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Epigenetic Drugs as Pleiotropic Agents in Cancer Treatment: Biomolecular Aspects and Clinical Applications

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In the last three decades huge efforts have been made to characterize genetic defects responsible for cancer development and progression, leading to the comprehensive identification of distinct cellular pathways affected by the alteration of specific genes. Despite the undoubtable role of genetic mechanisms in triggering neoplastic cell transformation, epigenetic modifications (i.e., heritable changes of gene expression that do not derive from alterations of the nucleotide sequence of DNA) are rapidly emerging as frequent alterations that often occur in the early phases of tumorigenesis and that play an important role in tumor development and progression. Epigenetic alterations, such as modifications in DNA methylation patterns and post-translational modifications of histone tails, behave extremely different from genetic modifications, being readily revertable by "epigenetic drugs" such as inhibitors of DNA methyl transferases and inhibitors of histone deacetylases. Since epigenetic alterations in cancer cells affect virtually all cellular pathways that have been associated to tumorigenesis, it is not surprising that epigenetic drugs display pleiotropic activities, being able to concomitantly restore the defective expression of genes involved in cell cycle control, apoptosis, cell signaling, tumor cell invasion and metastasis, angiogenesis and immune recognition. Prompted by this emerging clinical relevance of epigenetic drugs, this review will focus on the large amount of available data, deriving both from in vitro experimentations and in vivo pre-clinical and clinical studies, which clearly indicate epigenetic drugs as effective modifiers of cancer phenotype and as positive regulators of tumor cell biology with a relevant therapeutic potential in cancer patients.
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In the last decades different genetic mechanisms involved in cancer development and progression have been elucidated, leading to the identification of the "classical players" of tumorigenesis: activated oncogenes and inactivated tumor suppressor genes, as well as to the definition of the various cellular pathways affected. More recently, this context is being enriched and integrated by the recognition of epigenetic modifications as additional powerful players in human carcinogenesis, that frequently affect cellular pathways identical to those that are targeted by genetic alterations. Epigenetics refers to heritable changes of gene expression that do not derive from alterations of the nucleotide sequence of DNA, and DNA methylation and histone post-translational modifications represent the most widely characterized epigenetic modifications so far identified in mammals (Strahl and Allis, 2000; Klose and Bird, 2006).

DNA methylation is mediated by DNA methyltransferases (DNMT) and occurs at the C5-position of the cytosine in the context of CpG dinucleotides. To date 4 human DNMT have been described: DNMT1 preferentially methylates hemimethylated DNA and seems to be mainly responsible for the maintenance of DNA methylation patterns (maintenance DNMT); DNMT3a and 3b do not show preference for hemimethylated DNA and are thus being implicated in the generation of new methylation patterns (de novo DNMT); DNMT2 which is homologous to the other DNMT but shows only limited methyltransferase activity (Hermann et al., 2004). An additional member of the DNMT3 family, the DNMT3-like (DNMT3L) protein, lacks DNMT activity but is required for the methylation of imprinted genes in germ cells, and interacts with DNMT3a and 3b stimulating their de novo methyltransferase activity (Kaneda et al., 2004; Chen et al., 2005). DNA methylation inhibits gene expression either by directly blocking the binding of transcriptional activators to the target DNA, or by binding methyl-CpG-binding proteins (MBP) that silence gene expression by recruiting chromatin remodeling

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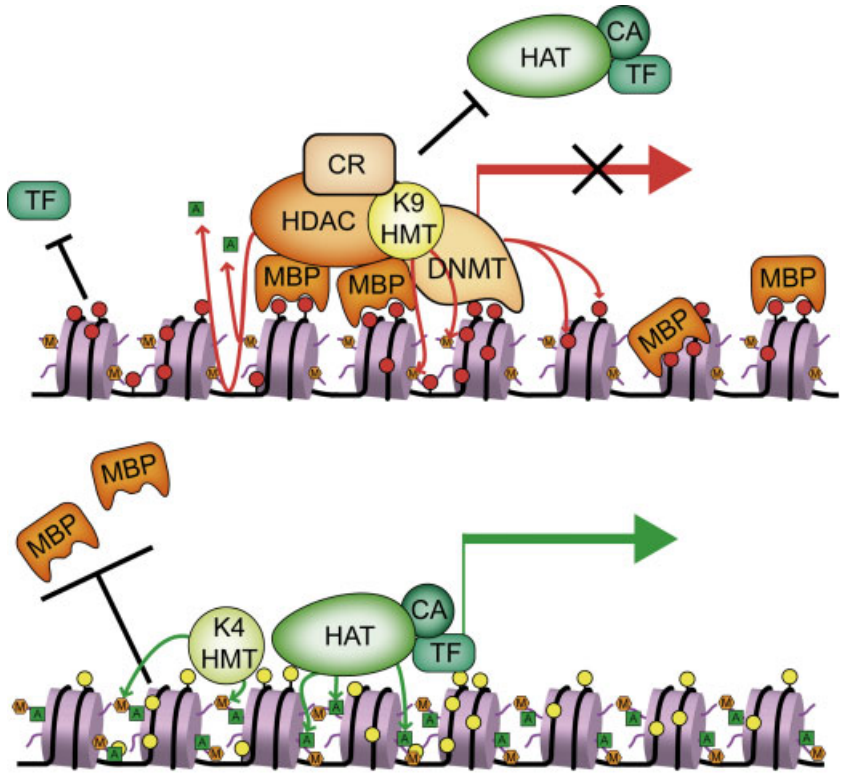

Fig. 1. Epigenetic regulation of gene transcription. Transcriptionally inactive chromatin (upper panel) is characterized by the presence of methylated cytosines within CpG dinucleotides (red circles), which is carried out and sustained by DNA methyl transferases (DNMT). Inhibition of transcription (crossed red arrow) may directly derive from methylated recognition sequence preventing the binding of transcription factors (TF) or may be a consequence of the binding of methyl-CpG-binding proteins (MBP) which are part of multiproteic complexes exhibiting transcriptional repressor activities (CR, co-repressor). The final effect of DNA methylation is the generation of a repressive chromatin status characterized by deacetylation of core histone proteins and methylation of H3 lysine 9 (M, orange hexagon), which are carried out, respectively, by histone deacetylases (HDAC) and histone methyl transferases (HMT) incorporated in the multiproteic repressor complex. On the other hand, demethylated promoters (yellow circles) prevent the binding of MBP and repressor complexes, and are occupied by complexes including TF and co-activators of transcription (CA). The presence within these activation complexes of histone acetyl transferases (HAT), which mediate acetylation of histones (A, green squares), and HMT, which methylate H3 at lysine 4 (M, light green hexagon), finally results in a transcriptionally active state of chromatin (green arrow). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

co-repressor complexes (Fig. 1) (Klose and Bird, 2006). The effect of DNA methylation on gene expression is finally sustained by modifications in the chromatin structure that are mediated by the ability of MBP and DNMT to bind histone deacetylase (HDAC) and histone methyltransferase (HMT) complexes (Klose and Bird, 2006).

Post-translational modifications of core histone proteins have been closely linked to the transcriptional status of chromatin, and the better characterized modifications are represented by histone tails acetylation and methylation. The acetylation status of histones is controlled by the balanced action of histone acetyltransferases (HAT) and HDAC, and acetylated histones have constantly been associated with transcriptionally active chromatin. On the other hand, histone methylation, accomplished by HMT, exerts different effects on gene expression depending on the target residue. In fact, while histone H3 lysine 9 (H3-K9) methylation marks transcriptionally inactive chromatin, methylation of H3-K4 is associated with transcriptionally active chromatin (Fig. 1) (Cheung and Lau, 2005). Rather than acting separately, the different epigenetic modifications so far identified clearly appear as different players of the same team that mutually cooperate and interact in establishing and maintaining gene expression patterns (Razin, 1998; Hashimshony et al., 2003).

The constrains posed by epigenetic marks to gene expression are so powerful and reliable that are utilized in the normal embryonal development to establish cell- and tissue-specific programs of gene expression (Morgan et al., 2005). Therefore, it is not surprising that cancer cells often undergo aberrant epigenetic reprogramming to acquire selective advantages. Gametogenesis and embryonal development also teach another fundamental characteristic of epigenetic marks: despite being fixed and heritable in differentiated cells, they can also be plastic and reversible during gametogenesis and embryonal development, being subjected to extensive modifications that include erasure of pre-existing marks and establishment of new ones (Morgan et al., 2005). This inherent characteristic of epigenetic alterations, and the need of specific enzymatic activities to propagate the epigenetic marks to the cell progeny, renders epigenetic alterations extremely different from genetic abnormalities, being potentially reversible through the use of specific pharmacologic inhibitors of DNMT and HDAC.

Epigenetic Abnormalities in Cancer

Cancer cells concomitantly display different, and apparently contradictory, epigenetic modifications, as observed from the co-existence of global genomic DNA hypomethylation and gene-specific promoter hypermethylation (Weber et al., 2005).

Genomic DNA hypomethylation has been the first epigenetic modification described in neoplastic cells (Feinberg and Vogelstein, 1983; Gama-Sosa et al., 1983) and it can influence the biology of cancer cells in different ways. Its role in gene activation has been linked to the aberrant expression of genes involved in different processes. Among these are: (i) the re-expression of tumor antigens belonging to the Cancer Testis Antigens family (De Smet et al., 1996, 1999; Sigalotti et al., 2002); (ii) the expression of the putative oncogene γ-synuclein (Gupta et al., 2003); (iii) the increased expression of the metastasis-associated genes S100A4 (Nakamura and Takenaga, 1998) and urokinase-type plasminogen activator (uPA) (Pakneshan et al., 2003). Besides these gene-specific effects, the global genomic DNA hypomethylation observed in cancer cells is primarily due to the loss of methylation in repetitive and parasitic elements of the genome (Yoder et al., 1997), which has been linked to the chromosomal instability commonly associated to cancer (Widschwendter et al., 2004; Deng et al., 2006).

