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NEW ANTI-CANCER STRATEGIES: EPIGENETIC THERAPIES AND BIOMARKERS

Curtis Balch [1], John S. Montgomery [1], Hyun-Il Paik [1], Sun Kim[2,3], Tim H-M. Huang [4], and Kenneth P. Nephew [1,5]

[1]*Medical Sciences, Indiana University, Bloomington, IN, 47405,*[2]*School of Informatics, Indiana University, Bloomington, IN, 47408,* [3]*Center for Genomics and Bioinformatics, Indiana University, Bloomington, IN, 47405,* [4]*Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, OH, 43210,* [5]*Indiana University Cancer Center, Indianapolis, IN, 46202*

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1. ABSTRACT

Epigenetics is the study of chromatin modifications that affect gene expression without altering DNA nucleotide sequences. This review highlights a prominent role for epigenetic therapies, particularly those that reverse aberrant DNA methylation and histone acetylation, in the potential treatment of cancer. Administration of such therapies to reverse epigenetic "silencing" of tumor suppressors, including genes involved in chemotherapy responses, could prove useful in the management of cancer patients. In this review, we summarize recent advances in the use of methyltransferase and histone deacetylase inhibitors and possible synergistic combinations of these to achieve maximal tumor suppressor gene re-expression. Moreover, when used in combination with conventional chemotherapeutic agents, epigenetic-based therapies may provide a means to resensitize drug-resistant tumors to established treatments. As specific, aberrant epigenetic modifications are frequently associated with distinct cancer types, and likely occur early in tumorigenesis, these have potential utility as biomarkers. Finally, future directions are addressed, including alternative epigenetic targets, gene-specific modifications, and the use of bioinformatics.

2. INTRODUCTION

Cancer is a group of over 200 distinct, often fatal, diseases affecting over 60 human organs**.** Despite this diversity, it is now accepted that all cancer cells possess six characteristic hallmarks: 1) unlimited potential for cell division; 2) invasion and metastasis; 3) resistance to anti-growth signaling; 4) self-sufficient growth; 5) evasion of apoptosis; and 6) sustained angiogenesis (1) (Figure 1). Emergence of these six traits is believed to result, probably through a multistage (2, 3) process, from altered expression of genes that modulate growth/apoptosis (4), angiogenesis (5), or metastatic (6) pathways.

Most current chemotherapeutics target the rapid, uncontrolled growth characteristics of tumor cells. To exploit this trait, DNA damaging drugs take advantage of increased cancer cell DNA replication; these include alkylating agents (carboplatin, melphalan), and DNA repair inhibitors (nitrosoureas). Microtubule modifiers target the mitotic spindle in M-phase cells and include paclitaxel, vinblastine, and vincristine (7). Antimetabolites, such as gemcitabine and 5-fluorouracil, interfere with DNA or RNA replication (8), while steroids, such as dexamethasone, preferentially inhibit the growth of

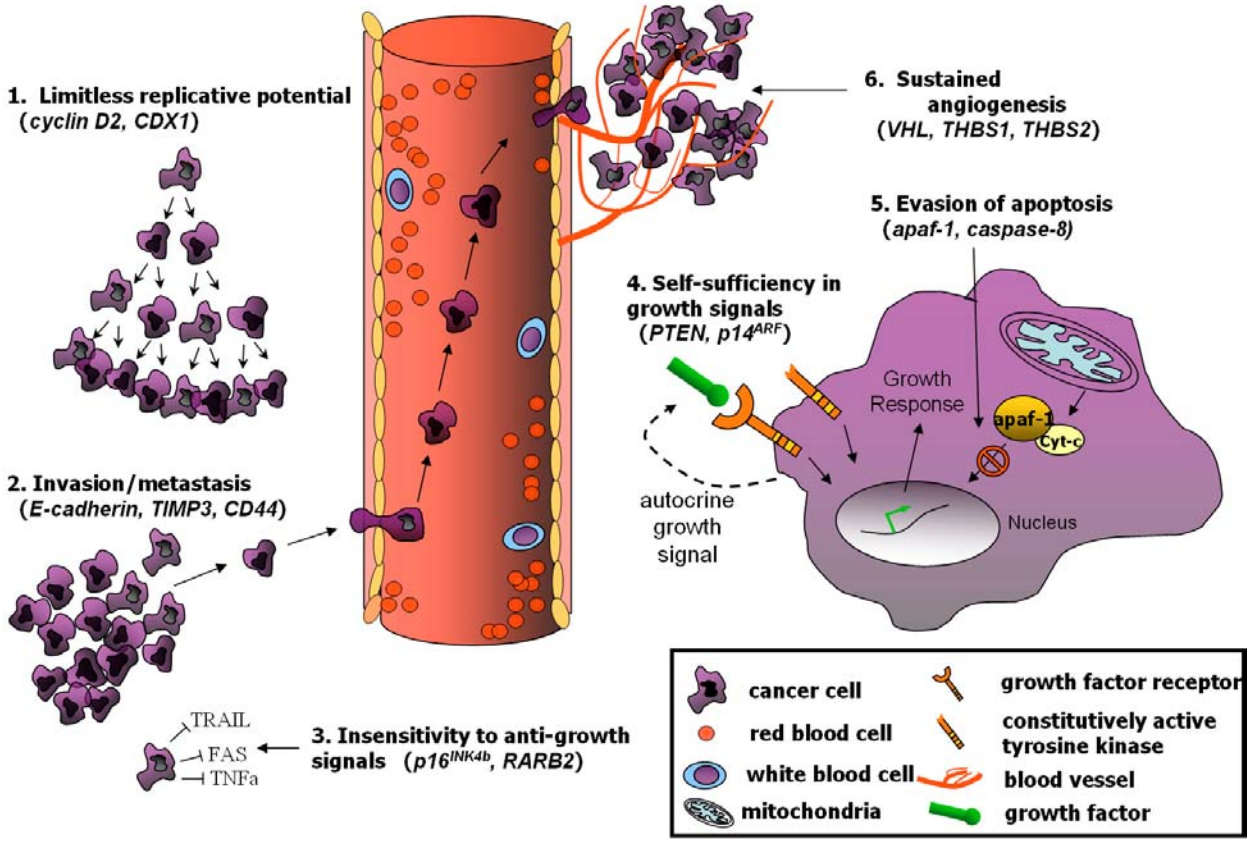

Figure 1. Six hallmarks of cancer. Epigenetic silencing of tumor suppressor genes (examples in parentheses) plays a significant role in the development of each of these six tumor traits.

hematopoietic cells in hematological malignancies (9). Hormonal therapies, including the anti-estrogens (tamoxifen, fulvestrant) (10) and anti-androgens (bicalutamide, flutamide) (11), slow the growth of hormone-responsive cancers of the breast (10) and prostate (11), respectively.

While older chemotherapeutics target the increased cell division trait of tumor cells, newer therapies are directed at specific signaling molecules, inhibiting the tumor characteristics of growth signaling (trastuzumab, gefitinib) (12), angiogenesis (2-methoxyestradiol, anti-VEGF) (13), and invasion (metalloproteinase inhibitors) (14). Other therapies currently in clinical trials include immunotoxins, antibody-toxin fusion proteins that kill tumor cells after being co-internalized with a cell surface antigen (15), and tumor vaccines, which enhance immune responses to specific tumor antigens (16). Such targeted therapies offer increased specificity and thus reduced toxicity to normal cells. However, their effectiveness is generally contingent upon elevated expression of the intended target by the tumor (e.g., overexpression of Her2/neu for herceptin or high tumor antigen levels for vaccines) (17, 18).

While most patients initially respond to these therapies, chemoresistance often occurs (19, 20). Drug resistance arises from a myriad of causes, including decreased uptake/increased effusion, cell cycle-dependent susceptibility (quiescent cells are generally more resistant), altered tumor microenvironments (i.e., hypoxia or acidity), or, in the case of targeted therapies, intrinsic or extrinsic absence of the intended target (21-24). Tumor drug resistance can be inherent or acquired but is generally a result of positive selection of clonal cells that possess a phenotype that allows survival from the intended insult (DNA or mitotic spindle damage, etc.) (19, 25, 26). Thus, one underlying goal of drug discovery research is to find replacements, or supplements, for many current chemotherapeutic agents that frequently lose their effectiveness. An emerging strategy in clinical trials, therefore, is to use a novel drug in conjunction with a proven chemotherapeutic to determine if the combination results in synergistic action. Although the discovery of a new agent effective enough to displace a proven chemotherapeutic is rare, a novel drug would be considered useful if it could be administered in combination with a reduced, less toxic concentration of the antineoplastic. A novel drug would also be valuable if it resensitized drug-resistant tumors to an established chemotherapeutic. The purpose of this review is to summarize recent research in novel epigenetic therapies and describe current findings regarding their mechanisms and clinical potential.

3. EPIGENETIC MODIFICATIONS IN CANCER

3.1. DNA Methylation

Altered gene expression is present in all tumors, allowing the emergence of phenotypes such as increased

proliferation, cell motility, matrix degradation, immune system evasion, and a capability for colonization at distant sites (metastasis). Downregulation of neoplasia-limiting genes, or tumor suppressors, has been well established over the last 20 years (27). Tumor suppressor genes (TSGs) include well-known examples such as *p53*, involved in cell cycle arrest or apoptosis induction due to cellular stresses, (28, 29), and *PTEN*, responsible for modulation of kinase signaling (30).

Downregulation of tumor suppressors is often a result of genetic deletion or mutations that abolish gene product function. Deletions or mutations in *p53* have been demonstrated in 75 – 100% of small cell lung (31) and >76% of esophageal (32) cancers. Loss of heterozygosity (LOH), abrogation of expression from one functional allele, has been well established for the *RB* gene in the childhood cancer retinoblastoma (33, 34). Similarly, imprinted genes, those developmentally silenced on one allele, can subsequently lose expression of the single functional allele in a process known as loss of imprinting (LOI). LOI has been demonstrated in genes such as *ARHI*, in ovarian cancer (35, 36), and *PEG1*, in breast cancer (37). The occurrence of both LOH and LOI in cancer led to Knudson's "two-hit" hypothesis, originally developed to explain retinoblastoma development, which states that two functional "hits" must occur in a tumor suppressor gene to negate its antitumor effect (34).

While genetic mutations or deletions are frequently known to elicit one or both hits to a TSG, several examples of LOH have been found where neither of these insults is present (38, 39). In many of those instances, loss of TSG expression was found due to *epigenetic*, *i.e.,* noncoding sequence, mutations (40). The most common epigenetic modification in cancer is methylation of the C5 position of cytosine within the nucleoside deoxycytosine (41, 42). The resulting *methylcytosine* can be spontaneously deaminated to form thymine, which, unlike the foreign base uracil, is repaired only by the inefficient mismatch repair system, and can thus be stably integrated into the genome (43). Due to such thymine replacement, the actual frequency of the CG dinucleotide is five-fold less than its expected frequency (43). Specific, isolated CG-rich regions, however, are often found unmethylated, in association with coding sequences (41). These regions, known as "CpG islands," were recently defined as sequences >500 base pairs in length, >55% GC content, and having an observed/expected CG ratio of 0.65 (44). Based on its total CG content, it was estimated that the human genome contains approximately 29,000 CpG islands (45); however, this number may be an overrepresentation, as it now appears that the genome contains many fewer genes than previously thought (46).

