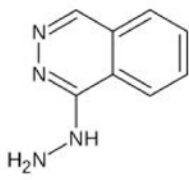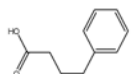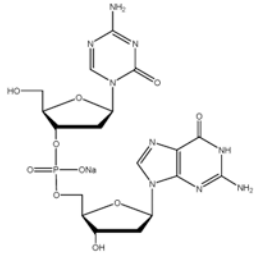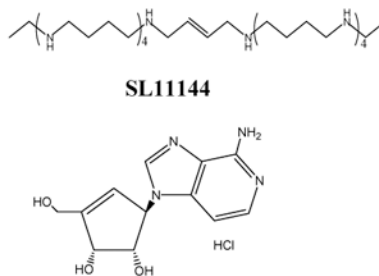
epigenetic alterations during transformation and gray arrows represent the reversion of this alteration by epigenetic therapy.

Figure 2. Chemical structure of selected compounds that target epigenetic modifications
At the present time, several molecules, which target epigenetic alterations in pathological states are at different stages of drug development. The nucleoside analogs 5-Azacytidine and 5-Aza-2′-deoxycytidine are FDA approved to treat high-risk myelodysplastic syndromes (MDS) patients and successful clinical results have been reported. The drug Hydralazine is currently being investigated in clinical trials as a putative demethylating agent against solid tumors and S110, a dinucleotide containing 5-aza-CdR, has been shown, *in vitro*, to demethylate DNA with a decreased deamination by cytidine deaminase. Targeting histone acetylation has also been a successful example of epigenetic therapy. Several histone deacetylase inhibitors are FDA approved such as the hydroxamic acid-based compound SAHA and the depsipeptide, Romidepsin, while others are currently in clinical trials for cancer (Phenylbutyrate and Entinostat) and neurologic diseases (Entinostat) and new molecules targeting specific HDACs are under pre-clinical investigations (e.g. PCI-34051, which targets HDAC8). More recently, significant effort is underway to find new molecules able to target histone methylation. To our knowledge there are no drugs targeting histone methylation which are FDA approved or in clinical trials. However, pre-clinical trials suggest anti-tumor activity of the oligoamine analogue SL-11144, a LSD1 inhibitor and the

S-adenosylhomocysteine hydrolase inhibitor 3-Deazaneplanocin A (DZNep), a drug that depletes cellular levels of PRC2 components.

Table 1

Selected Examples of Known Epigenetic Alterations Associated with Diseases

Epigenetic Aberration	Enzyme Responsible	Disease	Epigenetic Alteration	Comments	Ref
DNA Methylation	DNMT1, DNMT3A, DNMT3B and DNMT3L	Rett Syndrome	Inability to "read" DNA methylation	Mutation in MeCP2	6, 7, 8
		Diabetes	Hypermethylation of *PPARGC1A* gene promoter		3
		Cancer	Global Hypomethylation, hypermethylation of some CpG island promoters, including CIMP		1, 2, 6
		Systemic Lupus Erythematosus	Hypomethylation of CpG islands at specific promoter regions	Decreased DNMT1 and DNMT3B expression	4
		ICF Syndrome	Hypomethylation at specific sites	DNMT3B Mutation	6, 7, 8
		ATR-X Syndrome	Hypomethylation of specific repeat and satellite sequences	ATRX Mutation	6, 7
Histone Acetylation	Histone Acetyltransferases (HATs) and HDACs	Rubinstein-Taybi Syndrome	Hypoacetylation	Mutation in CBP, a known HAT	6, 7, 8
		Diabetes	Hyperacetylation at inflammatory genes promoters		3
		Asthma	Hyperacetylation	Increased HAT activity and decreased HDAC activity	5
		Cancer	H4K16 acetylation loss	Hypomethylation of DNA repetitive sequences	1
		Cancer	H4K20 tri-methylation loss	Hypomethylation of DNA repetitive sequences	1
Histone Methylation	Histone Methyltransferases (HMTs) and Histone Demethylates (HDMs)	Sotos Syndrome	Decreased H4K20 and H3K36 tri-methylation	Loss of function of NSD1, a histone methyltransferase	98
		Huntington's Disease (HD)	Increased H3K9 tri-methylation and possibly increased H3K27 tri-methylation	Increased HMT (ESET) expression and enhanced PRC2 activity	7
miRNA Expression	N/A	Cancer	Decreased miR-101	Increased EZH2, H3K27 trimethylation	61, 74
			Decreased miR-143	Increased DNMT3A	75
			Decreased miR-29	Increased DNMT3A and 3B	76
			Increased miR-21	Decreased PTEN	83
			Increased miR-155	Lower survival rates	82

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Table 2

Selected Clinical Trials of Epigenetic Therapy in Cancer

Epigenetic Target	Agent	Phase of Study	Disease	Findings	n	Reference
DNMT inhibitor alone						
DNMTs	5-Aza-CR	II/III	MDS and AML	Complete remission in 10-17% and hematological improvement in 23-36%	309	99
	5-Aza-CR	III	MDS	Better overall survival (24.5 vs. 15 months) than conventional care	358	29
	5-Aza-CdR	II	MDS and CMML	Anti-MDS and anti-CMML activities with a safe toxicity profile. 34% of patients achieved complete response and 73% had an objective response	95	100
HDACi Alone						
HDAC	Phenylbutyrate	I	MDS and AML	Well tolerated. No patients achieved complete or partial remission, although 4 achieved hematological improvement.	27	101
	Vorinostat (SAHA)	I	Relapsed or refractory AML, CLL, MDS, ALL, CML	Seven out 31 AML patients showed hematological improvement, including 2 complete response and 2 complete response with incomplete blood count recovery	41	102
	Vorinostat (SAHA)	I	Advanced solid and hematologic malignancies	One complete response (diffuse large B-cell lymphoma), three partial responses (cutaneous T-cell lymphoma)	73	103
Combination therapy						
DNMTs and HDAC	5-Aza-CR and VPA	I	Advanced solid cancers	The combination is safe; 25% of the patients showed stable disease (median, 6 months)	55	95
	5-Aza-CR and Phenylbutyrate	I	Refractory solid tumors	The combination is safe. No clinical benefit	27	97
HDAC	Vorinostat (SAHA) and Doxorubicin	I	Solid tumors	Two out of 24 partial responses (breast and prostate cancer) and 2 stable disease for more than 8 months (melanoma)	32	104
	Vorinostat (SAHA) plus Carboplatin and Paclitaxel	II	Advanced non-small-cell lung cancer	Better response ratio (34% vs. 12.5%), progression-free survival (6 months vs. 4.1) and overall survival (13 months vs. 9.7) than placebo plus Carboplatin and Paclitaxel	94	93