Despite the relatively limited insights available on the role of DNA hypomethylation in cancer development and progression, many studies have addressed the role of promoter hypermethylation in the aberrant suppression of gene expression in cancer cells (Baylin, 2005). Aberrant promoter methylation affects almost every cellular function that has been recognized to provide cancer cells with a selective growth/invasive potential; among these are genes involved in cell cycle regulation, DNA repair, cell signaling, apoptosis, angiogenesis, tumor cell invasion and adhesion (Table 1) (Widschwendter and Jones, 2002). The frequency of these epigenetic abnormalities seems to be at least equal if not higher than that of the respective genetic abnormalities and they appear in an initial stage of tumor transformation (Belinsky et al., 1998; Chan et al., 2002; Lee et al., 2004), being already detectable in the earliest precursor lesions, thus suggesting for their active involvement in cell transformation (Chan et al., 2002). The genomic frequency of aberrant DNA hypermethylation can be itself utilized to segregate tumors into two classes: those displaying a high incidence of CpG island methylation (defined as CpG island methylator phenotype-positive, CIMP+) and those not (CIMP−). Interestingly, CIMP+ and CIMP− tumors are characterized by different clinical and molecular features, and the existence of CIMP has suggested the presence of an underlying molecular defect that leads to aberrant DNA hypermethylation and epigenetic instability in cancer cells (Issa, 2004).

As seen with DNA methylation, histone post-translational modifications are widely altered in tumor cells compared to normal tissues (Fraga et al., 2005). A comprehensive examination of the post-translational modifications of histone H4 identified a global loss of H4-K16 acetylation and of H4-K20 trimethylation, which appears in the early phases of cell transformation and increases with tumor progression (Fraga et al., 2005). These alterations are associated with the hypomethylation of DNA repetitive sequences and are shared by tumor tissues of different histologic origin, representing a common mark of neoplastic transformation (Fraga et al., 2005). The alterations in the post-translational modifications of histones that are observed in cancer cells may be sufficient per se to shut-down gene expression (Richon et al., 2000), or may require the additional contribution of promoter hypermethylation (Cameron et al., 1999).

Along with the above-depicted series of complex and generalized deregulations of the epigenetic marks in cancer cells find place the alterations of the expression of imprinted genes. Genomic imprinting refers to the epigenetic regulation of gene expression that results in the expression of the gene from only one of the two parental chromosomes (Reik and Walter, 2001). Loss of imprinting (LOI) is frequently observed in cancer and may affect both growth-promoting genes, which became up-regulated being expressed from both alleles, and growth-inhibitory genes, which are shut-down through the

Table 1. Selected genes silenced by aberrant promoter methylation in cancer cells

Function	Gene[a]	Reference
Cell-cycle regulation	CDKN2A/p14^{ARF}	Robertson and Jones (1998)
	CDKN2B/p15^{INK4B}	Herman et al. (1996)
	RB1, RB2	Stirzaker et al. (1997), Tosi et al. (2005)
	FHIT	Tanaka et al. (1998)
	p73	Corn et al. (1999)
	14-3-3σ	Ferguson et al. (2000)
DNA repair/detoxification	MLH1	Herman et al. (1998)
	MGMT	Esteller et al. (1999)
	BRCA1	Dobrovic and Simpfendorfer (1997)
	GSTP1	Lee et al. (1994), Singal et al. (2001)
Apoptosis	DAPK1	Katzenellenbogen et al. (1999)
	RASSF1A	Dammann et al. (2000)
	XAF1	Byun et al. (2003)
	Caspase-8	Teitz et al. (2000)
	APAF-1	Soengas et al. (2001), Furukawa et al. (2005)
	TMS1	Levine et al. (2003)
	BIK	Kim et al. (2006)
Cell signaling	AR, ER, PR	Ferguson et al. (1995), Jarrard et al. (1998), Sasaki et al. (2001)
	CRBP1, RARβ, TIG1	Cote et al. (1998), Esteller et al. (2002), Youssef et al. (2004a)
	SOCS-1, SHP-1	Zhang et al. (2000), Galm et al. (2003)
	SFRPs	Suzuki et al. (2002)
	APC	Esteller et al. (2000b)
	IGFBP3	Hanafusa et al. (2002)
Tumor-cell invasion, metastasis and angiogenesis	CDH1, 13	Graff et al. (1995), Toyooka et al. (2001)
	TIMP-2, -3	Bachman et al. (1999), Ivanova et al. (2004)
	TFPI-2	Sato et al. (2005)
	VHL	Herman et al. (1994)
	THBS1	Li et al. (1999)
Immune recognition	HLA class I antigens	Nie et al. (2001)
	CIITA	Satoh et al. (2004)

[a]APAF-1, apoptotic protease activating factor 1; APC, adenomatosis polyposis coli; BRCA1, breast cancer 1; BIK, BCL2-interacting killer; CDKN2A, cyclin-dependent kinase inhibitor 2A; CDKN2B, cyclin-dependent kinase inhibitor 2B; DAPK1, death-associated protein kinase 1; FHIT, fragile histidine triad gene, GSTP1, glutathione *S*-transferase P 1; HLA, human leukocyte antigens; MGMT, *O*-6-methylguanine-DNA methyltransferase; MLH1, mutL homolog 1; RASSF1A, Ras association domain family 1; RB1, retinoblastoma 1; RB2, retinoblastoma 2; THBS1, thrombospondin 1; TMS1, target of methylation-induced silencing-1; XAF1, XIAP associated factor-1.

silencing of the sole normally active allele. LOI at the IGF2/H19 locus is the best characterized and more diffuse LOI event recognized in cancer. IGF2/H19 locus encodes the IGF2 growth factor, which is normally expressed from the paternal allele, and the H19 non-coding RNA, which has growth-suppressive properties and is normally expressed from the maternal allele. LOI at IGF2/H19 locus is the most common alteration observed in Wilms' tumor, affecting 50–70% of the lesions, and is associated with methylation of H19 gene. This alteration silences H19 gene and allows bi-allelic expression of the reciprocally imprinted IGF2 gene, whose up-regulation is thought to be responsible for the associated susceptibility to neoplastic transformation (Feinberg et al., 2002; Feinberg and Tycko, 2004). Examples of growth-inhibitory genes undergoing LOI in cancer are represented by the cyclin-dependent kinase inhibitor 1C (CDKN1C, $p57^{kip2}$) that is down-regulated in 10% of Wilms' tumors (Thompson et al., 1996), and by the RAS-related tumor suppressor gene ARHI that is down-regulated through methylation in 15–20% of breast cancers (Yuan et al., 2003).

Epigenetic Drugs

Epigenetic defects in cancer cells can be efficiently reverted by means of pharmacologic inhibitors of the enzymes that are responsible for establishing/maintaining the epigenetic marks (Yoo and Jones, 2006).

Aberrant promoter hypermethylation can be successfully targeted by inhibitors of DNMT, which can be divided into nucleoside analogues and non-nucleoside analogues.

The first, most widely utilized and more powerful DNMT inhibitors are represented by nucleoside analogues of cytidine in which the cytosine ring has been modified to give them DNMT inhibitory activity; they include 5-azacytidine, 5-aza-2′-deoxycytidine (5-AZA-CdR), 5-fluoro-2′-deoxycytidine, 5,6-dihydro-5-azacytidine and zebularine (Yoo and Jones, 2006). Once taken up by the cell, these nucleosides are converted by kinases to nucleotides that are then incorporated, directly (deoxyribonucleosides) or following ribose reduction (ribonucleosides), into the DNA during the S phase of the cell cycle (Momparler, 2005). The cytosine analogues incorporated into the DNA behave as optimal substrates for the DNMT during the S phase; however, the presence of a modified cytosine ring leads to the formation of a stable covalent bond between the enzyme and the ring, which ends up in the irreversible inactivation of the DNMT. The resulting cellular depletion of DNMT activity eventually leads to the synthesis of hypomethylated DNA (Zhou et al., 2002; Momparler, 2005).

Non-nucleoside inhibitors of DNMT are characterized for exerting their activity without being incorporated into the DNA. They may function by disturbing the interaction between the DNMT and its target sites, as proposed for procaine (Villar-Garea et al., 2003), or by directly blocking the catalytic site of DNMT1, as proposed for the main polyphenol compound of the green tea (−)-epigallocatechin-3-gallate (Fang et al., 2003), for hydralazine (Arce et al., 2006) and for the synthetic compound RG108 (Brueckner et al., 2005). Antisense oligonucleotides (i.e., MG98) have also been successfully utilized to induce hypomethylation and gene re-expression in cancer cells by interfering with DNMT1 mRNA translation and by causing mRNA degradation (Goffin and Eisenhauer, 2002). A recent work has compared the activity of different nucleoside and non-nucleoside inhibitors of DNMT revealing a functional diversity among them. Azanucleoside analogues, particularly 5-AZA-CdR, displayed the strongest demethylating activity and were the only able to cause promotorial demethylation and re-expression of the tissue inhibitor of metalloproteinases (TIMP)-3 tumor suppressor gene (Stresemann et al., 2006). In view of these results, recent efforts are focusing on the three-dimensional modeling of DNMT catalytic pocket to obtain new specific inhibitors of DNMT, which may prove as effective as 5-AZA-CdR in inducing DNA hypomethylation and may be characterized by a lower toxicity (Siedlecki et al., 2006).

