Review

Hydralazine target: From blood vessels to the epigenome

Claudia Arce[2], Blanca Segura-Pacheco[1], Enrique Perez-Cardenas[1], Lucia Taja-Chayeb[1], Myrna Candelaria[2] and Alfonso Dueñas-Gonzalez*[1]

Address: [1]Unidad de Investigación Biomédica en Cáncer, Instituto de Investigaciones Biomédicas (IIB)/Instituto Nacional de Cancerología, Universidad Nacional Autónoma de Mexico, Mexico City, Mexico and [2]Division of Clinical Research, Instituto Nacional de Cancerología, Mexico City, Mexico

Email: Claudia Arce - haydeearce@hotmail.com; Blanca Segura-Pacheco - myrnac@prodigy.net.mx; Enrique Perez-Cardenas - ba0512@prodigy.net.mx; Lucia Taja-Chayeb - zperez@salud.gob.mx; Myrna Candelaria - chayeb@salud.gob.mx; Alfonso Dueñas-Gonzalez* - alfonso_duenasg@yahoo.com

* Corresponding author

Abstract

Hydralazine was one of the first orally active antihypertensive drugs developed. Currently, it is used principally to treat pregnancy-associated hypertension. Hydralazine causes two types of side effects. The first type is an extension of the pharmacologic effect of the drug and includes headache, nausea, flushing, hypotension, palpitation, tachycardia, dizziness, and salt retention. The second type of side effects is caused by immunologic reactions, of which the drug-induced lupus-like syndrome is the most common, and provides clues to underscoring hydralazine's DNA demethylating property in connection with studies demonstrating the participation of DNA methylation disorders in immune diseases. Abnormalities in DNA methylation have long been associated with cancer. Despite the fact that malignant tumors show global DNA hypomethylation, regional hypermethylation as a means to silence tumor suppressor gene expression has attracted the greatest attention. Reversibility of methylation-induced gene silencing by pharmacologic means, which in turns leads to antitumor effects in experimental and clinical scenarios, has directed efforts toward developing clinically useful demethylating agents. Among these, the most widely used comprise the nucleosides 5-azacytidine and 2'deoxy-5-azacytidine; however, these agents, like current cytotoxic chemotherapy, causes myelosuppression among other side effects that could limit exploitation of their demethylating properties. Among non-nucleoside DNA demethylating drugs currently under development, the oral drug hydralazine possess the ability to reactivate tumor suppressor gene expression, which is silenced by promoter hypermethylation *in vitro* and *in vivo*. Decades of extensive hydralazine use for hypertensive disorders that demonstrated hydralazine's clinical safety and tolerability supported its testing in a phase I trial in patients with cancer, confirming its DNA demethylating activity. Hydralazine is currently being evaluated, along with histone deacetylase inhibitors either alone or as adjuncts to chemotherapy and radiation, for hematologic and solid tumors in phase II studies.

Review

Hydralazine as an antihypertensive

Hydralazine, a potent arterial vasodilator that reduces peripheral resistance directly by relaxing the smooth muscle cell layer in arterial vessels, has long been used for management of hypertensive disorders and heart failure [1,2]; nonetheless, its current use is limited nearly to hypertensive disorders during pregnancy [3,4]. Despite numerous studies with the drug, its mechanism of action has remained unknown but it is suggested that hydralazine may function by either modulating the effect of purine-like compounds released from sympathetic nerve

endings, and/or by producing an altered Ca^{2+} balance in vascular smooth muscle cells [5-7]. The majority of its effects are confined to the cardiovascular system. Decrease in blood pressure after hydralazine administration is associated with a selective decrease in vascular resistance in coronary, cerebral, and renal circulation, with a lesser effect in skin and muscle. Hydralazine lowers peripheral vascular resistance equally in supine and upright positions; it also lowers pulmonary vascular resistance and increases cardiac output causing mild pulmonary hypertension. [1,2].

Hydralazine is well absorbed through the gastrointestinal tract, but systemic bioavailability is low. Because the acetylated compound is inactive, the dose necessary to produce a systemic effect is higher in fast acetylators. N-acetylation of hydralazine occurs in bowel and/or liver. Hydralazine's half-life is 1 h and systemic clearance of the drug is approximately 50 mL/kg/min. Hydralazine rapidly combines with circulating α keto-acid to form hydrazones, and the major metabolite recovered from the plasma is hydralazine piruvic acid hydrazone. This metabolite possesses a longer half-life than hydralazine but does not appear to be very active. Systemic metabolism is dependent on hydroxylation followed by conjugation with glucoronic acid in liver, which is not dependent on acetylation rate; therefore, half-life does not differ to a great degree between slow and fast acetylators [8]. Hydralazine peak concentration in plasma and peak hypotensive effect of the drug occurs within 30–120 min of ingestion. Although its half-life in plasma is approximately 1 h, duration of the hypotensive effect can last as long as 12 h; there is no clear explanation for this discrepancy. The antihypertensive effect of hydralazine has no clear dose-response effects. The dose varies from 10 mg four times a day to 50 mg four times daily. After stabilization with multiple daily doses, a twice-daily dose regimen can be effective. Slow acetylators require a lower dose. For heart failure, recommended doses are higher (up to 800 mg daily or more); as a rule, 10–100 mg four times a day can be effective [9].

Two types of side effects occur after hydralazine use. The first type, an extension of hydralazine's pharmacologic effect, includes headache, nausea, flushing, hypotension, palpitation, tachycardia, dizziness, and angina pectoris; hydralazine can produce salt retention with congestive heart failure development. The second type of side effect is caused by immunologic reactions, of which the drug-induced lupus-like syndrome is the most common [8,9].

Cancer epigenetics
Epigenetics can be defined as the study of mitotically and/or meiotically heritable changes in gene function that can not be explained by changes in DNA sequence. There are two epigenetic systems that affect animal development and fulfill the heritability criterion: DNA methylation, and the polycomb-trithorax group (Pc-G/trx) protein complexes; nevertheless, post-transductional histone modification possesses some attributes of an epigenetic process and as such are studied within this field [10]. Epigenetics is a well-established phenomenon that plays a major role in a diversity of biological processes such as embryonic development, cancer biology, and immune system response, among many others. The two most widely studied epigenetic changes are DNA methylation and histone acetylation; however, the picture is much more complicated than this, with new players coming into the scenario; these include, among others, the RNA interference phenomenon, proven to be implicated in transcriptional silencing through small duplex RNA molecules that recruit silencing complexes to the chromatin, and the BORIS (Brother of the Regulator of Imprinted Sites)/CTCF gene family, which is involved in epigenetic reprogramming events [11,12].

DNA methylation
DNA methylation is a covalent chemical modification that mainly occurs at the cytosine ring, resulting in the addition of a methyl^{CH3} group at the carbon 5 position. Because DNA is made up of four bases, 16 possible dinucleotide combinations can occur; hence, the CpG dinucleotide should have a frequency of 6%. Nonetheless, actual presence is only 5–10% of its predicted frequency. The human genome is not uniformly methylated and contains regions of unmethylated interspersed with methylated segment regions. In contrast to the remainder of the genome, smaller regions of DNA – denominated CpG islands, ranging from 0.5–5 kb, and occurring on average every 100 kb – possess distinctive properties. These regions are unmethylated, GC-rich (60–70%), have a ratio of CpG:GpC of at least 0.6, and thus show no suppression of dinucleotide CpG frequency. Approximately one half of all genes in humans have CpG islands, these present in both housekeeping genes and in genes with tissue-specific patterns of expression [13,14].

At least three functional DNA methyltransferases (DNMT) have been identified; the most abundant is DNMT1, or maintenance DNMT. This methyltransferase enzyme copies pre-existing methylation patterns onto the new DNA strand during DNA replication to ensure clonal transmission of lineage-specific DNA methylation patterns in a mammalian genome during replication. Thus, DNMT1 is targeted to replication foci, interacts with proliferating cell nuclear antigen, and favors methylating the hemimethylated form of CpG sites. It has been demonstrated that DNMT1 methylates hemimethylated DNA with high processivity and a fidelity of >95% on one strand of double-stranded DNA during a single processive run; never-

theless, DNMT1 methylates unmethylated DNA with much less processivity, suggesting that these inherent enzymatic properties of Dnmt1 play an essential role in the faithful and efficient maintenance of methylation patterns in the mammalian genome [15,16].

Other known functional methyltransferases include DNMT3a and DNMT3b, which are responsible for *de novo* methylation during embryogenesis; thus, these have been classified as *de novo* methyltransferases [17]. These enzymes are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites by at least three possible means: first, DNMT3 enzymes themselves might recognize DNA or chromatin via specific domains; second, DNMT3a and DNMT3b might be recruited through protein-protein interactions with transcriptional repressors or other factors, and third, the RNA-mediated interference (RNAi) system might target *de novo* methylation to specific DNA sequences [18].