DNA methylation is catalyzed by enzymes known as DNA methyltransferases (DNMTs), of which three appear active in humans: DNMT1, DNMT3a, and DNMT3b (47). DNMT1 is primarily involved in maintenance of methylation patterns, while DNMTs 3a and 3b effect *de novo* DNA methylation (47). DNMT1 acts primarily on hemimethylated DNA and is often present in DNA replication complexes (48). DNMTs 3a and 3b, by contrast, are primarily expressed during development (49). The function of DNMT2 is unknown, as it does not appear to possess methylation activity (50).

In normal cells, most repeated DNA sequences, heterochromatic regions, and imprinted genes are methylated (51, 52). It is hypothesized that DNA methylation acts as a defense mechanism to suppress the potential harmful expression (and retrotransposition) of "selfish" repetitive DNA sequences such as *Alu* and *LINE-1* elements (53). In cancer cells, however, such sequences, in addition to centromeres and microsatellites, are found hypomethylated, possibly leading to genomic instability (54). Such instability is exemplified by a genetic disorder known as *immunodeficiency, centromeric region instability, and facial anomalies* (ICF) syndrome, resulting from mutations in DNMT3B that lead to genomic hypomethylation; these patients often possess rearrangements in chromosomes 1 and 16 (55). CpG islands, by contrast, while normally unmethylated, become hypermethylated during tumorigenesis (41). Consequently, in tumors, DNA methylation patterns are the inverse of normal cells, with cancer genomes possessing overall hypomethylation accompanied by local hypermethylation (of CpG islands) (40, 56). Such patterns may develop normally during aging, eventually resulting in specific cells harboring growth advantages, during the process of tumorigenesis (51, 57).

As inferable by its presence in heterochromatin, DNA methylation is strongly correlated with gene silencing. Indeed, most imprinted genes (with exclusive expression of the paternal or maternal allele) and the X chromosome in normal (XX) females are densely methylated (58, 59). In many tumors, methylation of CpG islands has been correlated with the silencing of numerous TSGs implicated in all six aforementioned hallmarks of cancer (Figure 1). For example, the TSGs *PTEN* and $p14^{ARF}$ are methylated in breast and gastric cancers (60-63); their repression may allow unchecked tumorigenic signaling by the MAPK pathway, leading to the cancer hallmark of self-sufficient growth. For the trait of recalcitrance to antigrowth signaling, epigenetic silencing of the apoptotic response genes, *RAR-beta*, *Apaf1,* and $p16^{INK4b}$, has been demonstrated (64-66). Similarly, the genes *cyclinD2*, methylated in breast and prostate cancers (67, 68), and *CDX1*, methylated in colorectal cancer (69), play roles in restraining unlimited growth potential. In angiogenesis, *VHL*, *THBS1*, and *THBS2* are silenced in various cancers (70-72), likely negating their inhibitory role in that tumor process. Metastasis-suppressing genes such as *TIMP3*, *E-cadherin* and *CD44* are silenced by methylation (73-75), contributing to the development of the cancer hallmark of invasion/metastasis. These (and many other) tumor suppressor genes, while silenced primarily by cytosine methylation, may also be repressed by histone deacetylation, and inhibitors of both processes thus hold promise as antitumor therapies (see following sections).

3.2. Histone Hypoacetylation and Histone Methylation

Another epigenetic modification of silenced genes is histone hypoacetylation. Normally, in

transcriptionally active genes, epsilon-amines of lysines within the N-terminal "tails" of the core histones (H2A, H2B, H3, and H4) are acetylated (76). This modification neutralizes the positive charge of the basic histones and is thus believed to "decompact" DNA, allowing access of transcription factors and polymerases to coding sequences. Consequently, highly acetylated histones are associated with transcriptionally active sequences, while hypoacetylated histones are found in silenced chromatin. Acetyl groups are added to histone lysines by histone acetyltransferases (HATs) and are removed by histone deacetylases (HDACs). Correspondingly, HATs are associated with expressed genes and HDACs with repressed genes. HDACs are actually a family of 18 enzymes, consisting of three classes. Class I and Class II HDACs are zinc-dependent enzymes for which a number of inhibitors have been developed (see below). Class III HDACs, also known as sirtuins (homologous to the yeast Sir2 transcriptional repressor) are NAD-dependent and resistant to those same inhibitors (77).

Alterations in histone acetylation patterns are present in many tumors (78, 79). Evidence for the role of aberrant histone acetylation patterns in tumors is found in several translocations characteristic of specific cancers. One of these occurs in acute leukemia in which the *mixed lineage leukemia* (*MLL*) gene is fused to various possible partners. One of these partners is the histone acetyltransferase CBP (CREB-binding protein); the ensuing sequestration of CBP results in inappropriate gene silencing and disrupted histone acetylation (80). In a similar example, the *MOZ* gene, located at 8p11, can act as a translocation partner for CBP and its homolog p300 (81). Another fusion oncoprotein found in leukemias, that of the retinoic acid receptor (RAR) partnered with the PML gene, results in aberrant recruitment of HDACs and inappropriate gene silencing (82). Additionally, in non-Hodgkin's lymphoma, the transcriptional repressor LAZ3 is overexpressed and believed to induce inappropriate transcriptional repression, primarily through association with HDACs (83); such repression likely contributes to lymphomagenesis (83). Deacetylation-induced silencing of specific genes, such as *gamma-globin* (84-86), *p21*$^{CIP1/WAF1}$ (87-89), and *gelsolin* (88), has also been demonstrated. The histone deacetylase HDAC1, in particular, is overexpressed in a number of tumors (90-92) and likely contributes to tumorigenic gene silencing (91). Similarly, HDAC2 has been associated with the *wnt* pathway, a cascade strongly implicated in neoplasia (93). That association was demonstrated by RNAi silencing of HDAC2, which led to colon tumor apoptosis, implicating a possible role for HDAC2 in cancer cell survival (93).

As histone deacetylation, in parallel with DNA methylation, is associated with gene silencing, "crosstalk" between these two processes has been discovered (41, 56). *Methylcytosine-binding proteins* (MBDs) are found in repressive chromatin complexes containing HDACs and histone methyltransferases (94-96), enzymes also often associated with gene silencing (97). Additionally, it has been shown that DNA methyltransferases can directly bind HDACs (98, 99), further linking the processes of DNA methylation and histone deacetylation.

Besides altered cytosine methylation and histone deacetylation, another cancer-associated, epigenetic anomaly is aberrant *histone* methylation. Lysines within the tails of histones H3 and H4 are found methylated, in repressive chromatin, by histone methyltransferases (100). These enzymes, except for one example, all contain a conserved, 130-amino acid SET domain (101). In cancer, overexpression of SET enzymes, such as EZH1 and SMYD3, has been found (102-104); it is possible that these repressive enzymes act similarly to HDACs in tumorigenesis. Histone methylation at H3 lysine 9 is strongly correlated with DNA methylation; interestingly, treatment with a DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC, see below) can also reverse H3 lysine 9 methylation (105). A current controversy is whether histone methylation precedes or follows DNA methylation (51). Our group has recently demonstrated that transcriptional silencing (by RNAi) of the estrogen receptor alpha (ER-alpha) resulted in silencing, and repressive chromatin modifications of a downstream ER-alpha target gene, the *progesterone receptor* (*PR*) (106). In that study, ER-alpha silencing initially led to the recruitment of repressive histone-modifying complexes to the *PR* promoter, followed sequentially by methylbinding proteins and DNA methyltransferases (106). These findings support the theory that DNA methylation is the final "locking" step in the silencing of transcriptional expression (51). Such studies may offer insight into the development of DNA methylation, following a loss of gene expression, during the processes of tumorigenesis and the development of drug resistance.

3.3. Epigenetic Aberrations in Drug Resistance

As noted above, drug resistance may result from a number of determinants. Often, chemoresistance is elicited by nongenetic factors such as altered drug exposure, variations in tumor microenvironment, and altered drug metabolism. In many instances, however, altered gene expression is present in intrinsically or extrinsically resistant tumor cells (19, 21). As most chemotherapeutics inflict various stresses on rapidly dividing cells, the net effect of these stresses on sensitive cells is cell death, predominantly mediated by apoptotic pathways (19, 21, 23, 26). For example, taxanes stabilize tubulin, resulting in defective spindle formation, G2/M arrest, and apoptosis, probably by p53-dependent cascades (107). Similarly, alkylating agents cause extensive DNA damage (26). Recognition of such damage by the mismatch repair system culminates in activation of the apoptotic program, although the precise mechanism of this activation remains uncertain (24). Thus, as the vast majority of conventional anticancer drugs exert their effects by inducing apoptosis, it is now evident that chemoresistant tumor cells often harbor overexpressed antiapoptotic genes and/or downregulated (or mutated) proapoptotic genes.

While chemotherapy response genes are often silenced by deletion or mutation, an increasing number are now known to be downregulated by epigenetic mechanisms. One example of these is the gene encoding the DNA mismatch repair enzyme hMLH1. Methylation-induced silencing of *hMLH1* has been demonstrated in a

Table 1. DNA methylation inhibitors

Inhibitor	Mechanism of inhibition	Clinical Trials?	FDA Approval?	References
5-aza-2'-deoxycytidine 5-aza-dC, DAC, decitabine, Dacogen	primarily DNMT1	Phase III	Pending	119, 122, 403, 404
5-azacytidine, 5-azaC, azacytidine	primarily DNMT1	Phase II	No	119, 120, 404
hydralazine	unknown	No	Yes vasodilator	145, 147
MG98	antisense RNA to DNMT1	Phase II	No	119, 152
procainamide	unknown	No	Yes antiarrhythmic	147, 405
procaine	unknown	No	Yes anesthetic	151
zebularine	primarily DNMT1	No	No	141, 142

number of tumors, including those of the colon (108), stomach (109), and ovary (110, 111), and has been linked with resistance to platinum drugs (112). These drugs, such as carboplatin and cisplatin, alkylate and damage DNA by the formation of inter- and intrastrand adducts; it is believed that apoptotic responses to such adducts are initiated by recognition by the mismatch repair system (26). Epigenetic silencing of *hMLH1*, therefore, and the resultant decreased apoptotic response to DNA adducts, is one contributing factor to tumor resistance to platinum-based drugs.

Besides *hMLH1*, several other genes have been implicated in a positive (apoptotic) chemotherapeutic response; many of these are epigenetically silenced during the acquisition of chemoresistance (21-23, 113). In addition, however, negative (survival-promoting) apoptotic regulators are found upregulated in chemoresistant tumors (21, 23). Consequently, one predominant current hypothesis is that chemotherapy sensitivity depends on the apoptotic potential of the targeted tumor cell (21-23). Genes that inhibit apoptosis, and promote drug resistance, include *NF-kappaB*, *XIAP*, and *bcl-2* (21-23). Conversely, genes that promote apoptosis, and drug sensitivity, include *PTEN*, *caspase-9*, and *Apaf-1* (21); several of these are found epigenetically silenced in drug-resistant cancers (60, 62, 114-117). Thus, restoration of apoptotic pathways, by DNA methylation and/or histone deacetylase inhibitors, is one strategy for positively shifting the apoptotic balance of tumor cells to possibly chemosensitize drug-resistant tumors. Indeed, several epigenetic therapies are now being examined in combination with conventional chemotherapeutics for maximal antitumor effects (see below).