Inhibitors of HDAC (HDACi) can be divided into short chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides, most of which seem to act by blocking the HDAC catalytic site containing a Zn^{2+} ion (Vannini et al., 2004). HDAC inhibition provokes an accumulation of acetylated histones that became incorporated into the nucleosomes and may lead to the reversal of the aberrant epigenetic patterns observed in cancer cells. Since the action of HDACi is not restricted to histones, but also causes hyperacetylation of non-histone cellular proteins, the effects of HDACi may be mediated, at least in part, by mechanisms different from direct chromatin remodeling (Terui et al., 2003; Luo et al., 2004; Lin et al., 2006; Yoo and Jones, 2006).

Short chain fatty acids, comprising butyrate and valproic acid (VPA), have been the first compounds for which a HDAC inhibitory activity has been established; however, they are not specific and require elevated drug concentrations to achieve HDAC inhibition (Candido et al., 1978; Yoo and Jones, 2006). On the other hand, hydroxamic acid-based HDACi, such as the *Streptomyces*-derived Tricostatin A (TSA), and the synthetic suberoylanilide hydroxamic acid (SAHA), pyroxamide, PXD-101, LBH589 and NVP-LAQ824, are highly effective HDACi that are active at concentrations ranging from nM to μM (Yoo and Jones, 2006). A potency comparable to that of hydroxamic acid-compounds has been reported for cyclic tetrapeptides (e.g., apicidin, depsipeptide, trapoxin), which represent a rapidly expanding class of HDACi, growing by the addition of a series of new tetrapeptide analogues carrying functional groups targeting the Zn^{2+} ion in the catalytic site of HDAC (Yoo and Jones, 2006). Benzidamides, such as MS-275 and CI-994, are synthetic compounds with efficient HDAC inhibitory activity, which, at least for MS-275, is mediated by the targeting of the Zn^{2+} ion in the catalytic pocket of the enzyme. This class of HDACi is particularly attractive since they retain HDACi activity when administered orally (Yoo and Jones, 2006).

Pharmacologic Reversal of Epigenetic Abnormalities

As described above, epigenetic abnormalities in cancer affect a plethora of genes involved in different and fundamental cellular pathways including cell cycle control, apoptosis, immune recognition, angiogenesis and tumor cell invasion and metastasis. Consistent with the functional diversification of epigenetic alterations, epigenetic drugs are characterized by pleiotropic effects: their activity concomitantly affects different aspects of neoplastic cell and tumor biology, leading to an overall impairment of the neoplastic potential of cells that constitutes the rationale for their current or proposed use, alone or in combination therapies, as anticancer agents.

Cell cycle control

Different studies have demonstrated the efficacy of epigenetic drugs in the reactivation of genes that inhibit cell cycle progression. A clear example of the potential of epigenetic drugs in restoring a "physiologic" cell cycle control has been provided by CDKN2A/$p16^{INK4A}$, a cyclin-dependent kinase (cdk) inhibitor that is frequently down-regulated in cancer by promoter hypermethylation, and that physiologically inhibits cell cycle progression by preventing cdk activation and pRb phosphorylation, resulting in G1 growth-arrest (Auerkari, 2006). In line with its control by an altered epigenetic information, 5-AZA-CdR proved to be highly effective in re-establishing CDKN2A/$p16^{INK4A}$ expression in different cancer cell lines by inducing demethylation of its promoter. The re-gained CDKN2A/$p16^{INK4A}$ expression restored a functional

control of the cell cycle, leading to growth inhibition and to the expected G1 phase arrest (Merlo et al., 1995; Bender et al., 1998). Pharmacologic reactivation has been observed for other inhibitors of cyclin-dependent kinases such as CDKN2B/p15^{INK4B} (Herman et al., 1996) and CDKN1C/p57^{KIP2} (Shin et al., 2000) by using DNA hypomethylating agents (DHA), and/or HDACi as seen for p21^{WAF1} (Gui et al., 2004). Furthermore, a concomitant up-regulation of 21^{WAF1}, p27 and p53, and down-regulation of cyclin D1 and D2 was obtained in lymphoid cancer cell lines by treatment with the HDACi SAHA, which ended up in G1 or G2-M arrest and apoptosis (Sakajiri et al., 2005).
The effect of epigenetic drugs on cdk inhibitors may not be solely related to a direct effect on their promoters. In fact, besides promoter methylation correlates with p21^{WAF1} down-regulation in acute lymphoblastic leukaemia (ALL) (Roman-Gomez et al., 2002), and extensive histone acetylation associated to p21^{WAF1} promoter has been observed upon treatment with SAHA (Sakajiri et al., 2005), re-expression of p21^{WAF1} by DHA has been reported in acute myelogenous leukemia (AML) cells irrespectively to its promoter methylation, and it has been suggested to derive from re-activation of methylation-inactivated p73, an up-stream regulator of p21^{WAF1} (Schmelz et al., 2005; Tamm et al., 2005). Recent studies indicated that 5-AZA-CdR-induction of p21^{WAF1} may require p53 activation, which is triggered by a DNA damage caused by 5-AZA-CdR incorporation and not by the hypomethylating activity of the drug (Karpf et al., 2001; Zhu et al., 2004; Pulukuri and Rao, 2005). Nevertheless, 5-AZA-CdR may also induce p53 by relieving the silencing of methylated p14^{ARF} gene, which results in MDM2 nuclear localization and subsequent stabilization of p53 (Robertson and Jones, 1998; Esteller et al., 2001). HCACi may exert their effects even by inducing hyperacetylation of p53, event that has been proposed to play an important role in mediating HDACi-mediated p21^{WAF1} expression (Zhao et al., 2006).
Comprehensively, cell cycle regulators including the pRb (pRb/p16^{INK4a}/cyclin D1) and p53 (p14^{ARF}/*mdm2*/p53) *pathways*, are profoundly affected by epigenetic drugs both *in vitro* and *in vivo* (Bender et al., 1998; Butler et al., 2001; Cheng et al., 2003), with frequently observed synergistic activity of DHA/HDACi combinations (Cameron et al., 1999). The action of epigenetic drugs may either be directed to the promoters of cell-cycle regulating genes or may involve alternative mechanisms, such as induction of a DNA damage response, which ultimately lead to cell cycle arrest or apoptosis (Fig. 2). Support to an hypomethylation-independent mechanism for 5-AZA-CdR cytotoxicity derives from the observation that cells expressing high levels of DNMT are more susceptible to 5-AZA-CdR-induced cytotoxicity, which may be mediated by covalent trapping of DNMT into 5-AZA-CdR-modified DNA (Juttermann et al., 1994; Oka et al., 2005). Whether 5-AZA-CdR cytotoxic effects are mainly due to gene reactivation or to trapping of DNMT into 5-AZA-CdR-modified genomic DNA still remain an unsolved issue. Both mechanisms seem to play an important role and the prevalence of one or the other may be related to the target neoplastic cell (Ferguson et al., 1997).

DNA repair

Epigenetic inactivation frequently affects DNA repair genes and is thought to provide neoplastic cells with an increased genetic instability (Esteller, 2000). Inactivation of the DNA mismatch repair gene MLH1 by promoter hypermethylation has been frequently observed in different human cancers and it has been associated to microsatellite instability (Esteller et al., 1998; Herman et al., 1998). In neoplastic cells, restoration of MLH1 expression was effectively achieved by 5-AZA-CdR-treatment, alone or in combination with HDACi, and resulted in the reconstitution of the mismatch repair function (Herman et al., 1998; Cameron et al., 1999). Administration of non-toxic doses of 5-AZA-CdR in tumor-bearing mice was also demonstrated to restore MLH1 expression in ovarian and colon tumor xenografts, which were consequently sensitized to the cytotoxic activity of different chemotherapeutic drugs (Plumb et al., 2000). MGMT is another example of a DNA repair gene that is frequently inactivated by promoter methylation in human cancers (Esteller et al., 1999). Consistent with its role in protecting the genome from G to A transitions induced by alkylating agents, MGMT inactivation by promoter hypermethylation has been associated to G to A mutations in k-ras and p53 genes in colorectal cancer (Esteller and Herman, 2004). Despite DHA, alone or in combination with HDACi, have proven effective in re-expressing MGMT in cancer cells, the clinical advantage of the restored MGMT expression is doubtful (Bae et al., 2002; Danam et al., 2005). In fact, MGMT-methylated tumors, in view of their impaired ability in repairing drug-induced O^6-alkyl-guanine adducts, appear significantly more susceptible to the cytotoxic effects of alkylating drugs such as 1,3-bis(2-chloroethyl)-1-nitrosourea and temozolomide (Esteller and Herman, 2004). Along the line of restoring DNA repair cellular capabilities lies the ability of epigenetic drugs to re-establish the expression of BRCA1, which was found to be inactivated by promoter methylation in sporadic breast and ovarian cancers, and of its effector GADD45 that participates in the growth arrest triggered by DNA damage (Esteller et al., 2000a; Wang et al., 2005; Wei et al., 2005).