In addition to DNMTs, the machinery of methylation includes demethylases, methylation centers that trigger DNA methylation, and methylation protection centers [19]. The effect of DNA methylation on gene transcription can be observed only within the context of chromatin remodeling players. DNA methylation can interfere directly with transcriptional factor binding, thus inhibiting replication [20], in addition to the ability of DNA methyltransferases DNMT1, DNMT3a, and DNMT3b to repress transcription in a methylation-independent manner [21]. Methyl-CpG-binding proteins, which can recognize methylated DNA, have been shown to associate with large protein complexes containing HDACs and chromatin-remodeling activities. It has also been suggested that DNA methylation could produce gene silencing by methyl-binding domain proteins that recruit histone methyltransferases, which methylate lysine 9 in histone H3 and subsequently repress gene transcription [22]. As a result, histones are deacetylated and gene transcription repressed.

Histones and post-translational modifications
How double-strand DNA is packaged into the dynamic structure of chromatin is crucial for the transcriptional control process by regulating transcription factor accessibility to DNA regulatory sequences. Chromatin is constituted of nucleosomes, which are comprised of 146 base pairs of DNA wrapped around a core of two copies each of histones H2A, H2B, H3, and H4. The N-terminal tails of histones, which undergo post-translational modifications such as acetylation, methylation, ubiquitination, phosphorylation, and sumoylation, protrude from their originating nucleosome and may contact adjacent nucleosomes as well as chromatin-associated proteins. These interactions are responsible, at least in part, for reg-

ulating chromatin structure [23]. The most widely studied modification is acetylation, which plays diverse roles in nucleosome regulation. Lysine acetylation, for example, may decrease histone-DNA interaction and promote accessibility of DNA for transcription activation. Moreover, acetylation specifically can also regulate DNA replication, histone deposition, and DNA repair by mechanisms of recruiting proteins that have an acetyl-lysine binding module, the bromodomain, a 100-amino-acid conserved sequence found in many chromatin-associated proteins. Thus, bromodomains in histone acetyltransferases and nucleosome remodeling complexes orchestrate the succession of chromatin regulators at a target site [24,25]. Overall, lysine acetylation results in chromatin decompactation, greater access of DNA to transcription factors, and the presence of a transcriptionally active genomic locus. This post-translational modification depends on the net local balance between histone acetyl transferase (HAT) and histone deacetylase (HDAC) activities. In general, hyperacetylation is found in more decondensed euchromatin, whereas hypoacetylation is characteristic of more condensed heterochromatin [26,27].

Epigenetic alterations and cancer
Abnormalities in DNA methylation have long been associated with cancer. Both hypo- and hypermethylation play prominent roles in carcinogenesis, and their contribution exhibits poorly defined boundaries. It has been known for some time that both alterations co-exist in cancer cells: malignant tumors exhibit global hypomethylation and regional hypermethylation. Whether one must precede the other or whether both should begin at the same time remains to be elucidated. In terms of carcinogenesis, initial observations were conducted on hypomethylation [28 29]; later, discovery of regional hypermethylation as a means to silence tumor suppressor gene expression gained the most attention [30].

Silencing of tumor suppressor genes
Observations that tumor suppressor gene function can be disrupted not only through structural changes (mutation and deletion) but also by lack of promoter hypermethylation-associated expression positioned tumor suppressor gene epigenetic silencing as a well-established oncogenic process [31]. The first suppressor gene known to be hypermethylated and silenced was *Rb* [32]. This finding was soon followed by multiple publications describing similar findings for a variety of tumor suppressor genes, among them *p16*, *MLH1*, *VHL*, and *E-cadherin* [33]. Whether gene promoter hypermetylation is the cause or consequence of tumor suppressor gene silencing remains a matter of controversy; nevertheless, these views are not mutually exclusive. That DNA methylation is causal has been shown by the ability of diverse pharmacologic compounds and molecular techniques to reactivate gene expression upon

Table 1: Agents targeting DNA methylation and histone deacetylase in preclinical and clinical development

DNA methylation inhibitors

deoxycytidine analogs:
5-azacytidine, 5-aza-2-deoxycytidine, 1-β-D-arabinofuranosil 5-azacytosine, dihydro- 5-azacytidine
nucleic acid-based:
MG98 antisense oligonucleotide
cytidine deaminase analogs:
zebularine
non-nucleoside analogs:
(-)-epigallocatechin-3-gallate, procaine, procainamide, hydralazine, RG108, psammaplins

Histone deacetylase inhibitors
Small molecular weight carboxilates:
sodium butyrate, valproic acid, sodium phenylbutyrate and pivaloyloxymethyl butyrate
Hydroxamic acids:
SAHA, trichostatin A, SBHA
Benzamides:
CI-994, MS-275
Epoxyketones:
trapoxin B, 2-amino-8-oxo-9,10-epoxydecanoic acid
Cyclic peptides:
apicidin, depsipeptide
Hybrid molecules:
CHAP 31, CHAP50
Miscellaneous:
Cyclostellettamines, Carbamazepine

Adapted from reference 106.

inhibition of DNA methylation in cancer cells [34]. On the other hand, other findings suggest that hypermethylation-induced gene silencing could be associated with changes that determine gene expression, such as chromatin modification, so that methylation aids in maintaining the gene's silenced status. Strong support for the second view has derived from experiments showing that histone H3 lysine 9 methylation – that is, chromatin modification – occurred along with *p16* re-silencing in absence of DNA methylation in cells in which *p16* had previously been activated by DNA methyltransferase knock-out [35], and by data demonstrating *p16* silencing in mammary epithelial cells that had escaped senescence and demethylated the promoter [36].

Silencing of genes involved in tumor-host interaction
Although cancer cells are less immunogenic than pathogens, the immune system is clearly capable of recognizing and eliminating tumor cells. However, tumors frequently interfere with immune response development and function through several mechanisms such as loss of antigen processing and presentation, the Fas counterattacking system, escaping from death receptor signaling, engaging in inhibition-blocking activation, suppression of antitumor responses by regulatory T cells, and tumor-induced immune suppression [37]. Current research demonstrates that epigenetic defects are involved in at least some mechanisms that preclude mounting a successful host-antitu-

mor response, including the HLA system, tumor-associated antigens, and accessory/co-stimulatory molecules.

Among tumor-associated antigens, testis-associated genes (CTA) are a sub-group of tumor antigens with restricted expression in testis and malignancies; CTA include the MAGE, NYESO, and SSX gene families and the GAGE/PAGE/XAGE superfamilies [38]. Distinct CTA can encode for different antigenic peptides that are presented to the immune system in association with various human leukocyte antigens (HLA), HLA class I or HLA class II allospecificities, eliciting both cytotoxic T lymphocytes (CTL) and humoral immune responses. De Smet et al., first showed a link between genomic DNA hypomethylation and *MAGE-1* gene expression [39], and subsequent studies demonstrated direct correlation between demethylation of CpG at the promoter of several cancer testis antigen coding genes with their expression [40-43]. Surprisingly, 5-aza-2'-deoxycytidine demethylation treatment led to induction of *MAGE-*, *GAGE-*, *PAGE-*, and *XAGE*-type genes in tumor cells but not in normal cells [44].

Presentation of antigens within the context of HLA molecules is crucial both during T-cell priming and the effector phase of an adaptive immune response. Alterations in antigen processing and presentation are commonly observed in malignancies. In tumors, the antigen presen-

tation pathway is commonly disrupted as a consequence of the mutations and/or deletions of one or several genes encoding components of the antigen-processing machinery, including proteasomal subunits and transporters peptides; thus, complete HLA loss is a common event in several murine and human tumors [45]. As for CTA, DNA methylation participates in regulation of the expression of the three classes of human leukocyte antigen class I antigens: HLA-A; HLA-B, and HLA-C, which are CpG-rich at their gene promoters. Nie et al. showed down-regulation of HLA class I antigens in esophageal carcinoma as a common mechanism for transcriptional inactivation caused primarily by DNA hypermethylation [46], as well as in melanoma, where 5-aza-2'-deoxycytidine significantly enhances the constitutive expression of HLA class I antigens, of HLA-A1 and -A2 alleles, and of the co-stimulatory molecule, intercellular adhesion molecule-1, and lymphocyte function-associated antigen-3 [47]. Further, it was shown that 5-aza-2'-deoxycytidine leads to re-expression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells [48]; likewise, it has been shown that methylation-induced silencing of the class II transactivator (CIITA) leads to lack of HLA-DR induction in hematopoietic tumor cells and solid tumors such as gastric and colorectal carcinomas [49,50].