4. CURRENT EPIGENETIC THERAPIES FOR CANCER

4.1. DNA Methylation Inhibitors

Several compounds have now been described with DNA demethylating activity. These drugs (Table 1) predominantly act by inhibiting DNA methyltransferase enzymes (primarily DNMT1), resulting in genomic hypomethylation. Most of these are cytosine analogs that are phosphorylated and subsequently incorporated into nascent DNA strands. Following DNA replication, the analogs covalently and irreversibly bind to the active site of the DNMT, resulting in cellular depletion of these enzymes (118). Consequently, the mechanism of these drugs is replication-dependent, requiring several cell divisions to complete the demethylation of each DNA strand (119). The first of these inhibitors was the cytidine analog 5-azacytidine or 5-aza-C (120). As this drug is a ribonucleoside, it is likely primarily incorporated into RNA, as well as its activity-dependent incorporation into DNA (following intracellular conversion to its deoxyribose form) (119). This RNA incorporation adversely affects ribosomal assembly and tRNA function, inhibiting protein synthesis and resulting in considerable cytotoxicity (118). 5-aza-C is also relatively unstable, requiring regular fresh drug preparations during protracted infusions (121). Nonetheless, 5-aza-C remains under investigation as a therapy for some hematological malignancies (see below).

Due to its cytoxicity, instability, and limited potency, 5-aza-C has largely been replaced by its deoxyribose analog, 5-aza-2'-deoxycytidine (5-aza-dC). Subsequently, 5-aza-dC (Table 1) has become the most studied and best-known methylation inhibitor (119, 122). As 5-aza-dC is a deoxyribonucleoside, it is incorporated directly (following phosphorylation) into DNA, making it a potent DNMT inhibitor, effective at the submicromolar concentrations. 5-aza-dC has been shown to effect demethylation in numerous cancer cell lines (123-126) and restore the expression of several tumor suppressors, including (but not limited to) *HIC-1*, *E-cadherin*, *RASSF1A*, $p15^{INK4B}$, $p16^{INK4A}$, *Apaf1*, and *caspase-8* (127-130). In ovarian cancer, Plumb *et al.* demonstrated that 5-aza-dC treatment of the ovarian cancer drug-resistant cell line A2780/CP could restore hMLH activity, and cisplatin sensitivity, to both cultured cells and mouse xenografts (131). In another study, 5-aza-dC was shown to upregulate 13 genes in pancreatic cancer, some of which likely mediate disease progression (132). In breast cancer cells, a putative tumor suppressor, *SYK*, believed involved in metastasis/invasion suppression, was found induced by 5-aza-dC treatment, resulting in a less invasive phenotype (133). Under the clinical name decitabine (also Dacogen), 5-aza-dC is now in large-scale development by SuperGen (Dublin, CA, www.supergen.com) and MGI PHARMA (Bloomington, MN, www.mgipharma.com). Decitabine was recently approved by the Food and Drug Administration for the therapy of myelodysplastic syndrome (MDS) and is currently being examined in 18 separate clinical trials, with 12 for MDS and other hematological malignancies and six for solid tumors (clinicaltrials.gov) Specifically, phase I and II clinical trials are in progress for myelodysplastic syndrome (MDS), chronic myelogenous leukemia and pulmonary and pleural malignancies (122). In one phase II MDS study, decitabine elicited an overall 49% response rate within the patient cohort, and a 54% response within the highest risk group

Table 2. List of histone deacetylase inhibitors organized by chemical structure

HDAC Inhibitor	Category	Acetylated Non-histone Proteins	Clinical Phase	Reference
Phenylbutyrate	Short chain Fatty Acid	unknown	I, II	406
Valproic Acid	Short chain Fatty Acid	p53	I, II	140
Sodium Butyrate	Short chain Fatty Acid	NF-kappaB, Sp3, p53	*in vitro*	164
pivaloyloxymethyl butyrate AN-9, Pivanex	Short chain Fatty Acid	unknown	II	181
Trichostatin A	Hydroxamic Acid	NF-kappaB, alpha-tubulin, YY-1, Sp1, Sp3, E2F androgen receptor	*in vitro*	185
Suberoylanilide hydoxamic acid, SAHA	Hydroxamic Acid	Alpha-tubulin, ribosomal s3	II, III	193
Suberic bishydroxamic acid SBHA	Hydroxamic Acid	Unknown	*in vitro*	407
PXD101	Hydroxamic Acid	Unknown	I	200
NVP-LAQ824	Hydroxamic Acid	Hsp90	*in vitro*	195
Pyroxamide	Hydroxamic Acid	Unknown	I	408
Oxamflatin	Hydroxamic Acid	Unknown	*in vitro*	409
Scriptaid	Hydroxamic Acid	Unknown	*in vitro*	410
Depsipeptide FR901228, FK-228	Cyclic Peptide	Hsp90, p53	I	411
Apicidin	Cyclic Peptide	Unknown	*in vitro*	412
Trapoxin TPX	Epoxyketone	Unknown	*in vitro*	204
HC-toxin	Epoxyketone	Unknown	*in vitro*	317
Cyclic Hydroxamic-containing Peptide CHAP	TPX-hydroximate hybrid	Unknown	*in vitro*	229
MS-275	Benzamidine	Unknown	I	413
CI-994 N-acetyl dinaline	Benzamidine	Unknown	II	414

The acetylated proteins column lists those proteins that have been shown to have increased acetylation as a result of treatment with the specified HDAC inhibitor

(134). Decitabine therapy for MDS is also undergoing a randomized, safety/efficacy phase III study that is in progress at several locations (clinicaltrials.gov). Another (phase I) clinical trial is also examining decitabine induction of various genes/pathways in renal and lung carcinomas, including the silenced tumor antigen NY-ESO-1 (135) and the tumor suppressing Rb/CDKN2 pathway (136). Decitabine is also being studied clinically in combination with other drugs, including cyclophosphamide (137), retinoic acid (138), the abl kinase inhibitor imatinib mesylate (Gleevec) (139), and the histone deacetylase inhibitors (see below) valproic acid (140) and depsipeptide (139).

Another cytidine analog, zebularine, can be administered orally and has shown impressive antitumor effects in human bladder cancer xenografts, with lower cytotoxicity than 5-aza-dC or 5-aza-C (141). In that same study, zebularine was demonstrated to effect *in vitro* and *in vivo* demethylation and reactivation of a silenced *p16* gene (141). In a follow-up study, zebularine was shown to elicit complete depletion of DNMT1 in T24 bladder cancer cells (142). The relatively low potency of this drug in animal studies (500-1000 mg/kg in mice) could limit its use in the clinic (143); however, lower doses may allow demethylation over longer periods (up to 40-days) (142). In a microarray study, zebularine appears to be highly specific in its action, upregulating 12 genes common to three cancer cell lines but not to normal fibroblasts (144). It also appears that the preferential tumor cytoxicity of zebularine is due to its increased metabolism in tumors, with lower metabolism in normal cells (144).

Besides cytidine analogs, several non-nucleoside reagents have been shown to possess demethylating activities. These include the antiarrhythmic procainamide and the vasodilator hydralazine. Both of these widely used drugs were shown to induce demethylation in MDA-MB-231 breast cancer cell xenografts and in human head and neck cancer patients (145). In another study, procainamide was found to induce re-expression of the tumor suppressor *GSTPI*, encoding the detoxifying enzyme glutathione-S-transferase, in human prostate cancer cells and xenografts (146). These agents have, however, been linked to drug-induced autoimmunity (147, 148), although this effect is generally reversible upon cessation of treatment (149, 150). Another non-nucleoside, the anesthetic procaine, was recently shown to possess demethylation and growth inhibitory activities in MCF-7 breast cancer cells (151) while a genetic strategy, using an antisense oligonucleotide to DNMT1 known as MG98, is currently under phase I study for the treatment of solid and hematological malignancies (152) (www.methylgene.com, www.mgipharma.com). Our group recently performed a similar *in vitro* study and found that siRNA against DNMT1 was more effective than siRNA to DNMT3b, for growth inhibition and demethylation/re-expression of the silenced genes *TWIST*, *RASSF1A*, and *HIN-1* (153).

4.2. Histone deacetylase inhibitors

Another repressive epigenetic modification is histone deacetylation. Histone deacetylation, the removal of acetyl groups from positively charged lysines, facilitates nucleosome binding to the negatively charged DNA phosphate backbone. The net result of deacetylation, therefore, is a closed chromatin conformation associated with gene repression, likely due to obstructed access of transcription complexes to coding sequences (98, 154, 155). Inhibition of histone deacetylases, enzymes that mediate this process, is thus another approach for the re-expression of epigenetically silenced genes. Subsequently, several histone deacetylation inhibitors (HDACIs) are currently the focus of preclinical and clinical trials, based on their impressive efficacy for inducing differentiation and apoptosis in tumor cells, with little or no toxicity to normal cells (77, 156, 157). It has been suggested that the basis for HDACI tumor specificity is via p53-independent, death receptor-dependent pathways (158) and/or a defective G2 checkpoint (159, 160).

A list of several HDACIs, categorized by chemical family, is provided in Table 2. These families

include short-chain fatty acids, hydroxamic acids, cyclic peptides, benzamides, epoxides, and hybrids (157). One HDACI binding mechanism, put forth by Finnin *et al.*, describes an aliphatic chain of the HDACI (*e.g.*, trichostatin A) fitting into a binding pocket of the deacetylase, with enclosure by a bulky "cap," often a benzyl ring (161). In that model, enzyme inhibition is thought to result from chelation of the zinc cofactor within the active site (161); however, some HDACIs likely act by different mechanisms (157, 162, 163). All true HDAC inhibitors, by suppressing the deacetylation of lysine residues, elicit hyperacetylation of histones H3 and H4 (76, 77, 157), although they may also act on other substrates (see below).

The mechanisms of HDAC inhibition by short-chain fatty acid HDACIs are still being elucidated. These fatty acids, including sodium butyrate, phenylbutyrate, and valproic acid (VPA), likely inefficiently coordinate the HDAC active site zinc through their carboxylic acids (157, 161, 162, 164). Alternatively, Kramer *et al*. demonstrated that VPA induces proteosomal degradation of HDAC2, in addition to inhibiting other Class I HDACs (165). VPA, an FDA-approved drug for the treatment of epilepsy for many years but recently discovered with HDACI properties (140, 166), has invoked much research interest in the epigenetics field. VPA was shown to reduce lung metastatic tumor growth of MT-450 breast cancer cells (140) and induce apoptosis in human melanoma cells lines (167) and acute myeloid leukemia cells (168). Although VPA is not as potent as other HDACIs, it is well tolerated in humans at near-millimolar serum levels (140, 169). VPA, in combination with the demethylating agent decitabine, is currently under Phase I study for the treatment of hematologic malignancies and non-small cell lung cancer (www.clinicaltrials.gov). Besides VPA, the small-chain fatty acid phenylbutyrate is now under clinical study, in combination with other agents. One such trial is combining phenylbutyrate with azacytidine, a DNA-demethylating agent, for the treatment of refractory solid tumors (cancer.gov/clinicaltrials), due to the demonstrated synergy of combining HDACIs with DNA-demethylating agents (73). Other trials in progress combine phenylbutyrate with the chemotherapeutic fluorouracil, for treatment of colorectal cancer (170), and the differentiating agent all-trans retinoic acid (ATRA), for treatment of hematological malignancies (171).