Apoptosis

Epigenetic drugs sensitize neoplastic cells to apoptosis either by restoring the defective expression of apoptosis effector proteins, or by re-establishing the expression of signal transducing/mediators of the apoptotic signal both pertaining to the mitochondrial and to the death receptor pathways. The first evidence for a role of epigenetic drugs in the regulation of genes directly involved in the apoptotic pathways derived from the demonstration that 5-AZA-CdR was able to restore the expression of DAPK1 in selected bladder carcinoma and B-cell lines (Kissil et al., 1997), and that demethylation-induced re-expression of DAPK1 in Raji Burkitt's lymphoma cell line restored the susceptibility of the neoplastic cells to IFN-γ-triggered apoptosis (Katzenellenbogen et al., 1999). Similarly, 5-AZA-CdR-treatment sensitized NSCLC cells to TRAIL-induced apoptosis by inducing DAPK1 expression (Tang et al., 2004). Besides DAPK1, 5-AZA-CdR and DNMT1 antisense oligonucleotides are able to restore the sensitivity of cancer cells to IFN-triggered apoptosis even through the re-expression of the pro-apoptotic gene RASSF1A and of the prototypic apoptosis-associated IFN response gene XAF1, which are frequently silenced by epigenetic mechanisms in different neoplasms (Reu et al., 2006a, 2006b). Caspases themselves are not spared from epigenetic inactivation during neoplastic transformation: hypermethylation at caspase-8 promoter leads to its reduced or absent expression in neoplastic cells from different tumor types, and is responsible for their resistance to death receptor- and drug-induced apoptosis. However, treatment with 5-AZA-CdR has proven to be effective in re-establishing caspase-8 expression in cancer cells, restoring their sensitivity to TRAIL-, anti-FAS-, and drug-triggered apoptosis (Hopkins-Donaldson et al., 2000; Fulda et al., 2001). An extensive modulation of TRAIL pathway by 5-AZA-CdR has been observed in glioblastoma cells, in which the drug concomitantly up-regulated TRAIL receptor-1 and caspase-8, and down-regulated the death receptor inhibitor PED/PEA-15. The restored sensitivity to TRAIL-induced apoptosis, mainly mediated by caspase-8 re-expression, was proposed as the underlying mechanism responsible for the

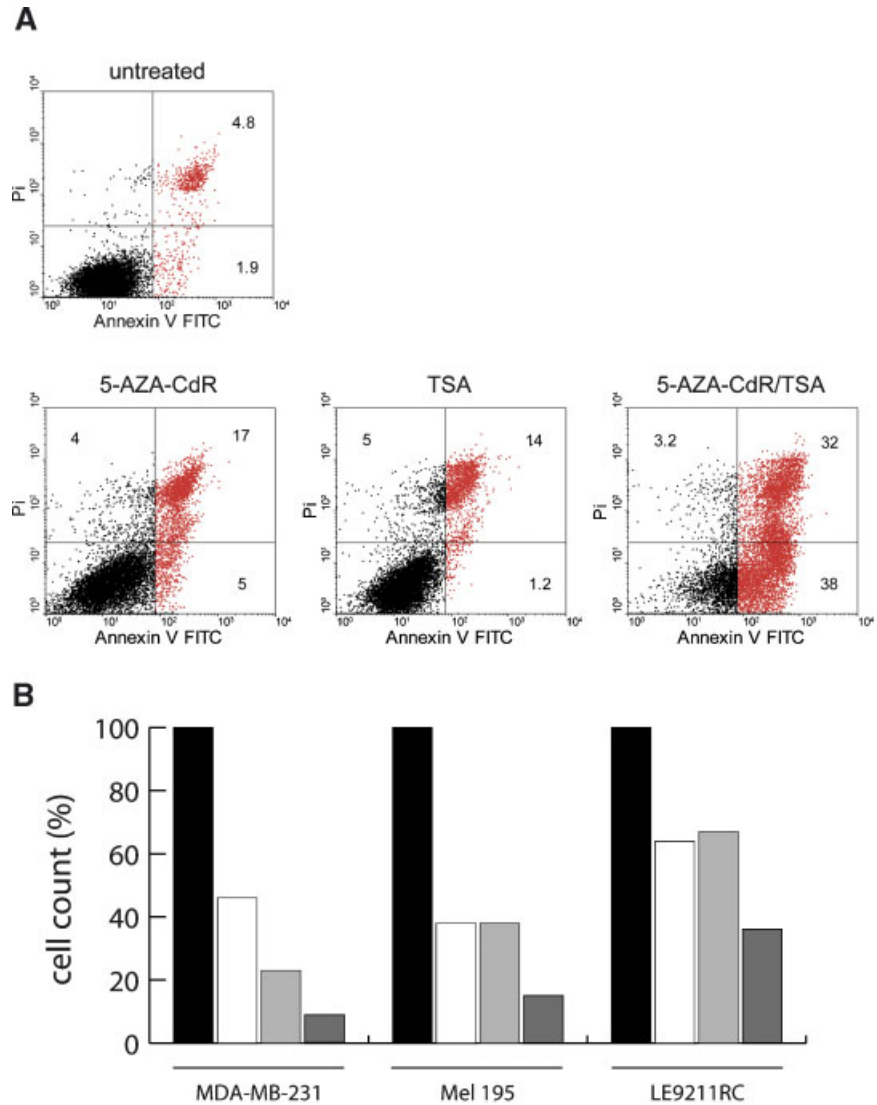

Fig. 2. Pro-apoptotic and antiproliferative activity of epigenetic drugs in cancer cells. Panel A: apoptosis versus necrosis assay following treatment with 5-AZA-CdR and/or TSA. MDA-MB-231 breast carcinoma cells were incubated for 24 h with 1 μM 5-AZA-CdR and then with or without 400 nM TSA for additional 24 h. Viable and dead cells were evaluated after the end of treatment by double staining with FITC-annexin-V and propidium iodide (PI) (BD Pharmingen) following the manifacturer's instructions. Analysis was performed by flow cytometry, with calculation of both the percentage of cells in early apoptosis (annexin V+/Pi−, lower right quadrant) and late apoptosis (annexin V+/Pi+, upper right quadrant) or necrosis (annexin V−/Pi+, upper left quadrant). Panel B: antiproliferative activity of 5-AZA-CdR and/or TSA treatment on neoplastic cells. MDA-MB-231 breast carcinoma cells, Mel 195 metastatic cutaneous melanoma cells and LE9211RC renal carcinoma cells were incubated for 24 h with 1 μM 5-AZA-CdR and then with or without 400 nM TSA for additional 24 h. The number of viable cells was assessed by trypan blue dye exclusion 2 days after the end of treatment, and cell counts of 5-AZA-CdR-treated (empty squares), TSA-treated (light-gray squares), and 5-AZA-CDR + TSA-treated (dark-gray squares) cells have been reported as a percentage of the count of untreated cells (black squares). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

observed in vivo synergism of 5-AZA-CdR and TRAIL combinations in apoptosis induction, caspase activation and reduction of the tumor mass in glioblastoma xenografts (Eramo et al., 2005). On the other hand HDACi demonstrated their activity in up-regulating death receptor 5 specifically in cancer cells, sensitizing them to TRAIL-triggered apoptosis (Nakata et al., 2004), and in inducing TNF-α expression through hyperacetylation of its promoter in myeloid leukemia cells, initiating an autocrine TNF-α loop which triggers programmed cell death (Sutheesophon et al., 2005). Lastly, 5-AZA-CdR-mediated re-expression of the cell-death effector APAF-1 was demonstrated to sensitize melanoma cells to chemotherapeutic drugs by rescuing the p53-dependent apoptosis pathway (Soengas et al., 2001).

As evidenced above, the large amount of data reporting direct effects of DHA in the re-expression of genes involved in apoptosis faces the limited data available on the direct role of HDACi-induced histone hyperacetylation on promoter activity and gene expression of apoptosis-related genes. Along this line, recent studies proposed that triggering of apoptosis by HDACi, besides relying on re-expression of epigenetically inactivated genes, may arise from hyperacethylation of heterochromatic regions (e.g., centromeres), which leads to aberrant mitoses and subsequent activation of programmed cell death programs (Johnstone and Licht, 2003). Irrespective of what is the triggering mechanism, the induction of apoptosis still represents a common effect in neoplastic cells treated with epigenetic drugs, and the combined treatment with DHA and

HDACi shows a potent synergistic effect on programmed cell death, suggesting that the two drugs act through complementary mechanisms (Fig. 2) (Zhu et al., 2001).

Cell signaling

Epigenetic inactivation of genes involved in cell signaling is frequently observed in cancer cells and it may result in their unresponsiveness to growth inhibitory signals and to therapeutic growth factor antagonists, or may generate an aberrantly sustained signaling caused by the down-regulation of signaling inhibitors.

Steroid hormone signaling pathways appear to be particularly targeted by epigenetic inactivation that acts mainly by shutting-down the transcription of hormone receptor genes, thus rendering neoplastic cells unresponsive to hormones and to their antagonists. In this scenario, epigenetic drugs consistently proved effective in restoring the expression of different steroid hormone receptors in cancer cells, including estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR) (Ferguson et al., 1995; Jarrard et al., 1998; Sasaki et al., 2001). The combination of DHA and HDACi seems to be particularly effective in restoring the expression of these genes; in fact, a 10-fold and above synergistic increase in ER expression was observed in breast cancer cell lines following combined treatment with 5-AZA-CdR and TSA, as compared to 5-AZA-CdR alone (Yang et al., 2001). Epigenetically induced receptors were readily able to mediate their native functions being able to trigger the transcription of hormone responsive genes, as seen with PSA induction following 5-AZA-CdR-mediated re-expression of AR in prostate cancer cells and with PR induction following re-expression of ER in breast carcinoma cells (Jarrard et al., 1998; Yang et al., 2001). This behavior is particularly intriguing from a clinical perspective since it suggests the possibility to apply highly effective hormonal therapies also to patients with tumors displaying hormone-independent growth. This hypothesis has recently gained support by the demonstration that re-expression of functional ER in ER-negative breast cancer cells by a 5-AZA-CdR/TSA combination results in neoplastic cell response to tamoxifen, which is characterized by a transcriptional repression of estrogen-responsive genes and inhibition of cell growth (Sharma et al., 2006).