Current epigenetic therapy agents

Reactivation of tumor suppressor genes silenced by an epigenetic mechanism such as gene promoter methylation is a very attractive molecular target for cancer therapy. There are several demethylating agents currently being evaluated in pre-clinical and clinical studies (Table 1). The classical demethylating agents comprise the analogs of deoxycytidine, including 5-azacytidine, 5-aza-2-deoxycytidine, 1-β-D-arabinofuranosil-5-azacytosine, and dihydro-5-azacytidine. 5-azacytidine and its analog 5-aza-2-deoxycytidine are the most frequently studied and were developed over 30 years ago as classical cytotoxic agents, but were subsequently discovered to be effective DNA methylation inhibitors [51]. These were tested as such in several phase II studies against solid tumors, demonstrating very modest activity [52]. Contrariwise, their antileukemic activity was very promising, and both are being revived due to their demonstrated inhibitory activity upon DNA methylation and gene-reactivating function.

Currently, 5-azacytidine is Federal Drug Administration (FDA)-approved for use against myelodysplastic syndrome, and 5-aza-2-deoxycytidine is also being tested in myelodysplastic syndrome and in a variety of solid tumors due to its DNA demethylating properties [53]. In particular, 5-aza-2-deoxycytidine when infused for 10 days proved to be well-tolerated and induced responses in 65% of 50 patients with acute myelogenous leukemia, myelodysplasia, chronic myelogenous leukemia, and acute lymphocytic leukemia [54]. Despite poor activity against solid tumors of these nucleoside analogs, it is remarkable that proof of the concept concerning the ability of DNA methylation compound inhibitors to demethylate and reactivate tumor supressor gene expression has been shown in solid tumors [55]. Whether or not the reactivating effect can translate into a clinical response on its own or in combination with classical cytotoxic therapies remains to be demonstrated.

As a second category of demethylating agents, there is the antisense oligonucleotide MG98 against the 3' untranslated region of *DNMT1* mRNA that codes for the enzyme DNA methyltransferase 1, which is responsible for DNA methylation maintenance [56]. This agent has shown an ability to inhibit *DNMT1* gene expression without affecting other *DNMTs* and to cause demethylation with *p16* re-expression in bladder and colon cancer cell lines, as well as to produce tumor growth inhibition in nude mice bearing human lung and colon xenografts [57]. MG98 has been evaluated in a phase I trial in patients with advanced or refractory solid tumors and has demonstrated its tolerability, despite the fact that dose-limiting toxicities of transaminitis, thrombocytopenia, and fatigue prohibited higher doses. MG98's molecular efficacy was demonstrated by producing a *DNMT* mRNA level decrease in the peripheral blood cells in six of 10 patients [58].

The fact that deoxycytidine analogs, like current cytotoxic agents, are not only carcinogenic but also exhibit neutropenia as their dose-limiting toxicity even when used at doses required for demethylation [59] has renewed interest in finding effective and less toxic demethylating agents. Zebularine is a new oral cytidine analog originally synthesized as a cytidine deaminase inhibitor that has shown to cause demethylation and reactivation of a silenced and hypermethylated *p16* gene in human bladder tumor cells grown in nude mice. Zebularine was also shown to be minimally cytotoxic *in vitro* and *in vivo* and can be administered continuously at a lower dose to maintain demethylation for a prolonged period, possible only because of its low-toxicity profile; to date, there are no results of clinical trials with this agent [60]. Within this class of so-called "non-toxic and orally administered agents", there is the green tea major polyphenol (-)-epigallocatechin-3-gallate (EGCG) that demonstrated to be an effective *DNMT* activity inhibitor at micromolar concentrations and that was able to demethylate and reactivate expression of several tumor suppressor genes such as *p16*, *RAR-β2*, and *MGMT* in cancer cell lines [61].

There is another class of so-called "old drugs" that includes hydralazine. The demethylating activity upon gene promoters of tumor suppressor genes of these compounds was recently demonstrated. Procainamide, a non-
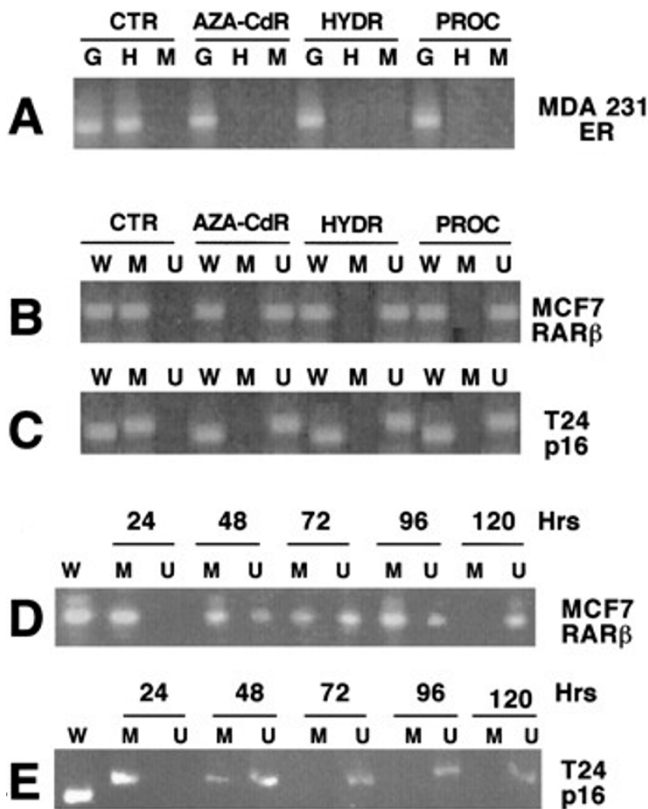
Figure 1
Methylation analysis of the ER, RARβ, and p16 genes. **A**, G is undigested genomic DNA as positive control, H is HpaII, and M is MspI. The band in H of control lanes indicates lack of digestion with HpaII and, therefore, methylation of untreated MDA-231 cells. **B** and **C** show the analysis by methylation-specific PCR of RARβ and p16 genes in MCF-7 and T24 cells. W is wild type, M is methylated primers, and U is unmethylated primers. D and E show a time course experiment of RARβ and p16 gene demethylation over 5 days on the MCF-7 and T24 cells, respectively. (Reproduced with permission from the AACR. Ref. 83. *Clin Cancer Res* 2003, **9**:1596–1603).
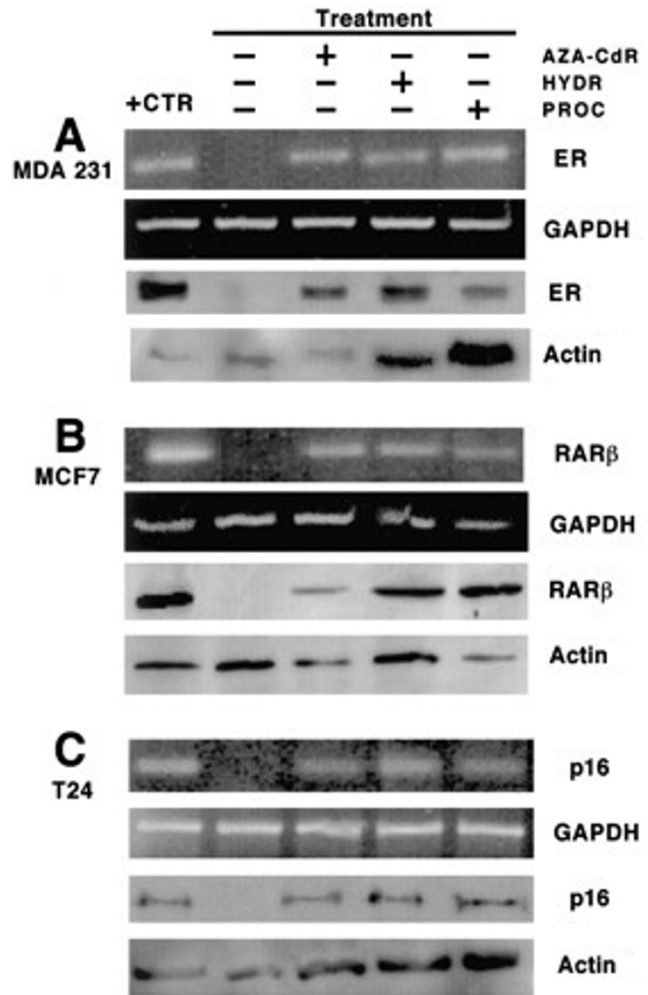
Figure 2
Expression of the mRNA and proteins of **A**, ER (MDA-231); **B**, RARβ (MCF-7); and **C**, p16 (T24) genes by RT-PCR and Western blot. The positive control (+ctr) for A is MCF-7, for B is MDA-231 exposed to 5-aza-CdR/atRA, and for C is HeLa cells. (Reproduced with permission from the AACR. Ref. 83. *Clin Cancer Res* 2003, **9**:1596–1603).

nucleoside inhibitor of DNA methyltransferases approved for treatment of cardiac arrhythmias, can demethylate the *GSTP1* promoter, a common somatic genome change in human prostate cancer, and reactivates expression of the gene *in vitro* and in nude mice [62]. A related drug, procaine, possesses the ability to demethylate and reactivate tumor suppressor gene expression, such as the *RARβ2* gene, in a breast cancer cell line, an effect accompanied by growth-inhibitory actions [63].