As the short chain fatty acids exhibit relatively low potency, likely due to instability (172) and inefficient coordination of the HDAC zinc cofactor (157, 173), several derivatives of these compounds are being examined. One of these is an ester derivative of butyrate, pivaloyloxymethyl butyrate (AN-9, also Pivanex) (174, 175). AN-9 is more potent, *in vivo*, than butyrate (174, 176), to which it is hydrolyzed by intracellular esterases (176, 177). AN-9 demonstrated growth inhibitory activity against cultured human (176, 178, 179) and murine cancer cells (175) and highly metastatic Lewis lung carcinoma mouse tumors (180). In Phase I trials for various advanced solid tumors, and a Phase II trial (alone and with other agents) for non-small cell lung cancer, AN-9 was well tolerated (181, 182). In a follow-up phase IIb study, however, in combination with the antimitotic docetaxel, significant safety issues were revealed by an independent data monitoring committee. That trial, and another for melanoma, were subsequently terminated by its manufacturer, Titan Pharmaceuticals (www.titanpharm.com). Besides ester derivatives, other modified short chain fatty acids have been synthesized to increase stability and potency (172). One of these hybridizes a fatty acid to a hydroxamate (see below), to enhance zinc chelation (173), and another, retinoyloxymethyl butyrate, creates a prodrug for both butyrate and retinoic acid (183).

A second category of HDACIs is the hydroxamic acids (Table 2). Trichostatin A (TSA), an antibiotic from the organism *S. hygroscopicus* (184), was the first hydroxamate found to inhibit histone deacetylase activity (185). Although TSA is potent at nanomolar concentrations, toxicity has limited its clinical efficacy; TSA does, however, yet serve as a research benchmark to which many novel compounds are compared. As such, TSA exhibits potent, relatively nonspecific inhibition of most Class I and II HDACs (Table 2) and has been utilized to examine mechanisms of HDAC inhibitor-mediated apoptosis in mastocytoma (186), prostate (187), osteosarcoma (188), and melanoma cells (167).

One of the best-known HDAC inhibitors, currently in several clinical trials, is the hydroxamate suberoylanilide hydroxamic acid (SAHA). SAHA has been demonstrated to induce growth arrest and differentiation in acute promyelocytic leukemia (APL) (189) breast cancer (190) (191), and multiple myeloma cells. In another study, SAHA was used to sensitize Jurkat leukemia cells to TRAIL-induced apoptosis (192). A phase I, now-completed clinical trial for intravenously-administered SAHA demonstrated the drug to be well tolerated, up to 300 mg/m^2/day, and to exhibit antineoplastic effects in solid and hematological malignancies (193). Currently, phase II/III trials are underway for advanced cutaneous T- and B-cell lymphomas, using an oral formulation of SAHA (clinicaltrials.gov).

In addition to SAHA, other hydroxamates appear promising as clinical candidates. NVP-LAQ824 is a novel hydroximate HDACI with activity against human acute myelogenous leukemia (AML) (194), colon carcinoma (HCT116), and non-small cell lung carcinoma (A549) cells (195). Additionally, NVP-LAQ824 has demonstrated efficacy in combination with an angiogenesis inhibitor (196), the apoptotic cytokine Apo2L/TRAIL (197), and the tyrosine kinase inhibitor imatinib (198). Another hydroxamic acid, PXD101, exhibited antitumour activity *in vitro* and *in vivo* and is currently in Phase I trials in Scotland (199, 200). Other hydroxamates and derivatives include sulfonamide hydroxamates and anilides; these have demonstrated potent antiproliferative and differentiating effects against a number of human cell lines and xenografts (201-203).

Epoxyketones, such as trapoxin B and HC-toxin, act by covalent alkylation of a nucleophilic group within

the active site of the HDAC (204). Trapoxin is a microbial metabolite demonstrated to possess antiproliferative properties against transformed NIH-3T3 (205) and human breast cancer (206) cells. HC-toxin, originally isolated from a maize fungal pathogen (207), has demonstrated antiproliferative effects against the human breast cancer cell lines T47D (208) and MCF7 (209).

Cyclic peptide HDAC inhibitors include depsipeptide and apicidin. Apicidin was demonstrated to induce caspase-dependent apoptosis in human Bcr-Abl (210) and differentiation in HL-60 (211) leukemia cells. In transformed NIH-3T3 and human melanoma cells, apicidin was inhibitory to both angiogenesis and invasion (212). Depsipeptide, a naturally occurring compound from *Chromobacterium violaceum* (213), demonstrated proapoptotic effects on human lymphoma (214) and multiple myeloma (215) cells. In one phase I study for chronic lymphocytic and acute myeloid leukemias, depsipeptide induced constitutional toxicity at 13 mg/m^2/day; further, no partial or complete responses were observed (216). Nonetheless, depsipeptide is currently in seven separate clinical trials for solid and hematological cancers (clinicaltrials.gov), both as a single agent and in combination with other therapies (217) (also see below).

The *benzamides*, including MS-275 and acetyldinaline (CI-994), do not likely act in the same manner as other HDACIs (163). MS-275 treatment induced apoptosis in leukemia cells (215) and also demonstrated significant antitumor effects in mouse xenografts of several human tumor cell lines (218, 219). MS-275 is now orally available and is presently in two separate clinical trials for solid and hematological neoplasms. The benzamide CI-994 is an orally available HDACI originally discovered as a potent differentiating agent in a rat model of human acute myelocytic leukemia (220). In other animal studies, CI-994 demonstrated toxicity to lymphoid and hematopoietic tissues; (221-223) however, those negative effects might be reversible (224). A phase I clinical trial for solid tumors, including renal cell carcinoma and pancreatic cancer, was already ongoing when CI-994 was discovered to possess histone hyperacetylation activity in a colon cancer cell line (225). In that first clinical study, a maximum tolerable dose (limited by thrombocytopenia) of 8 mg/m^2/day was found, with 3/20 patients exhibiting stable disease and one patient a partial response (226). CI-994 has now been further evaluated in phase I trials, in combination with gemcitabine, and capecitabine; in both cases thrombocytopenia was the dose-limiting toxicity (227, 228). As a single agent, CI-994 is currently in a phase II trial for multiple myeloma, while in combination with gemcitabine, a phase II advanced pancreatic cancer study is now complete (217). A phase III non-small cell lung cancer trial is also ongoing (clinicaltrials.gov).

Hybrid HDAC inhibitors are those that possess a cyclic peptide linked to a hydroxamic acid (229). These are thus known as cyclic hydroxamic-containing-peptides (CHAPs). CHAPs exhibit very high (nM) potency and one of these, CHAP31, exhibited impressive antitumor properties against mouse melanoma (229, 230) and a number of human cancer (229) xenografts. In another study, CHAP31 was used to enhance transgene expression in adenovirus-infected mouse liver, suggesting possible utility for gene therapy (231).

In the same manner that HDACIs vary widely in structure, they also vary in their effectual gene re-expression patterns, with the exceptions of genes encoding the cyclin-dependent kinase inhibitor p21$^{cip1/waf1}$ and the oxygen transporting gamma-globin, upregulated by nearly all HDACIs (85-87, 89, 232, 233). Glaser *et al.* found a cluster of eight up-regulated genes, including *p21$^{cip1/waf1}$*, and five downregulated genes, when three HDACIs were tested in two different cell lines; otherwise, several hundred other genes were differentially expressed (234). Several HDACIs have also been demonstrated to induce expression of gelsolin, an actin-binding protein (214, 219, 235). SAHA induces *15-lipoxygenase-1* (236), *TBP-2* (237), *semaphorin III* (238), and *gelsolin* (235). Genes downregulated by HDACIs include *cyclin A* (201, 239), *cyclin D1* (239, 240), *hTERT* (241), and *her2/neu* (242, 243). By downregulating bFGF (244), HIF1-alpha (245), VEGF (244), and RECK (a regulator of metalloproteinases) (246), HDAC inhibitors may abate the tumor processes of angiogenesis and metastasis. Current research is thus focused on defining the specificity of activity against various HDACIs, as well as finding patterns of gene re-expression. Additionally, combination therapies are being used to exploit the apoptotic pathways reopened by HDACIs. For example, the DNA repair enzyme hMLH1 is re-expressed in ovarian cancer cells as a result of treatment with PXD101, a novel HDACI (200). As hMLH1 has been demonstrated to be involved in cisplatin response (247, 248), such re-expression might allow for resensitization of cisplatin-resistant malignancies. Thus, HDACIs possess great potential in the clinic as primary cytotoxic agents, as well as adjuvant therapies to conventional antineoplastics.

In addition to their effects on histone acetylation, several HDACIs have been shown to effect acetylation of nonhistone proteins (Table 2). One interesting example is the tumor suppressor p53. During cellular stress, such as DNA damage, p53, a transcriptional activator, is upregulated and induces the expression of several downstream effectors, resulting in apoptosis and/or cell cycle arrest (28, 29). In many tumors, p53 plays roles in chemotherapy response; p53-null tumors correlate strongly with poor prognosis (249, 250). Transactivating activity of p53 is increased by lysine acetylation by the histone acetyltransferase p300; conversely, p53 is deacetylated by HDAC1, with a subsequent decrease in transactivation (251). Correspondingly, the HDACIs sodium butyrate and trichostatin A have been shown to elicit hyperacetylation of p53 (252, 253), providing another possible pathway to tumor suppression. Another nonhistone protein hyperacetylated by treatment with several HDACIs is tubulin. Alpha-tubulin, a component of microtubules within the mitotic spindle, is a target of anti-mitotic drugs such as paclitaxel and vinblastine. Acetylated tubulin likely stabilizes microtubules (254), and increased acetylation by treatment with HDACIs, such as TSA and SAHA, has also been demonstrated (87, 255). Consequently, such

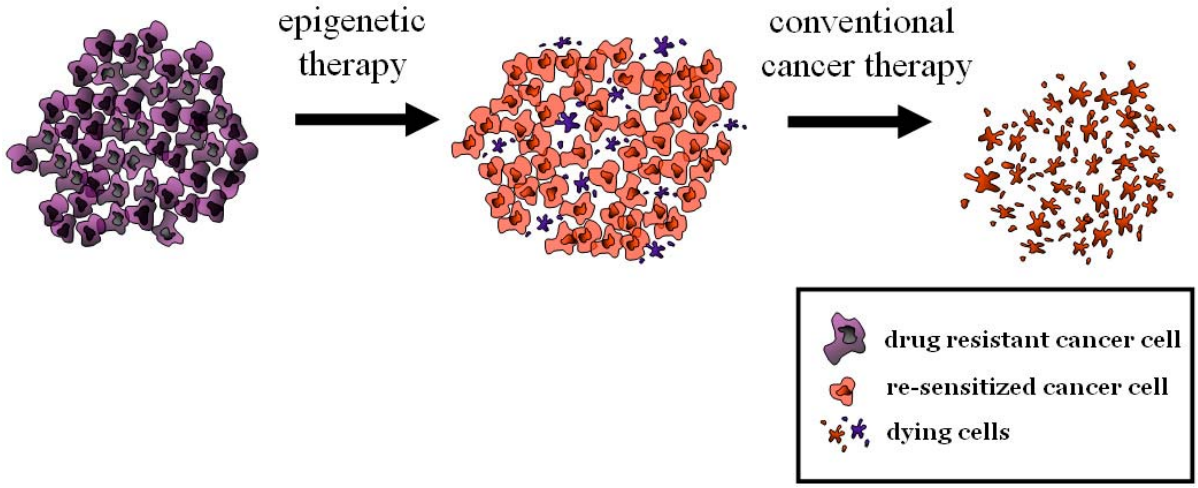

Figure 2. Epigenetic therapy can resensitize cancer cells to conventional therapies. In a heterogenous tumor cell population, a small percentage of cells may respond directly to epigenetic drugs. The remaining tumor cells, however, could then be resensitized (*e.g.*, by tumor suppressor gene re-expression) to conventional therapies.

treatments could potentialy resensitize drug-resistant cells to mitotic inhibitors. Other acetylated nonhistone proteins include *E2F*, *hsp90*, *Sp3*, and *ER-alpha* (157, 256, 257); several of these have also demonstrated hyperacetylation following HDACI treatment (157, 258). Thus, HDACI effects on nonhistone proteins, while still poorly understood, represent a nongenomic course of specificity of these agents.