Similarly, restoration of retinoid acid receptor-β_2 (RAR-β_2) expression in neoplastic cells of different histotype by 5-AZA-CdR or TSA, alone or in conjunction with all-*trans*-retinoic acid (ATRA), restored their sensitivity to ATRA, resulting in a higher inhibition of cell proliferation, increase in apoptosis and reduced growth of xenograft tumors as compared to each agent alone, suggesting the possible therapeutic use of associations of ATRA with DHA or HDACi in cancer treatment (Sirchia et al., 2002; Youssef et al., 2004b). Epigenetic treatment may also include DHA/HDACi combinations, as suggested by their demonstrated synergistic effect on RARβ induction in breast cancer cells (Bovenzi and Momparler, 2001), and may comprehensively target retinoid acid signaling through the demonstrated ability of 5-AZA-CdR to induce other members of the pathway, including cellular retinol-binding protein 1 (CRBP1) and tazarotene-induced gene 1 (TIG1) (Esteller et al., 2002; Youssef et al., 2004a).

Besides the well-characterized examples above, epigenetic drugs have proven effective in restoring-growth inhibitory signals in cancer cells by acting on many different pathways; among others are: (i) the restoration of the responsiveness of cancer cells to TGF-β following the re-expression of TGF-β receptor I and II (Ammanamanchi et al., 1998; Osada et al., 2001; Ammanamanchi and Brattain, 2004); (ii) the induction of insulin-like growth factor binding protein 1 (IGFBP1) and IGFBP3, which may inhibit the growth-promoting activities of IGF (Hanafusa et al., 2002; Ibanez de Caceres et al., 2006); (iii) the induction of secreted frizzled-related proteins (SFRP), which may inhibit a constitutively activated oncogenic WNT signaling (Suzuki et al., 2002; Suzuki et al., 2004); (iv) the potential abrogation of aberrantly persistent cytokine signaling through the restoration of negative regulators of signaling such as the suppressor of cytokine signaling-1 (SOCS-1) and SHP-1 (Zhang et al., 2000; Galm et al., 2003).

Tumor cell invasion, metastasis and angiogenesis

The contribution of epigenetic drugs in controlling cancer cell invasion and metastasis arises from their concomitant activity on adhesion systems and extracellular matrix modeling. Down-regulation of the cadherin adhesion system has long been associated with the invasive and metastatic potential of cancer cells, and the role of epigenetic alterations in the transcriptional inactivation of cadherins in neoplastic cells of different istotypes is a well-established phenomenon (Graff et al., 1995; Toyooka et al., 2001). Consistently, DHA have proven successful in re-establishing E-cadherin (CDH1) and H-cadherin (CDH13) expression in cancer cells of solid and hemopoietic origin by inducing demethylation at cadherin promoters (Graff et al., 1995; Corn et al., 2000; Toyooka et al., 2001; Nam et al., 2004). 5-AZA-CdR-induced re-expression of E-cadherin in cancer cells correlated with an increased in vitro cell aggregation and reduced cell motility; furthermore, systemic administration of the drug into mice with severe combined immunodeficiency grafted with human breast cancer cells resulted in the suppression of lung metastasis development, which was suggested to be at least in part attributable to the drug-induced re-expression of CDH1 (Nam et al., 2004). Interestingly, a decrease in matrix metalloproteinase (MMP)-2 and MMP-9 activity similar to that obtained by E-cadherin transfection was observed in SKOV3 ovarian cancer cells following 5-AZA-CdR-mediated up-regulation of E-cadherin, suggesting that the inhibition of cell invasion observed following DHA-treatment may also depend on a reduction in MMP activity (Yuecheng et al., 2006). On the other hand, MMP expression can be itself regulated by promoter methylation, and induction of MMP by 5-AZA-CdR was observed in pancreatic cancer and lymphoma cells, where it was associated to an MMP-mediated increase in the invasive potential of cancer cells (Chicoine et al., 2002; Sato et al., 2003). Increased invasiveness following DHA exposure was also observed in selected breast and prostate cancer cells where it was attributed to the induction of uPA, which is a member of the serine protease family that can breakdown various components of the extracellular matrix favoring tumor cell invasion (Xing and Rabbani, 1999; Pakneshan et al., 2003). The picture becomes even more complicated when considering that the TIMP, which antagonize MMP activity and suppress tumor cell invasion and metastasis, are frequently down-regulated in cancer cells by promoter methylation and are efficiently restored by DHA, even displaying a synergistic up-regulation by successive HDACi treatment (Bachman et al., 1999; Cameron et al., 1999; Gagnon et al., 2003; Ivanova et al., 2004; Galm et al., 2005). The same applies to the tissue factor pathway inhibitor-2 (TFPI-2), a wide spectrum inhibitor of proteases that down-modulates the malignant phenotype of cancer cells in different ways, including the inhibition of several MMP, and whose expression is effectively induced by DHA/HDACi combinations (Konduri et al., 2003; Sato et al., 2005; Steiner et al., 2005). HDACi on their own were proven effective in reducing the in vitro migration of cancer cells by concomitantly up-regulating TIMP-1 and TIMP-2 protein level and reducing MMP-2, MMP-9 and membrane type-1/MMP protein level and activity in uveal melanoma cells (Klisovic et al., 2005). In this scenario, the anti-invasive activity of HDACi seems to take a

particular contribution from their efficacy in up-regulating MMP-inhibitors such as RECK, which mediates a reduction in MMP-activity leading to a diminished cell invasion (Liu et al., 2003). Therefore, it is evident that the final effect of epigenetic drugs on the invasive potential of tumor cells results from the sum of the contributions of the different genes affected, and it is dependent on the epigenetic background of cancer cells.
Besides tumor invasion, epigenetic drugs also target angiogenesis, a critical requirement both for tumor growth and metastasis. This finding is consistent with the demonstrated epigenetic inactivation of different factors involved in the angiogenetic pathways. Among these, the *von Hippel-Lindau* (VHL) tumor suppressor gene is frequently inactivated through promoter hypermethylation in clear cell renal carcinoma (ccRCC), and its absence leads to a failing in degradation of hypoxia inducible factor (HIF)-1 whose accumulation produces a constitutive activation of hypoxia response pathways favoring tumor angiogenesis (Herman et al., 1994). This aberrant pathway activation has been successfully fixed by 5-AZA-CdR that, in addition to restoring VHL expression in VHL-methylated ccRCC cell lines, repressed the hypoxia response pathway as demonstrated by the down-regulation of the HIF-1 targets vascular endothelial growth factor (VEGF) and glucose transporter (GLUT)-1 (Alleman et al., 2004). The angiogenesis-inhibitor gene thrombospondin-1 is another target of aberrant epigenetic inactivation that is efficiently re-activated in cancer cells by a direct effect of 5-AZA-CdR (Li et al., 1999).
HDACi also play a profound role in inhibiting tumor angiogenesis by concomitantly up-regulating the anti-angiogenetic factors VHL, activin A, neurofibromin-2 and thrombospondin-1 and by down-regulating the pro-angiogenic factors HIF-1, VEGF, platelet derived growth factor and basic fibroblast growth factor in cancer cells (Liu et al., 2006).

Immunomodulation

Neoplastic cells adopt different strategies to evade host's immune surveillance, leading to tumor outgrow and to a reduced efficacy of immunotherapeutic strategies. Along this line, recent studies pointed to epigenetic alteration as important players in the down-regulation of different molecules involved in the immunological recognition of cancer cells (i.e., HLA class I antigens, co-stimulatory molecules and tumor antigens), concomitantly identifying a potent effect of epigenetic drugs as "positive" modulators of the immune profile and of the immunogenicity of neoplastic cells (Sigalotti et al., 2005).