Hydralazine as a demethylating agent
Clues to underscoring hydralazine's DNA demethylating property have derived from its ability to induce a lupus-like syndrome in connection with studies demonstrating

DNA methylation disorder participation in systemic lupus erythrematosus (SLE).

Lupus-like syndrome induced by hydralazine
More than 80 drugs have been associated with drug-induced lupus erythrematosus [64]; at least 39 of these are currently in use [65]. Only two of these, procainamide and hydralazine, are considered as high risk for induction of the syndrome, with up to 8 and 20% frequency respectively, during 1 year of therapy at currently employed doses [64,65]. Hydralazine was introduced as an antihypertensive drug in 1952, and the first case of hydralazine-induced lupus was reported soon thereafter [66]. Prevalence of slow acetylators among patient populations at
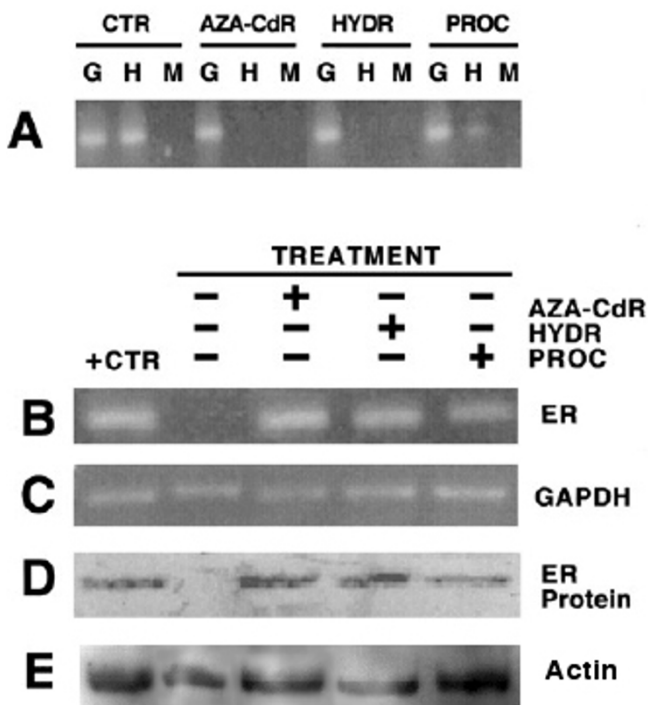
Figure 3
Analysis of methylation and product expression of the ER gene in nude mice. **A** shows that the tumor of control mice (CTR lanes) was methylated as shown by the band in the Lane H indicating no digestion with HpaII. The band disappeared with the treatment with 5-aza-CdR and hydralazine, which indicates gene demethylation. In PROC (procainamide) lanes, the band is absent in MspI but is very weak in HpaII, indicating that was only partially demethylated; **B** and **D** are the product expression by RT-PCR and Western blot. In both cases, the intensity of bands in procainamide is weaker, correlating with a partial demethylation, (**C** and **E** are the loading controls, GAPDH and actine respectively). G is undigested genomic DNA. (Reproduced with permission from the AACR. Ref. 83. *Clin Cancer Res* 2003, **9**:1596–1603).

risk for developing lupus erythrematosus is obviously much greater than the disease prevalence. Only 1–3% of populations of which at least 50% are slow acetylators develop hydralazine-induced lupus. In a study of 26 patients with hydralazine-induced lupus, 25 were slow acetylators, with a 4:1 ratio of females to males; this discrepancy may be explained by the fact that additional genetic traits are important in predisposing individuals to lupus [67,68].

Clinical features and laboratory manifestations of drug-induced lupus are similar to those of lupus erythremato-

sus. Symptom onset can be slow or acute, although an interval of 1–2 months typically occurs before diagnosis is made. As with idiopathic lupus, the most commonly observed abnormality is the presence of anti-nuclear antibodies, and these autoantibodies usually react with chromatin; however, unlike in patients with lupus, anti-chromatin antibodies rarely react with native (double-stranded DNA. In summary, neither the clinical nor the serologic picture are specific for drug-induced or idiopatic lupus; hence, the key (albeit retrospective) diagnostic tool is the observation that drug-induced lupus symptoms usually resolve within days to weeks after discontinuing the offending drug without introduction of anti-inflammatory medications [64].

Mechanisms of hydralazine-induced lupus syndrome
Early studies suggested that interaction between hydralazine with thymidine and deoxycytidine was at least partly responsible for initiating the syndrome [69], and that an immune response to hydralazine may be important in human hydralazine-induced SLE [70].

Additional recent studies have strongly supported that SLE pathogenesis is strongly related with disorders in DNA methylation, beginning with an observation by Richardson et al. that CD8 T cells treated with 5-aza-cytidine – but not with hydroxyurea, a DNA synthesis inhibitor – re-expressed CD4, implying that DNA methylation may be one of the mechanisms involved in thymocyte maturation [71]. Subsequently, the authors showed that the 5-azacytidine-treated cloned, CD4 IL-2- dependent antigen-specific T cells lost the requirement for antigen and could be activated by autologous macrophages [72]. Autoreactivity correlated with over-expression at a transcriptional level of lymphocyte function-associated antigen-1 (LFA-1) [73], and T cells overexpressing this molecule become auto-reactive and caused a lupus-like disease in syngeneic mice [74] as it did in mice injected with syngeneic normal murine T cells treated with demethylating drugs [75,76]. Latter, it was shown that 5-azacytidine, procainamide, and hydralazine induce CD11a promoter demethylation and re-expression [77]. Further, it has been demonstrated that patients with rheumatoid arthritis and SLE had DNA hypomethylation of T cells [78], which correlates with decreased DNA methyltransferase enzyme activity and DNMT1 mRNA due to inhibited ERK pathway signaling [79]. In addition, lymphocytes from patients with lupus had at least over-expression and promoter demethylation of genes coding for CD11a [80] and CD70, a B cell co-stimulatory molecule [81]. In summary, these studies indicate that DNA hypomethylation and deregulated expression of molecules including LFA-1 and CD70 are fundamental for the pathogenesis of both drug-induced and idiopathic human lupus.
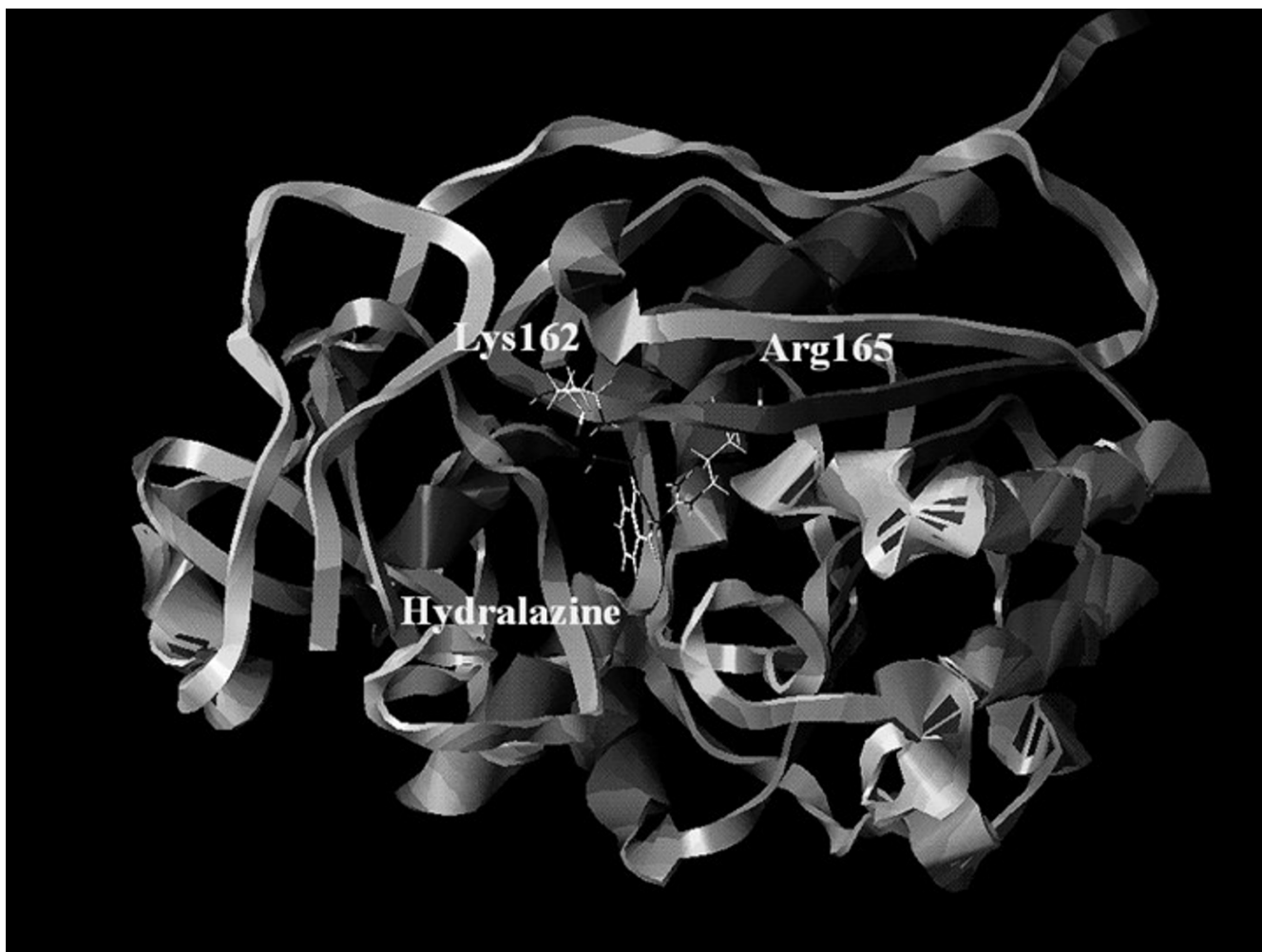
Figure 4
Docking between the enzyme DNA methyltransferase and hydralazine showing the residues at the pocket (Lys 162 and Arg 165) that interact with the hydralazine molecule (Modified from ref. 84. *Lett Drug Design Discov* 2005, **2**:282–286).