4.3. Combined methylation/HDAC inhibition

While both HDAC inhibitors and methyltransferase inhibitors have shown promising results in clinical trials as single agent therapies, many studies are now examining synergistic effects of combinations of these two classes of epigenetic drugs. Because cytosine methylation and histone hypoacetylation are both transcriptionally repressive, and DNA methyltransferases and histone deacetylases are found associated in repressive complexes, inhibition of both activities would likely cause maximal derepression of silenced genes (73). To this end, some methylation and HDAC inhibitors have been shown to produce "crossover" effects between histone and DNA modifications. For example, methylation inhibitors may also effect histone acetylation (259, 260) and histone demethylation (105), while one HDACI, valproic acid, was demonstrated to demethylate DNA in embryonal kidney cells (261). As this demethylation was independent of replication (261), it was unlikely mediated by DNMT inhibition alone. Similarly, TSA was shown to induce selective DNA demethylation in Neurospora (262). Despite these examples of crossover effects, however, the majority of studies demonstrate that combinations of HDACIs and demethylating agents actuate a synergistic effect on gene expression, compared to the use of either agent alone. For example, in the human leukemic cells HL-60 and KG1a, 5-aza-dC/TSA or 5-aza-dC/depsipeptide combinations were more effective than single agent treatments in inducing apoptosis (139). A 5-aza-dC/TSA combination also was synergistic in activation of *estrogen receptor (ER) alpha* in MDA-MB-231 breast cancer cells (263). In another study, in lung cancer cells, the apoptotic effects of TSA and depsipeptide were greatly enhanced following pretreatment with 5-aza-dC (264). Similarly, a 5-aza-dC/TSA combination was synergistic in the activation of a number of genes, including *PTGS2, hMLH1*, *TIMP3*, *CDKN2B*, and *CDKN*, in colorectal cancer cell lines (73, 259). We recently used this same combination in treatment of ovarian cancer cells and, taking a microarray approach, demonstrated synergistic upregulation of a greater number of genes than with either drug alone (265).

Due to the success of these combinatorial treatments *in vitro*, several HDAC/methylation inhibitor combinations are now being examined in animals and humans. One such combination, using 5-azacytidine and phenylbutyrate, is currently being studied in a phase I clinical trial for acute myeloid leukemia and myelodysplastic syndrome, while another combination, 5-aza-dC paired with depsipeptide, is under phase I study for the treatment of various lung cancers (clinicaltrials.gov). Additionally, 5-aza-dC is also being combined with the HDAC inhibitor valproic acid, in phase I and II trials, for the therapy of myelodysplastic syndrome, leukemias, and non-small cell lung cancer (clinicaltrials.gov).

5. EPIGENETIC RESENSITIZATION AND IMMUNOTHERAPY

As DNA methylation and histone hypoacetylation both contribute to silencing of drug sensitivity genes, reversal of these modifications, by epigenetic agents as a second-line therapy, could allow the re-expression of such genes. These treatments would then be combined with conventional first-line therapies to elicit tumor regression (Figure 2). *In vitro* studies with methylation inhibitors have firmly established the plausibility of this strategy. In one early study, 5-aza-dC pretreatment synergistically enhanced cytotoxicity of the topoisomerase inhibitor topotecan *in vitro* and *in vivo* (266). In another study, treatment with 5-aza-dC allowed

resensitization to all-trans retinoic acid and dihydroxyvitamin D3 in the human leukemic cell lines KOCL48 and KOPN-1 (123). In a comprehensive study of several tumor cell lines, including melanoma, medulloblastoma, and neuroblastoma, 5-aza-dC induced apoptotic resensitization to a variety of agents, including doxorubicin and cisplatin (115). Similarly, in colorectal cell lines, 5-aza-dC restored expression of the mismatch repair protein hMLH1 and sensitivity to the nucleoside analog 5-fluorouracil (267). In lung cancer cells, treatment with 5-aza-dC was found to upregulate the apoptosis promoter caspase-9 and allow resensitization to cisplatin and paclitaxel (130); a similar finding (increased cisplatin response) was made in the prostate cancer cell line DU-145 (125).

HDACIs have also been demonstrated, *in vitro*, to induce chemosensitization. In lung and esophageal cancer cells, the HDAC inhibitor depsipeptide allowed apoptotic sensitization to flavopiridol (268) and also sensitized the thyroid cancer cell line SW-1736 to doxorubicin (269). In another thyroid cancer cell study, depsipeptide administration resulted in increased uptake of radiolabeled iodine, due to upregulated thyroglobulin and the Na(+)/I(-) symporter, suggesting possible sensitization to this type of therapy (270). The cyclic tetrapeptide HDACI apicidin was recently shown, in human leukemia cells, to potentiate apoptosis by the abl kinase inhibitor imatinib (Gleevec) (271). In drug-resistant D54 glioblastoma cells, pretreatment with TSA or SAHA caused a 10-fold increase in cytoxicity by the topoisomerase inhibitor VP-16 (272). Furthermore, this effect was negated when VP-16 was administered *prior to* the HDACI, suggesting that the sensitization was epigenetic in nature (272). TSA treatment also restored tamoxifen sensitivity in ER-alpha-negative breast cancer cells, possibly through upregulation of *ER-beta* (273), while another hydroxamate, LAQ824, was shown to sensitize human breast cancer cells to a number of chemotherapeutics (242). Similarly, a methylation/HDAC inhibitor combination was synergistic in activation of ER-alpha in the ER-alpha-negative breast cancer cells MDA-MB-231 and MDA-MB-435, suggesting the plausibility of using this epigenetic combination for tamoxifen resensitization (263). In a study of multiple cell lines, including gliomas and breast cancer cells, SAHA and TSA were found to induce sensitization to camptothecin, ellipticine, cyclophosmamide, cisplatin, and VP-16 (272). In that study, the sensitization effect was attributed to a "loosening up" of repressive chromatin structure by the HDACIs, allowing accessibility of the various DNA-modifying drugs (272). Similarly, MS-275 sensitized leukemic cells to the antimetabolite fludarabine through increased reactive oxygen species (ROS) and caspase-dependent apoptosis (274). In a comparable study, sodium butyrate and SAHA were combined with a proteosome inhibitor, bortezomib, resulting in enhanced apoptosis (over either agent alone) in multiple myeloma cells, possibly due to mitochondrial dysfunction caused also by increased ROS (275). Sodium butyrate, in combination with interferon-alpha, also inhibited *in vitro* invasion of six human liver cancer cell lines, likely through downregulation of matrix metalloproteinases (276). In ovarian cancer cells, various HDACIs were found to effect resensitization to paclitaxel (277); such outcomes may be the result of increased acetylation of tubulin (87, 278). Another such combination, 5-aza-dC and LAQ824 (with each at low, nontoxic doses), was recently employed to resensitize drug-resistant ovarian cancer cells to cisplatin (279).

Besides chemosensitization, it is likely that HDAC inhibitors may increase the effectiveness of radiotherapies. In one study, MS-275 pretreatment resulted in increased radiation sensitivity, as compared to treatments with radiation or HDACI alone; furthermore, increased radiosensitivity correlated well with histone hyperacetylation (280). In another study, a number of HDACIs, including SAHA, TSA, and depsipeptide, enhanced radiation sensitivities of human squamous carcinoma cell lines, likely by a caspase-independent, nonapoptotic mechanism (281). TSA was also radiosensitizing to the human glioblastoma cell lines U373MG and U87MG (282). In addition to radiosensitizing tumor cells, it is possible that HDACIs can protect normal cells from radiation damage (283); such protection might allow increased tolerance to radiotherapy (283, 284).

Another emerging area is that of epigenetic-mediated immunotherapy. Members of the cancer/testis antigen (CTA) family, MAGE-1, MAGE-3, NY-ESO-1, and SSX-2, are not expressed in adult tissues but may be expressed in cancer cells (285). As the regulation of the corresponding genes is primarily epigenetic, re-expression of such antigens, by epigenetic inhibitors, is one strategy for increased humoral and T-cell anti-tumor responses (286). In several studies, 5-aza-dC induced both *de novo* and increased expression of CTA genes (135, 287) and one report demonstrated that, while individual clones are quite heterogeneous in CTA expression, 5-aza-dC could upregulate differentially-expressing cells to similar levels (288). Due to the replication-dependent action of methylation inhibitors, such re-expression can be long lasting, with detectable expression remaining several weeks following drug removal (289, 290). Additionally, epigenetic re-expression of co-stimulatory/accessory molecules, such as ICAM, CD80, CD86, and LFA-3, has been demonstrated (290, 291); such molecules play roles in nonspecific activation of T-cells (292). Thus, it is feasible to combine epigenetic therapies with immunotherapies such as vaccination or treatment with interferon gamma or interleukin-2.

Based on these *in vitro* studies, epigenetic chemosensitization strategies likely hold promise for many cancers, and possibly other diseases, in the clinic. For the treatment of tamoxifen-resistant breast cancer, the use of the methylation inhibitor procainamide has been proposed, based on the ability of such inhibitors to induce *estrogen receptor beta* (293). In a current phase II study, decitabine is being combined with imatinib for the treatment of chronic myelogenous leukemia (clinicaltrials.gov). Decitabine is also being investigated as a means to resensitize ^{131}I-resistant thyroid cancers. In another (phase I) clinical trial, phenylbutyrate is being combined with retinoic acid for differentiation therapy in various

hematologic malignancies (clinicaltrials.gov). Epigenetic chemosensitization in ovarian cancer is currently being studied in a European phase I trial combining decitabine with the conventional cytotoxics carboplatin and epirubicin (science.cancerresearchuk.org, also see reference (294)). A previous phase II trial of a 5-aza-dC plus cisplatin regimen in cervical cancer, however, proved discouraging, due to significant hematological toxicity, necessitating further investigation of possible dose combinations (295). The HDAC inhibitor valproic acid was recently studied, in a phase I trial, in combination with all-*trans*-retinoic acid for the treatment of myelodysplastic syndrome, eliciting a response in 44% of patients enrolled (296). An emerging clinical strategy, therefore, is to epigenetically activate apoptosis-related genes to restore chemosensitivity. Similarly, as several HDAC inhibitors enhance tubulin acetylation (87), these could augment sensitivity to taxanes or other microtubule modifiers. Indeed, in T-cell leukemia cells, decreased tubulin acetylation correlated with resistance to vincristine, another microtubule modifier (297). In another preclinical study, HDACI treatment was found to resensitize resistant ovarian cancer cells to paclitaxel (277).