Among the different tumor associated antigens (TAA) so far identified, Cancer Testis Antigens (CTA) are attracting growing interest as immunotherapeutic targets due to their in vivo immunogenicity, to their shared expression among tumors of different histotype and to their absence in normal tissues except testis and placenta, which makes them very close to be defined as tumor-restricted/specific antigens. Despite these biologic properties, therapeutic vaccination against CTA may be impaired by their constitutive expression in only a limited percentage of neoplastic lesions and by their heterogeneous intratumoral expression (Jungbluth et al., 2000; Maio et al., 2003; Scanlan et al., 2004; Sigalotti et al., 2004). The recent demonstration that promoter methylation is the critical factor responsible for regulating CTA expression in tumor cells, accounting for both the constitutive pattern of CTA expression in neoplastic cells and for their heterogeneous intratumoral expression within specific neoplastic lesions, has suggested the possibility to therapeutically modulate CTA expression in neoplastic cells through epigenetic drugs (De Smet et al., 1999; Sigalotti et al., 2002, 2004). Indeed, 5-AZA-CdR was consistently able to induce or to up-regulate CTA expression in solid and hematopoietic tumors of different histotype allowing their efficient immunological recognition and lysis by CTA-specific cytotoxic T lymphocytes (CTL) (Weber et al., 1994; Coral et al., 2002; Gattei et al., 2005; Sigalotti et al., 2005). 5-AZA-CdR was demonstrated to be effective also in reverting the intratumoral heterogeneous expression of CTA. In fact, exposure to 5-AZA-CdR of single cell clones generated from a primary culture of metastatic melanoma cells was able to homogenize their constitutively heterogeneous expression of the CTA MAGE-A3, allowing their homogeneous recognition by an anti-MAGE-A CTL (Sigalotti et al., 2004).
On the other hand, HDACi, when used alone, show negligible effects on CTA expression in human malignancies, though a recent report showed some minor effect of TSA on MAGE-A genes expression and an up-regulated transcriptional activity of both methylated and unmethylated MAGE-A2 and -A12 promoters following TSA treatment (Wischnewski et al., 2006). Confirming the major role of promoter methylation in determining the levels of CTA expression in neoplastic cells, combined treatment with DHA and HDACi produces a modest synergistic effect, which results in a 2–3 folds increase of NY-ESO-1 and MAGE-A3 CTA mRNA level over DHA treatment alone (Fig. 3) (Weiser et al., 2001a,b; Schrump and Nguyen, 2005). Furthermore, selected neoplastic cells result completely refractory to such synergistic modulation, suggesting that it is far from being a general phenomenon (Fig. 3) (Weiser et al.,

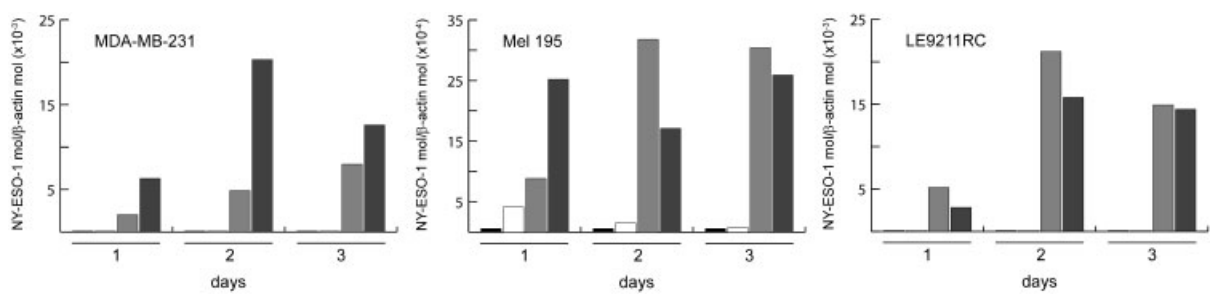

Fig. 3. Real-time quantitative RT-PCR analysis of NY-ESO-1 expression following treatment with 5-AZA-CdR and/or TSA. MDA-MB-231 breast carcinoma cells, Mel 195 metastatic cutaneous melanoma cells and LE9211RC renal carcinoma cells were incubated for 24 h with 1 μM 5-AZA-CdR and then with or without 400 nM TSA for additional 24 h. Cells were harvested for molecular analyses at days 1, 2 and 3 after the end of treatment, total RNA was extracted, subjected to reverse transcription and cDNA was utilized in quantitative real-time PCR using β-actin- and NY-ESO-1-specific TaqMan sets. Results are reported as NY-ESO-1 molecules/β-actin molecules. Black squares represent untreated cells, empty squares represent TSA-treated cells, light gray squares represent 5-AZA-CdR-treated cells, dark gray squares represent cells sequentially treated with 5-AZA-CdR and TSA.

2001a, b; Schrump and Nguyen, 2005). Even when a synergistic up-regulation of NY-ESO-1 was achieved using a combined 5-AZA-CdR/depsipeptide treatment, no significant enhancement of neoplastic cell recognition by a NY-ESO-1-specific CTL was observed as compared to 5-AZA-CdR treatment alone, supporting a primary role of 5-AZA-CdR in the functional modulation of CTA expression (Weiser et al., 2001a). In addition to the well-characterized role of epigenetics in regulating CTA expression, upcoming evidences are indicating its role in the inactivation of other TAA that are being utilized as immunotherapeutic targets. An example is represented by the high molecular weight melanoma associated antigen (HMW-MAA), which has been utilized as a target for melanoma immunotherapy with anti-idiotypic antibodies (Mittelman et al., 1992). Lack of HMW-MAA expression in acral lentiginous melanoma has recently been associated to methylation of HMW-MAA promoter, and reversal of promoter methylation by 5-AZA-CdR resulted in its re-expression both at mRNA and protein level, suggesting the possible use of epigenetic drugs to modify the phenotype of melanoma lesions constitutively lacking HMW-MAA, in order to render them suitable targets for HMW-MAA-directed immunotherapy (Luo et al., 2006).
Besides regulating TAA expression, epigenetic drugs clearly affect different molecular components involved in their presentation to the immune system and in the recognition and lysis of neoplastic cells by TAA-specific CTL.
HLA class I antigens are required both for the presentation of TAA-derived peptides to T cells and for the recognition and lysis of TAA-positive neoplastic cells by TAA-specific CTL. However, HLA class I antigens are frequently lost or down-regulated in cancer cells; nevertheless, DHA were consistently proven effective in up-regulating the basal expression of HLA class I antigens in melanoma cells both in vitro and in vivo (Coral et al., 1999, 2006), and in inducing their de novo expression in selected cell lines displaying complete loss of HLA class I antigens expression due to promoter hypermethylation (Nie et al., 2001; Serrano et al., 2001). Notably, re-expression of HLA class I antigens on MSR3-mel melanoma cells by 5-AZA-CdR allowed their recognition by an anti-MAGE CTL (Serrano et al., 2001), and up-regulated levels of HLA class I antigens and allospecificities induced by 5-AZA-CdR on Mel 275 melanoma cells resulted in their increased recognition by glycoprotein 100-specific HLA-A2-restricted CTL (Fonsatti et al., manuscript in preparation). TSA and SB also proved effective in up-regulating HLA class I antigens in the human neuroblastoma cell line SK-N-MC (Magner et al., 2000). Nevertheless, the more relevant influence that HDACi have on antigen presentation machinery may be regarded as their ability to induce HLA class II antigens expression in neoplastic cells either by acting directly or through the induction of the class II transactivator (CIITA) (Magner et al., 2000; Sigalotti et al., 2005; Gialitakis et al., 2006).
The immunomodulatory activity of epigenetic drugs is further sustained by their ability to up-regulate the expression of different accessory/co-stimulatory molecules on neoplastic cells. In fact, 5-AZA-CdR up-regulated the expression of intercellular adhesion molecule-1 (ICAM-1) on neoplastic cells of different histology (Coral et al., 1999; Arnold et al., 2001; Calabro et al., 2005; Sigalotti et al., 2005) and of leukocyte function-associated antigen-3 (LFA-3) on melanoma cells (Coral et al., 1999). In this scenario, HDACi play their part by being able to up-regulate the expression of CD40 on neuroblastoma cell lines and of CD86 and ICAM-1 in cell lines and leukemia blasts of acute myeloid leukemia (AML) (Maeda et al., 2000; Magner et al., 2000). These modifications may finally result in an increased recognition of neoplastic cells by the immune system, as suggested by the enhanced proliferation of allogeneic lymphocytes challenged with SB-treated HL60 myelomonocytic leukemia cells (Maeda et al., 2000).

The impact that epigenetic drugs may have in combined chemo-immunotherapeutic regimens is further strengthened by the demonstrated long-lasting in vitro and in vivo immunomodulatory activity of 5-AZA-CdR. Indeed, consistent with the physiologic inheritance of DNA methylation patterns, de novo induced CTA expression in neoplastic cells was maintained in vitro up to 7 months following 5-AZA-CdR-treatment (Calabro et al., 2005), and in vivo for at least 30 days after the end of treatment (Coral et al., 2006). Recent data pushed these observations further suggesting that the acquired expression of CTA may become a constitutive feature of 5-AZA-CdR-treated neoplastic cells. In fact, when analyzed at single cell level, cancer cells seemed to maintain the acquired CTA phenotype indefinitely, without manifesting the characteristic drop in levels of expression that is commonly observed when analyzing the whole cell population (De Smet et al., 1999). Similarly, the up-regulated expression of HLA class I antigens and co-stimulatory/accessory molecules induced in melanoma cells by the in vitro treatment with 5-AZA-CdR required 32 days to return to baseline levels (Coral et al., 1999), and up-regulated levels of HLA class I antigens were still detectable in human melanoma xenografts 40 days after the end of the systemic administration of the drug (Coral et al., 2006). These characteristically prolonged effects of DHA are in sharp contrast with the short-lived synergistic effect that HDACi have on the CTA expression induced by 5-AZA-CdR. In fact, the level of NY-ESO-1 expression induced in MDA-MB-231 breast carcinoma cells and in Mel 195 metastatic melanoma cells by the sequential treatment with 5-AZA-CdR and TSA rapidly returned to the 5-AZA-CdR-baseline, suggesting that the immunomodulatory activity of DHA/HDACi combinations, when present, may be of limited clinical advantage in the immunotherapeutic setting (Fig. 3).
Besides the background information provided above, functional pre-clinical studies in vivo have recently posed a major basis for the use of epigenetic drugs in designing combined chemo-immunotherapeutic regimens: (i) immunization of BALB/c mice with 5-AZA-CdR-treated human melanoma cells was able to generate high titer circulating antibodies against the de novo induced NY-ESO-1 protein (Coral et al., 2006); (ii) systemic treatment of BALB/c mice with 5-AZA-CdR induced the expression of the murine CTA P1A in 4T1 mammary tumors, which determined a significant reduction in the number of 4T1-derived lung metastases upon adoptive transfer of anti-P1A CTL (Guo et al., 2006).