Hydralazine in demethylating and reactivating tumor suppressor genes

These observations, unrelated to the field of DNA methylation in cancer and demonstrating that hydralazine possesses DNA demethylating activity [82], led to testing the hypothesis that hydralazine, by virtue of its DNA methylation inhibitory activity, could restore tumor suppressor gene re-expression by relieving hypermethylation at the gene promoter. To that end, demethylation and re-expression of tumor suppressor genes, estrogen receptor, and *p16*, *RARβ*, which are methylated in MDA-231, T24, and MCF-7 cell lines, respectively, were studied. Treatment of these cell lines with hydralazine at 10 μM for 5 days led to gene promoter demethylation at these genes, which correlated with gene re-expression at the level of RNA and protein levels (Figures 1 and 2); in addition, it was shown that the reactivated gene product was functional. Hydrala-

zine's gene-demethylating and -reactivating effects were also observed for the estrogen receptor gene in estrogen receptor-negative MDA231 cell-xenographed mice treated for 7 days with hydralazine by intraperitoneal (i.p.) route at doses equivalent to those observed in hypertensive patients [83] (Figure 3).

With the aim of explaining hydralazine's demethylating effects, we assessed the structural and electronic properties between hydralazine and methyltransferase by modeling the most stable conformation of hydralazine and the pocket at the active site of the enzyme. Results indicate the existence of a high-affinity interaction between hydralazine and the DNA methyltransferase. The residues Lys 162 and Arg 240 within the enzyme active site demonstrated four interacting points with hydralazine (Figure 4) at distances between these residues and hydralazine nitrogen

Figure 5
In *in vitro* methyltransferase assay of hydralazine. There was no digestion with HpaII (lanes H, with 0 and 5 µM of hydralazine) indicating full methylation. Starting at 10 µM of hydralazine, bands in the lanes digested with HpaII (H) are visible, and at 20 µM the pattern of bands in HpaII (H) and MspI (M) digestions is similar indicating full demethylation. (MW: molecular weight). The substrate DNA for the *in vitro* methylation assay was a 1112 bp fragment of the type I Human Herpes Simplex virus tymidine kinase gene which has a high GC content. The methylation reaction contained 1 µg of substrate DNA and 10 units of M.SssI methylase (0.5 µmol/L, New England Biolabs, Beverly, MA) in a final volume of 30 µL. Hydralazine was added to final concentrations of 0, 5, 10, 15, and 20 µmol/L starting two hours before adding the M.SssI enzyme for the methylation reaction. Reactions were done at 37°C for 2 hours. After completion, the reaction the DNA was purified using the rapid PCR Purification system (Ijamsville, MD). Then, equal volumes of extracted DNA for each sample were placed in two separated eppendorf tubes for restriction enzyme digestion (one tube with HpaII and the other with MspI) both from New England Biolabs. Each reaction contained 10 U of the enzyme, in a final reaction volume of 50 µL at 37° for 3 hours. After digestion, samples were reduced to dryness by speedvac concentration and redissolved in 10 µL of ddH20 and then loaded into a 2% Trisborate EDTA agarose gels for electrophoresis.

atoms not exceeding 4A°. These interactions are energetically stable, supporting that hydralazine may inhibit DNA methyltransferase [84]. These results from modeling have been confirmed by experiments demonstrating the ability of hydralazine at concentrations between 10 and 20 µM to inhibit DNA methylation in an in vitro DNA methyltransferase assay (Figure 5). It remains debatable whether or not hydralazine directly inhibits DNA methyltransferase enzymatic activity. In fact, Richardson's group failed to demonstrate enzymatic inhibition in vitro with hydralazine; instead, the authors reported that hydralazine decreased RNA methyltransferase 1 and 3a expression in a similar manner to PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor, this suggesting that

hydralazine does not directly inhibit DNA methyltransferase enzymatic activity [85]. These discrepancies with regard to hydralazine's precise mechanism of action as DNA methylation inhibitor may stem from the different DNA methyltransferase assays used. Richardson's group employed a methyltransferase assay with a partially purified enzyme obtained from Jurkat T cell lysates assayed upon hemimethylated double-stranded oligonucleotides and 3H-AdoMet [85], whereas our group utilized an assay based on lack of DNA restriction after the DNA substrate was treated with M. SssI methylase enzyme; this latter assay has been used recently to characterize the demethylation inhibitory activity of newer demethylating compounds [86]. Nevertheless, the issue concerning this direct inhibition by hydralazine on the enzyme needs to be further addressed. It is remarkable, however, that our group likewise Richardson's group, also have observed decreased expression of DNMT1 and DNMT3a, but no DNMT3b mRNA by RT-PCR in MCF-7 cells after treatment with hydralazine (Figure 6).

A recent study from the Jones group compared the DNA-methylating and gene-reactivating activity of 5-aza-2'-deoxycytidine against non-nucleoside inhibitors (-)-epigallocatechin-3-gallate, hydralazine, and procainamide. These authors found that 5-aza-2-deoxycytidine is far more effective both in its DNA methylation inhibitory activity and in its ability to reactivate methylation-silenced genes in cancer cells [87]; nonetheless, the authors failed to reproduce the findings obtained by the three independent researcher groups who reported the effect of these non-nucleosides upon DNA methylation. Contrariwise, in a recent report the ability of hydralazine to demethylate and reactivate estrogen receptor gene expression was confirmed [88].