6. POTENTIAL DETRIMENTAL EFFECTS OF EPIGENETIC THERAPIES

As epigenetic therapies have been demonstrated to induce re-expression of a number of tumor suppressor genes (73, 79, 298) and resensitize resistant tumors to conventional drugs (115, 123, 131), several concerns remain regarding possible global demethylation and hyperacetylation. Global hypomethylation is a common feature of cancer cells and is thought to result in genomic instability (299, 300). In fact, transgenic mice with 90% decreased DNA methyltransferase developed genomic instability, with widespread tumorigenesis and growth defects (301). Similarly, hyperacetylation has been linked to chromosomal instability, due to the misregulation of mitotic checkpoints (302). Hypomethylation induced by procainamide, an antiarrhythmic, and hydralazine, a vasodilator, has been linked to T-cell autoreactivity (147, 148). However, this syndrome is generally reversible upon discontinuation of such treatments (149, 150). Tumor induction of *multidrug resistance gene 1* (*MDR-1*), encoding the membrane transporter P-glycoprotein, is believed due, in large part, by demethylation (303). In addition, metastasis-related genes such as *SNCG* (304), *uPA* (305, 306), and *HPSE* (307), have been found demethylated and overexpressed in aggressive tumors and/or cancer cell lines. Thus demethylation could, conceivably, result in the induction of silenced oncogenes. It is also possible that global demethylation could result in deleterious expression of X chromosome (308) or imprinted genes (309) or harmful transcription (or retrotransposition) of repetitive elements such as *LINE-1* and *Alu* sequences (53).

While demethylating agents may elicit a global genomic hypomethylation, several reports have demonstrated that only a small percentage (1-3%) of silenced genes are actually upregulated by such treatment in tumors (259) and only 0.4% in normal fibroblast cell lines (126). It is possible that tumor cells lack transcription factors for other silenced genes, or that chromatin condensation may be dominant. Additionally, it has been shown that removal of methyltransferase inhibitors results in rapid remethylation (310). DNMT-knockdown mice (301), which suffer from genomic stability and tumorigenesis, undergo about 90% reduction in overall methylation; it is unclear, however, that this level would ever be achieved with therapeutic methylation inhibitors (311). Moreover, Eads *et al*. found that mice with greatly reduced DNMT levels could completely suppress colon polyp formation in mice predisposed to such polyps by germline *APC* mutations (312). In another study of breast cancer cell lines, it was found that treatment with 5-azacytidine, while resulting in overall hypomethylation, left specific regions unaffected (313). That finding led to a hypothesis that a cell might "protect" specific chromosomal regions from demethylation that might lead to deleterious gene activation or genomic rearrangements (313, 314). Additionally, a recent study by our group found that siRNA-induced methylation of a specific gene, *progesterone receptor*, could not be reversed by DNA methylation inhibitors alone, in the absence of estrogen signaling (106). Furthermore, in clinical trials of 5-aza-dC, few detrimental effects have been observed. In leukemia patients treated with 5-aza-dC, no significant demethylation of repetitive elements was found, nor were any cases of secondary malignancies (311). Intriguingly, in a clinical trial of myelodysplastic syndrome patients, chromosomal abnormalities were actually *reversed* in 19/61 patients (315).

Histone deacetylase inhibitors (HDACIs) likely suffer from the same shortcomings as methyltransferase inhibitors, *i.e.*, nonspecific epigenetic alterations that could reactivate silenced oncogenes. In one study, it was found that valproic acid sensitized human endometrial adenocarcinoma cells to estrogen-induced proliferation (316). Similarly, sodium butyrate was found to activate the MEK-ERK-SP1 pathway in K562 cells (317). Hyperacetylation has also been linked to genomic instability (318, 319), although one study found no deleterious effects on mitosis following treatment with the HDACI TSA (320). Additionally, despite reports of possible proproliferative effects and genomic instability, it is interesting to note that HDACIs are much more frequently linked to the induction of cell cycle arrest, apoptosis, or cell differentiation (for review see references (76, 321)). Moreover, these effects seem specific to tumor cells (77, 156). Similar to methylation inhibitors, HDACIs upregulate only a small subset of loci (2%) and are very specific in their activation and repression of distinct genes (322). This may be due to HDACI inhibition of specific HDACs that act on distinct genes and/or pathways (323). Furthermore, HDACIs can also inhibit deacetylation of *non-histone* proteins, such as transcription factors, including p53 (324) and E2F (325), and tubulin (87) (Table 2), allowing other possible courses of specificity.

7. ANALYTICAL METHODS FOR EPIGENETIC BIOMARKER DISCOVERY

7.1. Global and high-throughput analyses

To examine genome-wide methylation changes in tumors, several effective methods have now been developed. One array-based method, known as differential

methylation hybridization (DMH), involves hybridization of distinctively labeled tumor and non-tumor DNA to an array of CpG islands (326, 327). Differences in hybridization are detected by utilizing different fluorophores (*i.e.,* red versus green) for normal and tumor samples. This technique has been successfully used to generate methylation patterns for breast (328, 329) and ovarian (330) tumors. In a study by our group, DMH was used to show that CpG island hypermethylation is widespread in ovarian cancer genomes and also identified individual loci correlated to poor prognosis in ovarian cancer patients (331). Those results thus indicate that distinct, hypermethylated CpG islands may be important prognostic markers for this disease (332).

Another array-based technique utilizes methylation-specific oligonucleotides (MSO) to determine methylation levels of specific CpG sites. MSO has been successfully used to discriminate between two types of non-Hodgkin's lymphomas (333) and two types of B-cell leukemias (334). In this technique, arrayed oligonucleotides, corresponding to methylated and unmethylated CpG dinucleotides, are hybridized with (differentially labeled) tumor and non-tumor genomic DNA. This method is fully quantitative and can provide exhaustive maps of individual CpG islands in several tumor types (335, 336).

A common, non-array, PCR-based method known as methylation-specific PCR (MSP) is frequently used for examining qualitative methylation changes of specific genes (337). This technique utilizes primers that are unique to methylated and unmethylated forms of specific alleles, following bisulfite-mediated deamination of unmethylated cytosines (methylcytosine is protected) (337). By combining MSP with quantitative PCR methods (such as SYBR Green fluorescence or TaqMan technology), a technique known as "MethyLight," one can quantitate the degree of methylation of the allele under study (338). This technique has been successfully used to establish panels of methylated genes highly prognostic of colorectal (339), breast (339), esophageal (340) and pancreatic (341) neoplasms. MSP has been used to successfully detect methylated genes in the serum of ovarian (342), cervical (343), and breast (344) cancer patients. In ovarian tumors, we recently determined, using MSP, the methylation status of several markers in ovarian tumors that correlated significantly with clinical outcome (331).

A fourth technique, known as restriction landmark genome scanning (RLGS), has also been used for assessing the methylation status of multiple differentially methylated loci in specific tumors (345). RLGS relies on the use of the methylation-sensitive restriction enzyme *NotI*, which cuts within the recognition sequence GC^GGCCGC. Methylation within the CG cut site abrogates *NotI* digestion; two-dimensional electrophoresis of digested genomic DNA can then allow discrimination between digestion patterns of normal and tumor DNA (346). RLGS has been used to successfully identify aberrantly methylated loci in astrocytomas (347), squamous cell carcinomas (348), and liver cancers (349).

A recently developed technique, known as **m**ethylated **C**pG island **a**mplification/**r**epresentational **d**ifferential **a**nalysis (MCA/RDA) (350) relies on the use of a methylation-sensitive endonuclease, *Sma1* and its methylation-insensitive isoschizomer, *Xma1*. In MCA/RDA, tumor and normal genomic DNA is first digested with *Sma1*, which cuts only at unmethylated CCC^GGG sites, producing blunt ended products. The remaining DNA is then cut with *Xma1*, which cuts at C^CCGGG, leaving a CCGG overhang ("sticky end"). Linkers are then ligated to the sticky ends and PCR performed to amplify CpG islands (350). For the RDA step, tumor and normal DNA are hybridized to subtract the common sequences and the resulting sequences are those that are differentially methylated; these can then be cloned and sequenced (56). MCA/RDA has been used successfully to determine 33 differentially methylated CpG islands in colon tumors. (350). In another study, MCA/RDA was used to discover seven distinctly methylated sequences in pancreatic cancer cell lines (351), while an analysis of prostate cancer yielded four tumor-specific CpG islands; two of these resided in a gene encoding a tyrosine kinase receptor (352).

While aberrant histone acetylation is another frequent characteristic of tumors, high throughput analyses of such aberrations remain more complicated than similar methylation analyses. One method, **Ch**romatin **I**mmuno**P**recipitation (ChIP) is used for epigenetic examination of specific loci. In this technique (353), antibodies to specific chromatin proteins are used to coimmunoprecipitate DNA associated with such proteins. PCR is then used to detect (and possibly quantitate) distinct sequences precipitated by the specific antibody. For example, ChIP, using an antibody to acetylated lysine 9 of histone H3, can be used to determine specific genes associated with that acetylated residue. A more recent technique, known as ChIP-on-chip, is a high throughput technique in which the immunoprecipitated DNA sequences are subsequently amplified, fluorescently labeled, and hybridized to an arrayed library (354). Recently, our group used ChIP-on-chip in a technology known as "chromatin landscaping" to determine downstream chromatin modifications induced by silencing of the gene encoding the nuclear steroid receptor ER-alpha (355).

One promising profiling method, recently developed by our group, combines DNA methylation, gene expression, and chromatin modifications on one array. In this "triple array", total cDNA and ChIP-amplified DNA are sequentially hybridized to an array of CpG islands (265). Positively hybridized spots indicate loci that are both expressed and epigenetically modified (265). While our initial triple array study identified aberrantly methylated and acetylated loci specific to ovarian cancer, it is likely that this method will also be useful for epigenetic profiling of other tumor types.

7.2. Epigenetic biomarkers

As several promising high throughput methods are now available, ongoing efforts aim to determine epigenetic "signatures," unique DNA methylation and histone acetylation patterns, for the diagnosis, stratification, and/or staging of specific cancers (356). As several methylated genes are now correlated with various tumor types, one could, theoretically establish a "panel" of discriminative biomarkers for any one particular cancer and its specific subtypes and/or stages (357, 358). For example, ovarian cancer has been associated with methylation of the genes *LOT1*, *OPCML*, *BRCA1*, *ARHI*, *RASSF1A*, and *hMLH1* (359, 360). Similarly, prostate cancer has been correlated with methylation of *E-cadherin*, *androgen receptor*, *cyclin D2*, *CD44*, *HIC1*, and *RASSF1A* (361), while lung cancer has been linked to methylation of *caspase-8*, *DAPK*, *MGMT*, $p16^{INK4}$, and *RASSF1A* (362). In theory, the methylation status of these genes could be used as a biomarker for these tumor types. For example, increased methylation of *HIC-1* correlated with short survival in lung cancer (363). Methylated *RASSF1A* also associated with poor clinical outcome in neuroblastoma (364), but not in kidney cancer, although it was more frequent in high-grade tumors (365). In prostate cancer, increased methylation of *RAR-beta*, *RASSF1A*, *GSTP1*, and *CDH13* correlated with higher Gleason scores, which indicate aggressiveness (366). Also in prostate cancer, Sidransky *et al*. demonstrated that hypermethylated *GSTP1*, in particular, is present in needle biopsies, can discriminate between normal prostate tissue from adenocarcinomas (367, 368), and also correlates with Gleason score (369). That same group further demonstrated that increased methylation of another gene, *RAR-beta*, also correlates with prostate malignancy and stage (370). Monitoring the methylation status of such genes could thus provide accurate determinations of staging and/or treatment response.