Epigenetic Therapy of Cancer

The impressive amount of available pre-clinical in vitro and in vivo data generated in the last decade points to epigenetic drugs as efficient modulators of gene expression acting on different pathways potentially important in the clinical control of cancer. Thus, in recent years we have assisted to the development of therapeutic strategies exploiting the chromatin remodeling activities of new agents, as well as of drugs previously utilized in cancer treatment for their cytotoxic activity. The latter was the case of 5-AZA-CdR (decitabine), which has been first introduced into clinical development based on its cytotoxic effects on neoplastic cells of hematologic origin. In this setting, phase I studies defined 1500–2250 mg/m^2 per course as the maximum tolerated dose (MTD), and phase II studies using high-dose schedules reported encouraging results in AML, myelodysplastic syndrome (MDS) and chronic myelogenous leukemia (CML) patients (for review see Santini et al., 2001; Issa, 2005). Based on these results, and on the demonstrated activity of low concentrations of 5-AZA-CdR in inducing cellular differentiation in vitro (Pinto et al., 1984), two large phase II studies evaluated the activity and toxicity of lower doses of 5-AZA-CdR (from 135 mg/m^2 to 1000 mg/m^2 total dose per

course) in MDS and CML patients (Wijermans et al., 2000; Kantarjian et al., 2003). Results showed response rates from 28 to 63%, depending on the type and phase of disease, and no significant correlation between dose of 5-AZA-CdR and clinical response rate. Furthermore, the mean number of treatment cycles required to reach the best-observed response was 3 (Wijermans et al., 2000; Kantarjian et al., 2003). Two intriguing features of 5-AZA-CdR treatment derived from these studies, the relatively long time to achieve best clinical responses and the fact that these were often seen at doses well-below the MTD. According to these observations, a phase I study was conducted in patients affected by hematologic malignancies, testing multiple low-dose longer exposure schedules (5, 10, 15, or 20 mg/m^2/d for 5 days a week, for 2 consecutive weeks; or 15 mg/m^2/d for 10, 15, or 20 days). Results showed that the highest number of responses was observed at 15 mg/m^2/d for 10 days (65%) and suggested that a prolonged low-dose administration of 5-AZA-CdR was optimal in generating clinical responses in hematologic malignancies (Issa et al., 2004). The requirement for a prolonged exposure to 5-AZA-CdR to achieve clinical responses also derived from the results of the first phase III randomized trial of 5-AZA-CdR versus supportive care, performed in patients with MDS (Kantarjian et al., 2006a). In fact, even though 5-AZA-CdR was clinically effective in the treatment of patients with MDS, providing durable responses and improving time to AML transformation or to death, the observed response rate (17%) was lower then that reported in the previous phase II studies in which the drug was delivered for longer time periods (Wijermans et al., 2000). Furthermore, a recent study in MDS patients who received low-dose 5-AZA-CdR as re-treatment at the time of disease recurrence showed that 45% patients were still responsive, indicating a persistent sensitivity to the drug. Upfront resistance to the second treatment was also noted, suggesting that continued initial treatment beyond 6 to 8 courses might delay or prevent secondary resistance, and that continuation of the initial treatment might result in an increased clinical benefit (Ruter et al., 2006). Based on these data, a recent study was performed to formally test mechanism-based approaches to optimize low-dose prolonged therapies with 5-AZA-CdR in 95 patients with MDS and chronic myelomonocytic leukemia (CMML). To this end a reduced dose of 5-AZA-CdR (from 135 mg/m^2 to 100 mg/m^2) was utilized in a randomized study of three treatment schedules: (1) 10 mg/m^2 intravenously (i.v.) over 1 h daily $\times$ 10 days; (2) 20 mg/m^2 i.v. over 1 h daily for 5 days; (3) 20 mg/m^2 subcutaneously (s.c.) daily $\times$ 5 days. Each course of 5-AZA-CdR was delivered every 4 weeks and therapy was continued for at least three courses before evaluating response or failure of therapy. Overall, 32 patients (34%) achieved CR, and 69 (72%) had an objective response according to the International Working Group criteria. The 5-day i.v. schedule, which had the highest dose-intensity, was selected as optimal, since it gave the highest complete clinical response rate (39%), compared to 21% in the 5-day s.c. arm and 24% in the 10 days i.v. arm. This 5-AZA-CdR schedule also optimized epigenetic modulation, since it was superior in inducing hypomethylation at day 5 and in activating p15 expression at days 12 and 28 after therapy (Kantarjian et al., 2006b).

Whether the clinical results obtained in hematologic malignancies predominantly rely on the DNA hypomethylating activity of 5-AZA-CdR is still an open question. 5-AZA-CdR is definitely able to induce DNA hypomethylation in peripheral blood mononuclear cells (PBMC) of treated patients, as evaluated both at gene-specific sites and at whole genome level (Yang et al., 2006). Nevertheless, demethylation of the p15 gene, despite being observed in patients who displayed high levels of pre-treatment p15 gene methylation, was not associated to the clinical response to the drug (Issa et al., 2004; Yang et al., 2006). On the other hand, percentage of demethylation of Alu repetitive elements, 5–14 days after the end of a low-dose schedule of 5-AZA-CdR, correlated with the clinical response in AML patients, suggesting a direct role of drug-induced demethylation in triggering the clinical response, and warranting the analysis of drug-induced reactivation of other tumor suppressor genes to provide a molecular explanation to the observed data (Yang et al., 2006). Furthermore, the observation that a single low-dose course of 5-AZA-CdR was able to induce a de novo expression of different CTA in AML and MDS patients suggests for the possibility that long-term disease control may be sustained, at least in part, by the activation of a CTA-specific immune response (Sigalotti et al., 2003).

In spite of the encouraging results observed in hematologic tumors, the experience with DNA hypomethylating agents in solid malignancies is rather limited, and clinical response rates have been generally low (Momparler et al., 1997; Yogelzang et al., 1997; Santini et al., 2001; Schrump and Nguyen, 2005). In a phase I trial, Aparicio et al. investigated the effect of decitabine (20, 30, and 40 mg/m^2 by 12 h continuous i.v. infusion over 72 h on days 1–3 of a 28-day cycle) on the methylation patterns of selected genes in tumor biopsies from patients with metastatic solid tumors (Aparicio et al., 2003). Though no objective clinical responses were seen in this study, changes in methylation were observed. However, no relationship was detected between the dose of decitabine and the effect on methylation (Aparicio et al., 2003). To test the potential utility of DNA methyltransferase inhibitors as part of a combination chemotherapy approach, Samlowsky et al. developed a phase I clinical protocol in patients with recurrent or metastatic solid tumors using a low-dose continuous infusion (2 mg/m^2/d for 7-day) of 5-AZA-CdR (Samlowski et al., 2005). Quantitative real-time PCR and a quantitative HPLC-based assay were performed on DNA from PBMC of treated patients to monitor the effect of 5-AZA-CdR on gene-specific (MAGE-A1) and on genomic changes in DNA methylation occurring in the course of treatment. Results showed that a 7-day continuous infusion of 5-AZA-CdR is well-tolerated and inhibits promoter-specific and genomic DNA methylation in vivo. However, no data pertaining to 5-AZA-CdR pharmacokinetics or target gene induction in tumor tissues were reported in this study. Recently, a phase I study was designed to identify the MTD of 5-AZA-CdR administered as a continuous 72-h infusion in patients with thoracic malignancies, and to set up 5-AZA-CdR exposure conditions that would simultaneously modulate CTA and tumor-suppressor gene expression in tumor tissues (Schrump et al., 2006). The MTD defined in this study was 60–75 mg/m^2. Although no objective responses were observed with this regimen, a molecular response was observed in 8 of 22 patients with tumor biopsies available for analysis, which exhibited induction of NY-ESO-1, MAGE-A3, or p16 following 5-AZA-CdR treatment. Post-treatment antibodies to NY-ESO-1 were also detected in three patients exhibiting induction of NY-ESO-1 in their tumor tissues.

The observed generation of an anti-CTA immunological response against CTA de novo expressed following 5-AZA-CdR strongly suggests for the use of 5-AZA-CdR in combined therapies with CTA-directed vaccines, or with other immunotherapeutic approaches. Along this line, a recent phase I trial was conducted in melanoma or renal carcinoma patients to evaluate the effects of DHA pre-treatment on the immune activating properties of a high-dose IL-2 schedule (Gollob et al., 2006). Subcutaneous daily injection of 0.1–0.3 mg 5-AZA-CdR/Kg for 5 days, on weeks 1 and 2 of a 12-week cycle, resulted in global genomic DNA hypomethylation and changes in the expression of immunomodulatory genes in PBMC, which preceded and were still present at IL-2 administration. The evaluation of the immunomodulatory activity of 5-AZA-CdR in this setting revealed both

upregulation (e.g.: IFN related genes, chemochines, and genes involved in IL-1, IL-17 and IL-22 signaling) and down-regulation (e.g.: IL-2Rα, CD3-ε, CD2 and genes involved in IL-12 signaling) of genes which may favor the activity of administered IL-2. Interestingly, a down-regulation of CTLA-4 was observed in PBMC of 5-AZA-CdR-treated patients, which may positively affect different immunotherapeutic strategies by reducing CTLA-4-mediated immune suppression. From a clinical perspective, autoimmune phenomena (i.e.: vitiligo and hypothyroidism) were frequently observed in responding patients. Furthermore, the 23% major responses rate observed with this combined 5-AZA-CdR/IL-2 treatment favorably compared to the 15% response rate observed in melanoma patients treated with high-dose IL-2 alone, and was suggestive for a negligible if any adverse effect of 5-AZA-CdR in the clinical activity of high-dose IL-2 (Gollob et al., 2006).