Antitumor efficacy in pre-clinical hydralazine and valproic acid models
Due to the well-known synergistic effect of DNA methylation and histone deacetylase inhibitors on gene re-expression and antitumor effects, the combined effect of hydralazine and valproic acid has been investigated in pre-clinical models. Results demonstrate that treatment with these drugs in combination possesses a strong growth inhibitory effect in several cell lines tested. Nevertheless, hydralazine alone at the concentration tested had no or small growth-inhibitory properties. With the exception of 5-aza-2'-deoxycytidine, a classical cytotoxic agent with demethylating properties [53], the *in vivo* or *in vitro* antiproliferative effects of other DNA demethylating agents such as procainamide, zebularine, tea polyphenol (-)-epigallocatechin-3-gallate, and RG108 [86] are low or absent at DNA demethylating concentrations. Interestingly, MCF-7 and SW480 were more sensitive to the combination of compounds, although the difference was
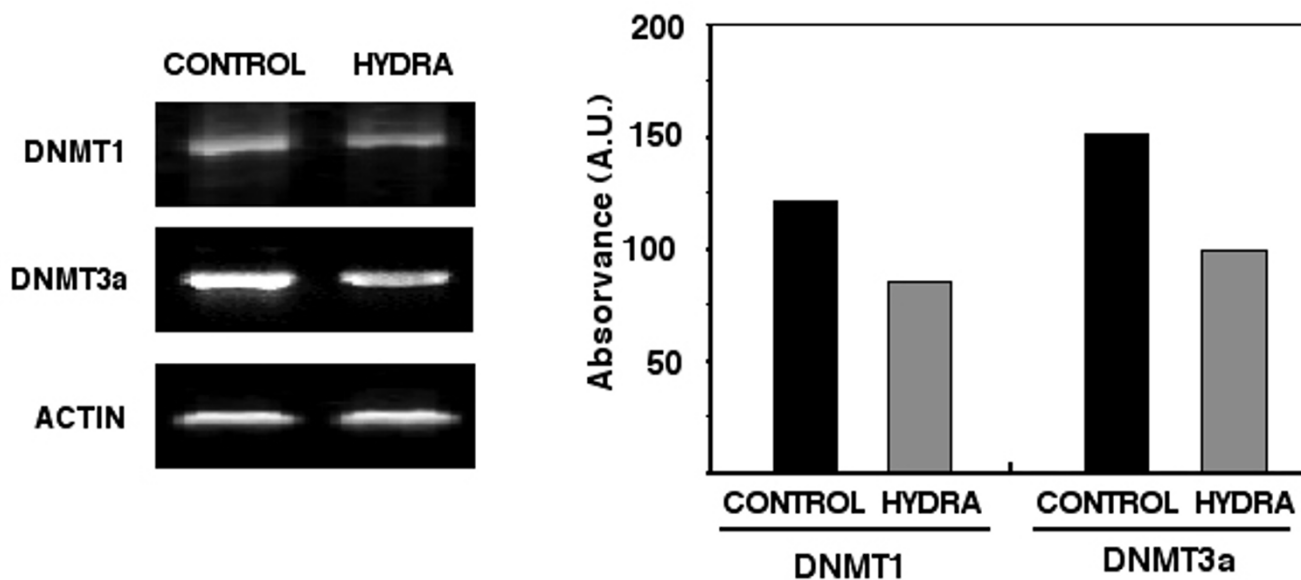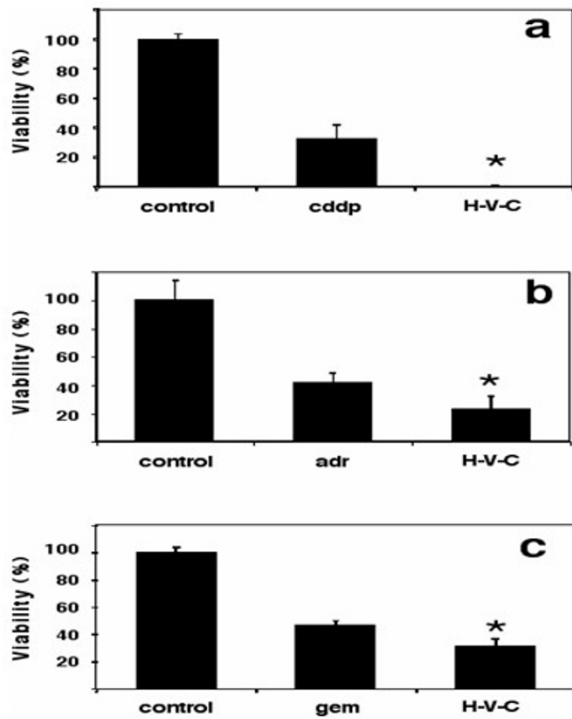
Figure 6
Expression of DNMT1 and DNMT3a in MCF-7 cells. The expression of DNMT1 and DNMT3a mRNA is decreased in MCF-7 cells treated with hydralazine. The lower band intensity was confirmed by densitometric (right) analysis, (control is untreated cells). MCF-7 cells were growth arrested for 48 hours by serum deprivation, then treated for 24 hours with hydralazine at 10 μM and then RNA extracted for analysis. Reverse transcription was carried out as previously described [110] using random hexamers, superscript II reverse transcriptase (Life Technologies) and 2.5 μg of total RNA as recommended by the manufacturer in a total volume of 50 μL. One microliter of RT reaction was used for subsequent PCR amplification for each of the desired transcripts with dNTPs and Amplitaq (Applied Biosystems). Primer sequences used were as follows: DNMT1 sense 5-gat cga att cat gcc ggc gcg tac cgc ccc ag-3 and antisense 5-atg gtg gtt tgc ctg gtg c-3. DNMT3a sense 5-ggg gac gtc cgc agc gta cac-3 and antisense 5-cag ggt tgg act cga gaa atc g-3. Amplification conditions were: 94 C° for 5 min 1 cycle; 94 C° 30 sec, (58°C for DNMT1 and 65°C for DNMT3a) annealing temperature for 1 min, and 72 C° for 30 sec, 35 cycles.

statistically significant only in SW480 cells [89] suggesting that the known synergistic effect on tumor suppressor gene re-activation by a DNA demethylating agent and a HDAC inhibitor [90] lead in some models to stronger antitumor effects [91,92].

Data on the synergy between a DNA methylation inhibitor and an HDAC inhibitor for gene re-expression led to exploration of the effect of hydralazine plus valproic acid on global gene expression. Microarray analysis utilizing the Amersham CodeLink system containing 55K genes probes demonstrated that 1,281 genes (651 up-regulated and 630 down-regulated) were differentially expressed at least three-fold differently from an untreated control. Among up-regulated genes, 153 were specifically induced by hydralazine and 178, by valproic acid; however, when cells were exposed for 4 days to hydralazine and then for 16 h to valproic acid the number increased to 352, demonstrating that these epigenomic active drugs have a synergistic effect upon gene expression [89]. Synergy between DNA methylation inhibitor 5-aza-2'-deoxycytidine and deacetylase inhibitor trichostatin A on gene re-expression
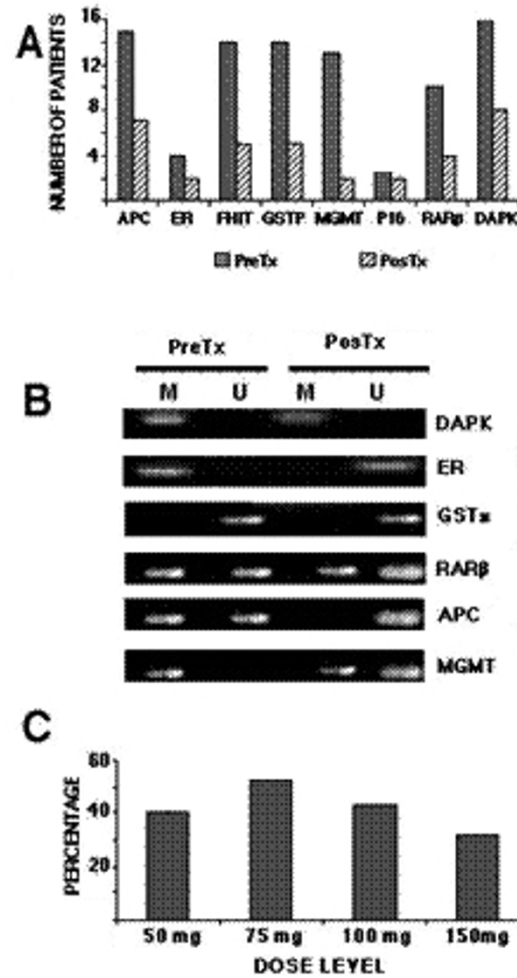
at a global scale is already known; it has been shown that the percentage of up-regulated genes with 5-aza-2'-deoxycytidine was 1.9, 1.1 with trichostatin A, but 10.4% with the combination [93], indicating that reversal of two epigenetic factors (i.e., DNA demethylation and histone hyperacetylation) synergizes gene re-expression [94]. Among genes up-regulated by hydralazine and valproic acid are those coding for major histocompatibility complex, class I-related and major histocompatibility complex, class II, DR alpha. This class I-related antigen is a powerful NKG2D ligand for NK cell-mediated antitumor immunity induction, whereas the DR alpha chain is crucial for antigen presentation within the context of class II MHC [95,49,50].

Upon any stimuli, cells must orchestrate a number of changes in gene expression, as well as in other dynamic processes such as protein phosphorylation, protein trafficking, and protein-protein interactions, to cope with cytotoxic stress [96]. Therefore, it is vital for cells to possess intact transcription regulation machinery. On this basis, it was hypothesized that malignant cells exposed to
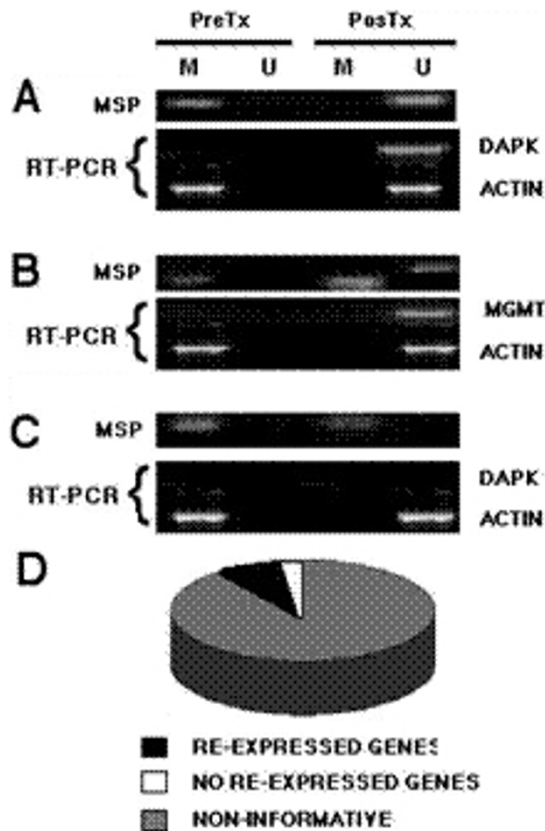
Figure 7
HeLa cells were treated for 24 hours with panel **a**: cisplatin
(cddp), panel **b**: adriamycin (adr) or panel **c**: gemcitabine
(gem) at or close to IC^{50} plus hydralazine (h) and valproic
acid (v). Afterwards medium was removed and fresh medium
containing only hydralazine and valproic acid was added for
additional 48 hours after which viability was measured. There
was a significant higher cytotoxicity of the chemotherapeutic
agent when used in combination with hydralazine plus valp-
roic acid. Cisplatin alone at 12 μM caused a reduction in via-
bility to 37% in HeLa cells which was essentially zero viability
when cells were co-treated with hydralazine plus valproic
acid (h-v-c). The increasing toxicity of the combination was
also seen with adriamycin and gemcitabine. Viability was 42%
and 45% in the chemotherapy drugs alone versus 27% and
37% respectively when hydralazine plus valproic acid were
added to chemotherapy drugs (h-v-c). (Reproduced from ref.
89. *Cancer Cell Int* 2006,**6**:2).