In addition to analyses of tumor tissue DNA, it also appears that many tumors "shed" DNA into the serum and other body fluids (371-373). Such serum DNA is amenable to epigenetic analyses and could thus potentially be a source of epigenetic biomarkers for early detection, tumor typing, or monitoring of response to therapy. In ovarian cancer, alleles of *BRCA1*, *RASSF1A*, $p16^{INK4a}$, and $p14^{ARF}$ were found methylated in serum and peritoneal fluid DNA from ovarian cancer patients (342). In another ovarian cancer biomarker study, methylation of serum *hMLH1* following chemotherapy was found to correlate significantly with tumor relapse (374). In serum from breast cancer patients, methylated alleles of *RASSF1A*, *APC*, and *DAPK* were found; of these, 94% of patients possessed methylation of at least one allele (365). In another breast cancer study, serum-present methylated *RASSF1A* or methylated *APC* correlated significantly with poor clinical outcome (344). In prostate cancer patients, methylated *GSTP1* has been detected in both serum and urine (375, 376). Similarly, in a cervical cancer study, 87% of patients possessed detectable serum methylation of one or more of the genes *CALCA*, *hTERT*, *MYOD1*, *progesterone receptor*, or *TIMP3* (343). A serum tumor marker study of gastric cancer revealed that 28% of patients (and 0/10 healthy volunteers) demonstrated aberrant methylation of *p16* or *E-cadherin* (377). Similarly, in renal cancer patients, 16 of 17 (94%) patients tested positive, in serum or urine, for hypermethylation of at least one of seven genes examined (378); in urine sediments from bladder cancer patients, 78% demonstrated methylation of *DAPK, bcl-2, and hTERT*, as compared to age-matched healthy individuals (379).

Despite these successes, one obstacle to the extensive use of early detection epigenetic biomarkers is the requirement for a high positive predictive value (PPR) for screening large populations (380). One discouraging example is that of ovarian cancer, for which no early detection methods exist. As ovarian cancer afflicts 50/100,000 women, to achieve a PPR of 10%, 99.6% specificity would be required of a biomarker (381). For this objective, it is unlikely that any single methylated gene would be a suitable biomarker. However, it might be possible to monitor the epigenetic status of a *panel* to increase both sensitivity and specificity; linear combinations of biomarkers could then be chosen to increase diagnostic accuracy (382). For example, for lung cancer, one could assay serum DNA for methylation of *RASSF1A*, *p16*, and *DAPK***.** Early detection, using epigenetic biomarkers, might be feasible for cancers that are highly prevalent (*i.e.*, colon, prostate, breast) and could thus supplement current screening approaches. In a prostate cancer study of 118 carcinomas, it was demonstrated that the combined use of methylated *GSTP1* and *APC* could achieve a theoretical sensitivity of 98.3%, as compared to benign prostatic hyperplasia, with a specificity of 100% (370). Thus, epigenetic biomarker-based early detection holds promise and it is also highly likely that these biomarkers will be useful for disease stratification, staging, and monitoring response. The accuracy of epigenetic (and other) biomarkers for classification and prediction was recently shown by Pepe and Cai to be amenable to methods previously used for evaluating diagnostic tests (383). In addition to serum detection (which may not be feasible in some tumor types), the tumor tissue itself may be amenable to DNA isolation for methylation-specific PCR and/or microarray analyses (see above).

Besides specificity and sensitivity, other obstacles remain regarding the successful establishment of epigenetic biomarkers. One such obstacle is a standard definition for methylation of an allele, *e.g.,* percent of CG sites that gain methylation. Other obstacles include standardization of methods/reagents used for epigenetic analyses (see below) and correlations of epigenetic data to clinical "gold standards" (384). Our group recently convened, in an international forum, to address these disparities and develop a consensus for standards for epigenetic cancer biomarkers (385). While this forum was specifically dedicated to ovarian cancer, one objective was for these standards to pertain to epigenetic biomarkers for other tumor types.

7.3. Bioinformatical analyses

To analyze the vast amount of epigenetic data produced by microarray studies, bioinformatical techniques

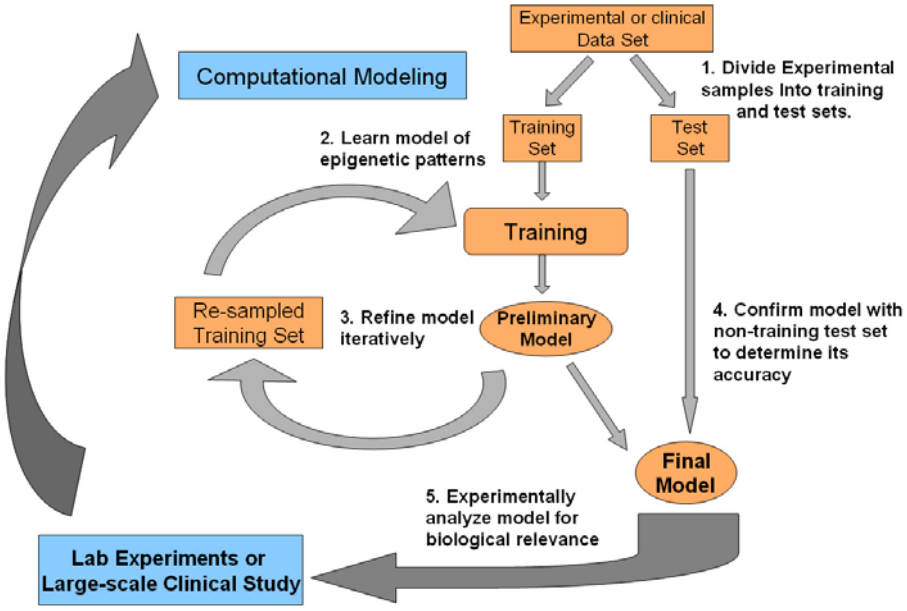

Figure 3. An overview of model building from experimental data. using an iterative process. The first step splits a dataset into a training set and a test set. To estimate the generalization error of the given model or to select the best model, cross validation is commonly used. A variety of methods, including support vector machine (SVM), artificial neural network, and linear discriminants, may be used. Data that is not reflected in the initial model can be collected and used for retraining to refine the model. Computational modeling is thus a powerful method to interpret laboratory or clinical epigenetic data.

are now increasingly used. Several algorithms have now been developed for the determination of epigenetic patterns as biomarkers specific to distinct tumor types and/or stages. To this end, the combination of pattern discovery and machine learning techniques is often used. For example, Feltus *et al*. discovered seven CpG island patterns using DNA pattern recognition and a supervised learning technique. This classification function, based on the frequency of the seven patterns, was capable of discriminating methylation-prone from methylation-resistant CpG islands, with significant accuracy (386). Recently, patterns of histone methylation were also shown as non-random, especially trimethylation of lysine 4 of histone H3 (387).

A recent study by our group investigated a correlation between gene function and patterns of CpG islands located in the promoter regions of specific genes. CpG islands, used as gene markers (388), were clustered, based on sequence similarities, using a clustering algorithm called BAG (389). According to gene annotations from GenBank (ncbi.nlm.nih.gov/Genbank), gene functions were referenced and compared. While not all CpG islands shared similar functions, some common motifs, with correlative patterns, were discovered to cluster together (unpublished). Thus, determining CpG island regions susceptible to DNA methylation, as related to tumor suppressor gene silencing during drug treatment, might allow clinicians to target demethylation of such CpG islands to elicit tumor resensitization.

In addition to providing models of methylation alterations in tumor progression, bioinformatical analyses of tumor DNA could be useful for early cancer detection, as methylation is an early event in the carcinogenic process (390). Although methylation of numerous protooncogenes and tumor suppressor genes is involved in cancer development, the network of expression of those genes, as it relates to methylation, remains poorly understood. While such analyses remain in infancy, machine-learning techniques hold promise for these types of determinations; one of these is entitled “feature gene selection” (FGS) and is now widely used. FGS involves a search for gene subsets able to discriminate tumors from normal tissue, and may allow clear biological interpretations or provide insight into the molecular mechanism of tumorigenesis. This method is thus able to reduce the number of genes and select an optimal (or near optimal) subset from an initial set of oncogenes and tumor suppressor genes related to early stages (391). FGS may also be capable of providing information regarding gene networking, by identifying methylated genes that play predominant roles in the development or progression of various tumors and/or stages. Thus, by classifying comprehensive networks of oncogenes and tumor suppressor genes, it may be possible to detect cancers in very early stages.

Finally, in combined microarray experiments, DNA methylation analysis can be used to predict cancer class. A recent study has demonstrated that cancer classification, based solely on DNA methylation analysis, is possible and can achieve results comparable to mRNA expression analysis (392). In that study, principle component analysis (PCA), Fisher criterion, and t-tests were utilized and compared for performance in CpG island selection for classification, with Fisher criterion performing best (392). Figures 3 and 4 show overviews of the process of building a prediction model and supervised learning