In contrast to the high number of clinical trials investigating the activity of the epigenetic modifications induced by DHA, the evaluation of the clinical activity of different HDACi is still in an initial phase. Phase I and phase I/II trials have been conducted utilizing different schedules of HDACi belonging to different classes (i.e., phenylbutyrate, depsipeptide, SAHA, VPA, MS 275, LBH589/LAQ824, PXD101, MGCD0103) and reported a limited clinical activity, with complete durable responses observed only in T-cell cutaneous lymphoma and leukemia patients, despite toxic effects were frequently observed (Garcia-Manero and Issa, 2005). These preliminary data, together with the large bulk of pre-clinical evidences indicating synergistic/sensitizing activities of HDACi with other

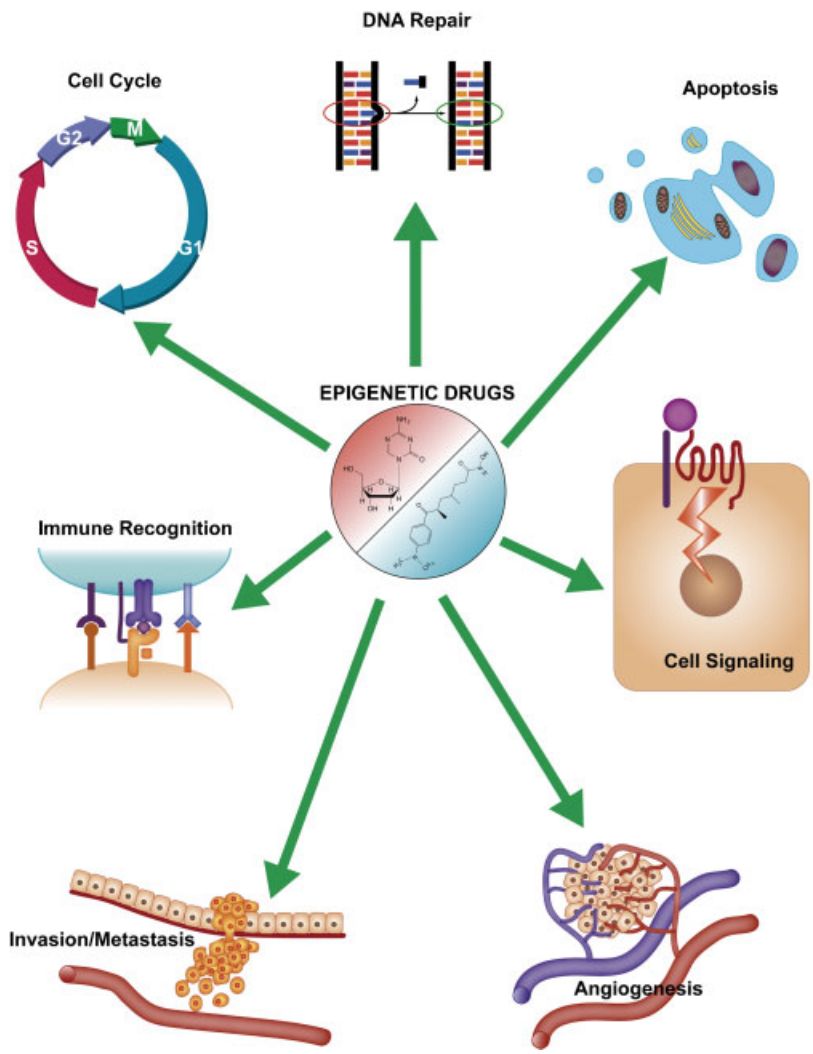

Fig. 4. Cellular pathways that can be concomitantly affected by epigenetic drugs. Epigenetic drugs can be envisaged as multifaceted anticancer drugs able to concurrently: (i) restore cell-cycle control and induce cell-cycle arrest by reactivating aberrantly inactivated CDK inhibitors (e.g., p15, p16, p21, p57), and down-regulating CDK; (ii) restore DNA repair machinery through the re-expression of DNA repair genes (e.g., MLH1, MGMT); (iii) restore physiological sensitivity to apoptotic stimuli by re-establishing the expression of apoptosis signaling/effector proteins (e.g., TRAIL receptors, DAPK1, XAF1, caspases, APAF1); (iv) restore physiological signaling pathways, enabling cells to become sensitive to growth inhibitory signals (e.g., through re-expression of RAR-β and ER) or attenuating aberrantly sustained growth promoting signals through restoration of negative regulators of signaling (e.g., IGFBP, SOCS-1, SHP-1, SFRP); (v) reduce the invasive and metastatic potential of cancer cells by re-establishing the physiological expression of adhesion molecules (e.g., CDH1) and inducing the expression of inhibitors of the matrix metalloproteinases (e.g., TIMP3 e TFPI-2); (vi) suppress tumor angiogenesis by up-regulating anti-angiogenic factors (e.g., VHL, thrombospondin-1) and down-regulating pro-angiogenic factors (e.g., HIF-1, VEGF); vii) increasing tumor cell recognition by the host's immune system through induction/up-regulation of TAA, HLA class I and II antigens and accessory/co-stimulatory molecules (e.g., ICAM-1, LFA-3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

anticancer agents, suggested that combination therapies would be more effective in the treatment of cancer patients. Along this line, a recent phase I/II study investigated the concomitant administration of 5-AZA-CdR and VPA in leukemia patients (Garcia-Manero et al., 2006). Fifty-four patients were treated with a fixed dose of 5-AZA-CdR (15 mg/m^2 i.v. daily for 10 days) in association with escalating doses of VPA (20–50 mg/kg orally daily for 10 days). Treatment induced global DNA hypomethylation and histone H3 and H4 acetylation in PBMC from patients; these epigenetic modifications were found to be associated with p15 reactivation, but not with clinical response. The observed response rate (22%) in this study was much lower than that observed in trials utilizing 5-AZA-CdR alone (Issa et al., 2004). This result may find a rationale on recent data demonstrating an antagonism in antineoplastic activity between the HDACi LAQ824 and 5-AZA-CdR when used simultaneously, but not when LAQ824 exposure followed 5-AZA-CdR treatment (Hurtubise and Momparler, 2006). This information suggests that sequential exposure to the drugs may be more effective in obtaining important clinical responses, and should be evaluated in the future planning of combination epigenetic therapies.

Final Comments

Despite few studies have suggested possible clinically unfavorable effects of epigenetic drugs in the biology cancer cells, such as the up-regulation of selected pro-metastatic genes (uPA, MMP), and the demonstration that treatment with hypermethylating compounds may result in a reduction of growth and invasive potential of prostate cancer cells (Shukeir et al., 2006), the vast majority of the available literature prompts to epigenetic drugs as efficient pleiotropic anti-cancer agents. The recent developing of high-density gene expression profiling technologies has reinforced this notion by allowing to concomitantly investigate the expression of genes involved in disparate cellular processes. It seems now well-established that DHA and/or HDACi are able to coordinately regulate the expression of different members within specific gene families, such as those involved in IFN, IGF or WNT signaling (Liang et al., 2002; Suzuki et al., 2002; Lodygin et al., 2005; Ibanez de Caceres et al., 2006). The coordinated effect of epigenetic drugs on cancer cell transcriptome goes beyond the regulation of specific pathways and seems rather to affect the whole cell physiology, leading to a less aggressive phenotype. In fact, SAHA and depsipeptide treatments have been recently reported to concomitantly modulate multiple genes within the Myc, type β TGF, cyclin/cyclin-dependent kinase, TNF, Bcl-2 and caspases pathways, in a manner that favored induction of apoptosis and decreased cell proliferation (Peart et al., 2005). The pleiotropic activities of epigenetic drugs, by acting on different pathways involved in tumor development and progression (Fig. 4), may represent ideal therapeutic weapons against the multifaceted cancer cells that notoriously take advantage of concomitant multiple defects in the physiologic regulation of cellular behavior. The composite biologic effect of epigenetic drugs may further offer the advantage of reducing the likelihood of tumor escape from their anticancer activities since multiple cellular pathways can be simultaneously targeted.

The above reported considerations, and recent data generated both in animal models and clinical trials, demonstrating the efficacy of epigenetic drugs in modifying human cancer phenotype in vivo (Coral et al., 2006; Gollob et al., 2006; Schrump et al., 2006), represent a strong driving background to further pursue the use of epigenetic drugs as in vivo epigenome modifiers to comprehensively address the aberrant epigenetic modifications responsible for cancer aggressiveness. In this view, the composite effects of epigenetic drugs may possibly require the design of multimodal therapeutic approaches to take full advantage of their biological properties, including their sensitizing activity to chemotherapy/radiotherapy (Kim et al., 2003; Camphausen et al., 2004; Zhang et al., 2004; Loprevite et al., 2005; Munshi et al., 2006; Segura-Pacheco et al., 2006) and active or adoptive immunotherapy (Coral et al., 2006; Guo et al., 2006; Schrump et al., 2006).

Overall, epigenetic drugs represent intriguing and promising drugs in cancer treatment, and their pleiotropic activities strongly suggest for their use in combination therapies using DHA and/or HDACi in conjunction with biologic and/or cytotoxic and/or radiation therapy to achieve optimal clinical results.

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