Figure 8
A. Pre- (dark bars) and post-hydralazine treatment (light
bars). The bars represent the number of patients that
showed methylation for each studied gene from each of the
16 patients. **B**. Representative cases of genes (M methylated,
U unmethylated; pre/post): M/M; M/U; U/U, M/U; MU/U; M/
U-M. **C**. Percentage of demethylation after treatment
according to the dose. Percentage was calculated considering
100% methylation the total number of pre-treatment methyl-
ated genes in each cohort of 4 patients (Reproduced from
ref. 74. *BMC Cancer* 2005, **5**:44).

cytotoxic chemotherapeutic drugs would have a deranged
response and that this would perhaps compromise sur-
vival if co-treated with drugs targeting two of the main
gene-regulating processes: DNA methylation and histone
acetylation. In fact, hydralazine and valproic acid
increased the cytotoxicity of chemotherapy agents cispla-
tin, doxorubicin, and gemcitabine (Figure 7) [89]; it is
noteworthy, however, that sensitization was higher for
cisplatin and doxorubicin, as could be expected because
loosening the chromatin structure by histone acetylation

increases the efficiency of anticancer drugs that target
DNA [97]. The observed chemosensitization *in vitro* was
evaluated in a murine model of fibrosarcoma using nude
mice xenografted with the sarcoma cell line HT1080.
Remarkably, while tumors receiving only adriamycin re-
grew 1 week after the adriamycin final dose, animals
treated with hydralazine plus valproic acid showed no

Figure 9
Representative cases correlating methylation and re-expression before and after hydralazine treatment. **A** is a patient treated with 75 mg/day that demethylated and re-expressed the DAPK gene. **B** corresponds to a patient receiving 150 mg/day who showed only the methylated band pre-treatment, but both bands after treatment, which correlated with re-expression of MGMT. **C** is a 50 mg/day patient which failed to demethylate the DAPK gene and therefore lacked expression. D represents the distribution of informative cases. From the 128 genes/cases, 116 were RT-PCR positive regardless of the methylation status, hence were not informative. In the remaining 12 cases, nine demethylated and re-expressed the gene. (Reproduced from ref. 74. *BMC Cancer* 2005, **5**:44).

tumor re-growth [89]. These results suggest that the combination's antitumor efficacy was higher and that therefore no microscopic residual remained or alternatively, that the treatment disabled cell proliferative capacity. This in principle may be particularly relevant for patients with minimal residual disease after curative attempt by surgery, chemotherapy, and/or radiotherapy.

Clinical studies of hydralazine and valproic acid in patients with cancer

These pre-clinical studies were the foundation for performing a phase I study to investigate whether hydralazine administered by oral route at doses commonly used for treatment of high blood pressure could demethylate and re-activate tumor suppressor gene expression in the tumors of patients with cervical cancer [98]. The study was performed in 16 newly diagnosed patients with cervical cancer with locally advanced disease receiving 50, 75, 100, or 150 mg a day for 10 days. The treatment was well-tolerated and methylation analysis of the biopsies taken prior to and after 10 days of hydralazine administration was performed in all patients. Overall, 70% (89 of 128) of pre-treatment samples analyzed (eight genes for each of the 16 pre-treatment biopsies) had at least one methylated gene, and all 16 patients had at least one methylated gene. Irrespective of the hydralazine dose administered, post-treatment biopsies showed a gene-variable demethylation rate varying from 15% (2/13 samples) for the *MGMT* gene to 67% of demethylation for the *p16* gene. Correlation between demethylation analysis and dose level revealed the following: at 50 mg a day, 40% of methylated genes suffered demethylation; at 75 mg, this was 52%; at 100 mg, the rate was 43%, and the rate was 32% for the 150-mg dose (Figure 8).

Regarding expression of the analyzed genes, 90% (116 of 128) of tumor samples expressed the messenger in the pre- and post-treatment biopsies regardless of methylation status; hence, these were not informative. Notwithstanding this, nine of 12 informative cases had the gene re-expressed after treatment: one case at 50 mg; four cases at 75 mg; one case at 100 mg, and three cases at 150 mg (Figure 9). Interestingly, at the doses tested there were no observed changes in the methylation status of imprinted and "normally methylated" genes in the DNA of peripheral blood cells. Additionally, hydralazine lowered global methylcytosine content by 1% in tumors, as evaluated by a capillary electrophoresis method [99].

It is widely accepted that cancer development has been associated with DNA methylation deregulation and aberrant histone deacetylase (HDAC) activity, which cooperate for inducing aberrant gene silencing [100]. Therefore, a number of investigation groups are currently exploring the clinical antitumor activity of a DNA methylation inhibitor plus a histone deacetylase inhibitor [101].

Among histone deacetylase inhibitors, the discovery that valproic acid, which belongs to the short-chain fatty acids category and which showed to be an effective HDAC inhibitor [102,103], encouraged investigation of this agent as a potential cancer therapy agent. We recently demonstrated, within the context of a phase I study, that

magnesium valproate when used at only slightly higher doses than those utilized as anticonvulsant, not only produces H3 and H4 histone hyperacetylation in PBMN cells, but also leads to hyperacetylation of H3 and H4 hyperacetylation and HDAC activity inhibition in primary tumors of patients with cervical cancer [104]. These data have led us to investigate the clinical efficacy of hydralazine and magnesium valproate in a series of on-going phase II studies.

Breast cancer exhibits a number of epigenetic alterations that potentially can be reverted by epigenetic therapies [105]; hence, in the first study newly diagnosed patients with locally advanced breast carcinoma receive four courses of neoadjuvant chemotherapy with adriamycin and cyclophosphamide plus hydralazine and magnesium valproate on a daily basis beginning 7 days before the first chemotherapy cycle and continuing through the end of chemotherapy, with pathologic response rate as the end point. Likewise, epigenetic alterations in cervical cancer are common [106]; hence we are performing a phase II trial in previously untreated FIGO stage IIIB cervical cancer. Patients receive standard weekly cisplatin plus radiation along with the combination of hydralazine and magnesium valproate with the aim of increasing clinical complete response rate.

On the other hand, studies have shown that DNA methylation and histone deacetylase inhibitors are able to resensitize drug-resistant tumors to established treatments [107]; hence, we are testing whether the combination of these epigenetic agents added to chemotherapy induces responses in common solid tumors such as breast, lung, cervical, ovarian, head & neck, prostate, and testicular carcinomas. In this trial, patients with measurable disease who exhibit non-response or tumor progression after three courses of palliative chemotherapy initiate with hydralazine and magnesium valproate plus the same chemotherapy scheme. Finally, we are also testing the clinical activity of the combination alone in myelodysplasic syndrome and refractory acute leukemia, because these tumor types are responsive to a combination of DNA demethylating and histone deacetylase inhibitors [108,109]. In all these trials, several parameters, including global and gene specific DNA methylation and histone acetylation, are being evaluated to gain insight into the predictive tumor response value of these molecular markers.

Conclusion
Targeting DNA methylation and histone deacetylase constitutes a promising antitumor treatment, which is also denominated epigenetic therapy. The fact that the most extensively studied nucleosides with known DNA methylation inhibitory activity, 5-azacytidine and 5-aza-2'-deox-

ycytidine, exhibit myelosuppression even when used at low doses has spurred the search for less toxic alternatives. Among non-nucleoside demethylating agents, hydralazine is a good candidate for clinical development mainly due to the fact that its extensive use for decades for cardiovascular conditions has demonstrated its tolerability and safety. Existing data show that this agent is able to demethylate and re-activate tumor suppressor gene expression in patients with cancer. Its good tolerability also permits its use in combination with an epilepsy treatment-approved histone deacetylase inhibitor. The antitumor efficacy and safety of this "epigenetic therapy" are currently being evaluated in phase II studies, along with chemotherapy and/or radiation for a variety of malignancies in different scenarios. If encouraging, results of these trials will dictate its further testing in randomized phase III trials.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
CA and MC participated in writing sections of the review, B S-P, and E P-C conducted the laboratory work, and A D-G conceived of the review and wrote the manuscript.