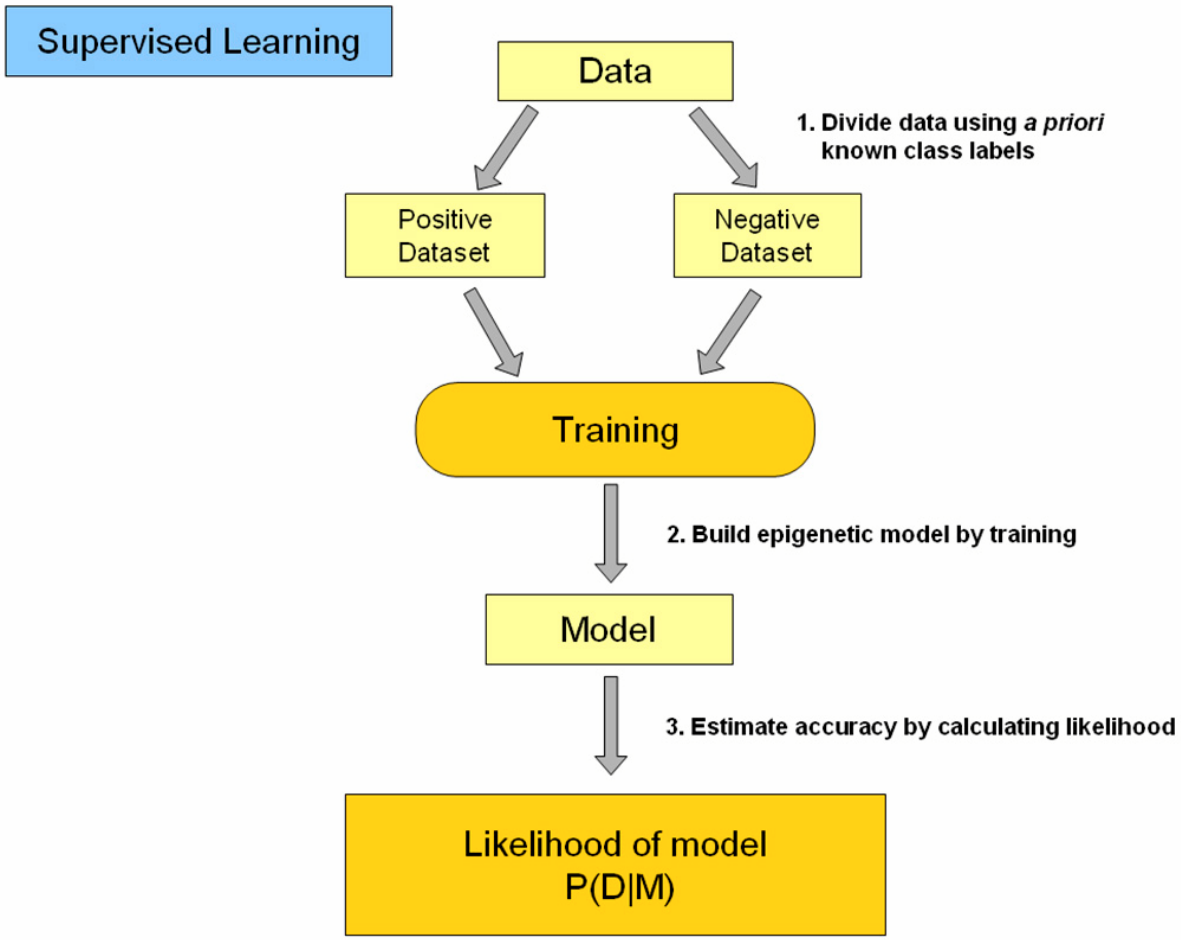

Figure 4. An example of a supervised learning process. Training is guided by *a priori* knowledge obtained experimentally. Accuracy is improved using positive and negative data rather than merely using positive data and random background information. The accuracy of the model is then estimated by calculating its likelihood, P(D|M) where P is the probability of the dependency model M using data D.

model, respectively. Combining these analyses, for selected CpG islands, with a support vector machine technique could classify acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) with about 85% accuracy (392).

8. CONCLUSIONS

Epigenetic alterations have been firmly demonstrated to play a role in tumor initiation, development, and progression. Aberrations in DNA methylation and histone acetylation/methylation almost certainly contribute to tumor development and progression to metastatic and drug-resistant states. As these aberrations occur in several genes involved in apoptotic and differentiation pathways, their correction might allow for resensitization of tumor cells to drugs that affect such pathways. Demethylating agents and histone deacetylase inhibitors are two classes of epigenetic therapies currently under considerable preclinical and clinical study. As shown in Figure 5, however, these agents represent only two points of intervention within the spectrum of repressive epigenetic modifications. While inhibition of HDACs results in increased histone acetylation, one could achieve the same effect by activating histone acetyltransferases (HATs). One such small molecule activator was recently reported; however, this compound was poorly permeable to cell membranes (393). Further, in addition to the well-established inhibitors of Class I and II (Zn-dependent) HDACs, inhibitors to Class III (NAD-dependent) deacetylases are now in development (394, 395). As histone methyltransferases, such as EZH2 and SMYD3, are overexpressed in many cancers (102, 103), these enzymes also represent inviting targets for inhibition. Indeed, one biotechnology company, Chroma Therapeutics (Abingdon, UK, chromatherapeutics.com), is currently developing and patenting several promising HMT inhibitors. Another epigenetic target could be methylcytosine-binding proteins (MBDs), also demonstrated to play a role in tumor suppressor silencing (354). In one study, antisense inhibitors of the methyl binding protein MBD2 suppressed tumor growth in both lung cancer lines and xenografts;

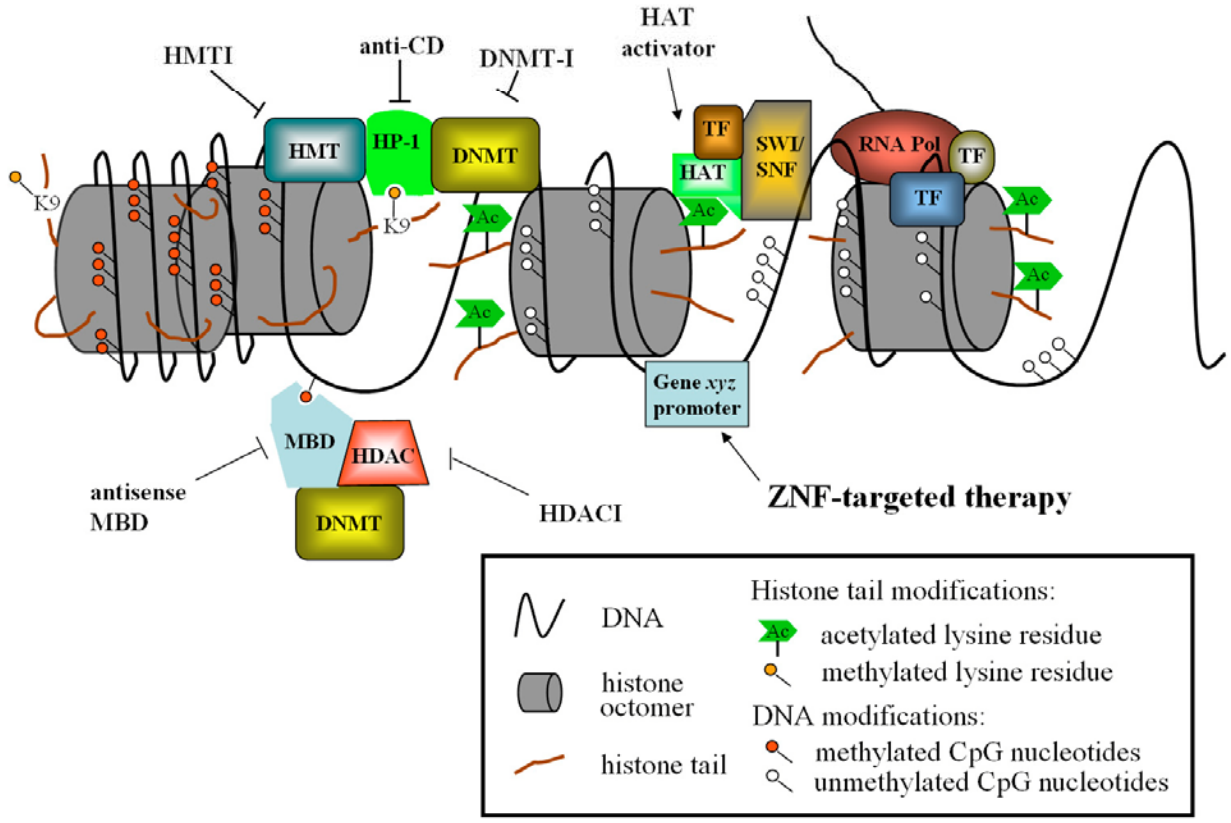

Figure 5. Possible points of epigenetic intervention in active and repressive chromatin. Antisense MBD antisense RNA against mRNA transcript for methyl-binding protein; HDACI = histone deacetylase (HDAC) inhibitor; anti-CD = antibody to chromodomain, preventing binding of repressive heterochromatin protein-1 (HP-1); MI = methylation inhibitor, repressing the activity of DNA methyltransferase (DNMT); HMTI = inhibitor of histone methyltransferase (HMT); a HAT activator increases the activity of histone acetyltransferase (HAT) enzymes; ZNF-targeted therapy = sequence-specifically-designed zinc finger fused to activating or repressive epigenetic modifiers. Other abbreviations: TF = transcription factor; RNAPol = RNA polymerase II; SWI/SNF = ATP-dependent nucleosome remodeling complex.

these inhibitors were not significantly toxic to normal cells (396). Such specific effects might be due to the finding of MBD assistance in the recruitment of repressive complexes to specific genes (354, 397). Recently, it was shown that *chromodomain* antibodies, which target methylated lysine 9 of histone H3, could inhibit binding of the repressive protein HP-1, resulting in apoptosis of cultured fibroblasts (398). The specific targeting of such inhibitory molecules to tumor cells would represent a powerful anti-cancer therapy. Another intriguing strategy is the use of liposomes to entrap and deliver epigenetic agents, allowing increased effective concentrations, sustained release, protection from clearance, and potentially, tumor-specific delivery (157).

The feasibility of targeted epigenetic therapies has also been demonstrated in several studies. One such targeted therapy is double stranded RNA (siRNA). As siRNA has been shown to induce DNA methylation in plants (399), it is likely possible to also perform targeted, gene-specific methylation in mammals. Indeed, siRNA against CpG island sequences mediated gene-specific, targeted methylation of the genes *E-cadherin* and *erbB* in normal human mammary and breast cancer cells (400). Zinc-finger proteins have also been demonstrated to specifically target distinct sequences; these proteins could thus be used as fusion partners for enzymes/inhibitors of epigenetic processes. For example, a Zn-finger protein was used to target the histone methyltransferases G9A and SUV39H1 to specific genes, including *MDR1*, *erythropoietin*, *erbB2*/*B3*, *VEGF*, and *PPAR-gamma*, resulting in directed epigenetic silencing (401). Similarly, it was found that zinc-finger proteins could target a DNA methylase (*Sss1*) to specific sequences in yeast, demonstrating the feasibility of gene-specific silencing by DNA methylation (402). Based on the latter two studies, one would predict that the opposite effects, targeted histone or DNA methylation *inhibition*, would also be feasible using zinc finger strategies.

In summary, epigenetic therapies hold promise for the treatment of a wide variety of tumors, including those that acquire drug resistance. Consolidation strategies combining histone deacetylase and methyltransferase inhibitors appear to be ideal to achieve maximal re-expression of silenced tumor suppressors. Such inhibitors, when combined with conventional chemotherapeutics, may

represent a powerful weapon for the management of both early and late stage cancers.

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Abbreviations: CHAP: cyclic hydroxamic-containing peptide; ChIP: chromatin immunoprecipitation; DNMT: DNA methyltransferase; ER: estrogen receptor; FGS: feature gene selection; HAT: histone acetyltransferase; HDAC: histone deacetylase; HDACI: histone deacetylase inhibitor; hMLH1: human mut L homolog 1; LOH: loss of heterozygosity; LOI: loss of imprinting; MBD:

methylcytosine-binding protein; MI: methylation inhibitor; MSO: methylation-specific oligonucleotide; MSP: methylation-specific PCR; RLGS: restriction landmark genome scanning; ROS: reactive oxygen species; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; TSG: tumor suppressor gene; TSA: Trichostatin A.

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Send correspondence to: Kenneth P. Nephew, Ph.D., 302 Jordan Hall, Medical Sciences Program, 1001 East Third Street, Bloomington, IN, 47405, Tel: 812-855-9445, Fax: 812-855-4436, E-mail: knephew@indiana.edu

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