Acknowledgements

References
1. Herting RL, Hunter HL: **The physiologic and pharmacologic basis for the clinical treatment of hypertension.** *Med Clin North Am* 1967, **51**:25-37.
2. Klein L, O'Connor CM, Gattis WA, Zampino M, de Luca L, Vitarelli A, Fedele F, Gheorghiade M: **Pharmacologic therapy for patients with chronic heart failure and reduced systolic function: review of trials and practical considerations.** *Am J Cardiol* 2003, **91**:18F-40F.
3. Magee LA, Cham C, Waterman EJ, Ohlsson A, von Dadelszen P: **Hydralazine for treatment of severe hypertension in pregnancy: Meta-analysis.** *BMJ* 2003, **327**:955-965.
4. Powers DR, Papadakos DJ, Wallin JD: **Parenteral hydralazine revisited.** *J Emerg Med* 1998, **16**:191-196.
5. Ellershaw DC, Gurney AM: **Mechanisms of hydralazine induced vasodilation in rabbit aorta and pulmonary artery.** *Br J Pharmacol* 2001, **134**:621-631.
6. Jacobs M: **Mechanism of action of hydralazine on vascular smooth muscle.** *Biochem Pharmacol* 1984, **33**:2915-2919.
7. Bang L, Nielsen-Kudsk JE, Gruhn N, Trautner S, Theilgaard SA, Olesen S-P, Beosgaard S, Aldershvile J: **Hydralazine-induced vasodilation involves opening of high conductance Ca²⁺-activated K⁺ channels.** *Eur J Pharmacol* 1998, **361**:43-49.
8. Jounela AJ, Pasanan M, Matilla MJ: **Acetylator phenotype and the antihypertensive response to hydralazine.** *Acta Med Scand* 1975, **197**:303-306.
9. Johnston GD: **Dose response relationship with antihypertensive drugs.** *Pharmac Ther* 1992, **55**:53-93.
10. Bird A: **DNA methylation patterns and epigenetic memory.** *Genes Dev* 2002, **16**:6.21.

11. Kawasaki H, Taira K: **Transcriptional gene silencing by short interfering RNAs.** *Curr Opin Mol Ther* 2005, **7**:125-131.
12. Klenova EM, Morse HC 3rd, Ohlsson R, Lobanenkov VV: **The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer.** *Semin Cancer Biol* 2002, **12**:399-414.
13. Bird AP: **CpG-rich islands and the function of DNA methylation.** *Nature* 1986, **321**:209-213.
14. Antequera F, Bird A: **Number of CpG islands and genes in human and mouse.** *Proc Natl Acad Sci U SA* 1993, **90**:11995-11999.
15. Vilkaitis G, Suetake I, Klimasauskas S, Tajima S: **Processive methylation of hemimethylated CpG sites by mouse Dnmt1 DNA methyltransferase.** *J Biol Chem* 2005, **280**:64-72.
16. Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR: **DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells.** *Nat Genet* 2003, **33**:61-65.
17. Klose RJ, Bird AP: **Genomic DNA methylation: the mark and its mediators.** *Trends Biochem Sci* in press. 2006 Jan 3;
18. Liu K, Wang YF, Cantemir C, Muller MT: **Endogenous assays of DNA methyltransferases: evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo.** *Mol Cell Biol* 2003, **23**:2709-2719.
19. Reik W, Dean W, Walter J: **Epigenetic reprogramming in mammalian development.** *Science* 2001, **293**:1089-1093.
20. Zhu WG, Srinivasan K, Dai Z, *et al.*: **Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter.** *Mol Cell Biol* 2003, **23**:4056-4065.
21. Nguyen CT, Gonzales FA, Jones PA: **Altered chromatin structure associated with methylation-induced gene silencing in cancer cells: correlation of accessibility, methylation, MeCP2 binding and acetylation.** *Nucleic Acids Res* 2001, **29**:4598-4606.
22. Geiman TM, Robertson KD: **Chromatin remodeling, histone modifications, and DNA methylation- how does it all fit together?** *Cell Biochem* 2002, **87**:117-125.
23. Chakravarthy S, Park YJ, Chodaparambil J, Edayathumangalam RS, Luger K: **Structure and dynamic properties of nucleosome core particles.** *FEBS Lett* 2005, **579**:895-898.
24. Khorasanizadeh S: **The nucleosome: from genomic organization to genomic regulation.** *Cell* 2004, **116**:259-272.
25. Eberharter A, Ferreira R, Becker P: **Dynamic chromatin: concerted nucleosome remodelling and acetylation.** *J Biol Chem* 2005, **386**:745-751.
26. Kuo MH, Allis CD: **Roles of histone acetyltransferases and deacetylases in gene regulation.** *Bioessays* 1998, **20**:615-626.
27. Davie JR: **Covalent modifications of histones: expression from chromatin templates.** *Curr Opin Genet Dev* 1998, **8**:173-178.
28. Feinberg AP, Vogelstein B: **Hypomethylation distinguishes genes of some human cancers from their normal counterparts.** *Nature* 1983, **301**:89-92.
29. Shivapurkar N, Wilson MJ, Poirier LA: **Hypomethylation of DNA in ethionine-fed rats.** *Carcinogenesis* 1984, **5**:989-992.
30. de Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB: **The short arm of chromosome 11 is a "hot spot" for hypermethylation in human neoplasia.** *Proc Natl Acad Sci USA* 1988, **85**:5693-5697.
31. Laird PW, Jaenisch R: **DNA methylation and cancer.** *Hum Molec Genet* 1994, **3**:1487-1495.
32. Greger V, Passarge E, Hopping W, Messmer E, Horsthemke B: **Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma.** *Hum Genet* 1989, **83**:155-158.
33. Santini V, Kantarjian HM, Issa JP: **Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications.** *Ann Intern Med* 2001, **134**:573-586.
34. Szyf M, Targeti NG: **DNA methylation in cancer.** *Ageing Res Rev* 2003, **2**:299-328.
35. Bachman KE: **Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene.** *Cancer Cell* 2003, **3**:89-95.
36. Clark SJ, Melki J: **DNA methylation and gene silencing in cancer: which is the guilty part?** *Oncogene* 2002, **21**:5380-5387.
37. Ahmad M, Rees RC, Ali SA: **Escape from immunotherapy: possible mechanisms that influence tumor regression/progression.** *Cancer Immunol Immunother* 2004, **53**:844-854.
38. Zendman AJ, Ruiter DJ, Van Muijen GN: **Cancer/testis-associated genes: identification, expression profile, and putative function.** *J Cell Physiol* 2003, **194**:272-288.
39. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T: **The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation.** *Proc Natl Acad Sci USA* 1996, **93**:7149-7153.
40. Coral S, Sigalotti L, Altomonte M, Engelsberg A, Colizzi F, Cattarossi I, Maraskovsky E, Jager E, Seliger B, Maio M: **5-aza-2'-deoxycytidine-induced expression of functional cancer testis antigens in human renal cell carcinoma: immunotherapeutic implications.** *Clin Cancer Res* 2002, **8**:2690-2695.
41. De Smet C, Lurquin C, Lethé B, Martelange V, Boon T: **DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter.** *Mol Cell Biol* 1999, **19**:7327-7335.
42. Bert T, Lubomierski N, Gangsauge S, Munch K, Printz H, Prasnikar N, Robbel C, Simon B: **Expression spectrum and methylation-dependent regulation of melanoma antigen-encoding gene family members in pancreatic cancer cells.** *Pancreatology* 2002, **2**:146-154.
43. Sigalotti L, Fratta E, Coral S, Tanzarella S, Danielli R, Colizzi F, Fonsatti E, Traversari C, Altomonte M, Maio M: **Intratumor heterogeneity of cancer/testis antigens expression in human cutaneous melanoma is methylation-regulated and functionally reverted by 5-aza-2'-deoxycytidine.** *Cancer Res* 2004, **64**:9167-9171.
44. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA: **Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine.** *Mol Pharmacol* 2004, **65**:18-27.
45. Seliger B, Maeurer MJ, Ferrone S: **Antigen-processing machinery breakdown and tumor growth.** *Immunol Today* 2000, **21**:455-464.
46. Nie Y, Yang G, Song Y, Zhao X, So C, Liao J, Wang LD, Yang CS: **DNA hypermethylation is a mechanism for loss of expression of the HLA class I genes in human esophageal squamous cell carcinomas.** *Carcinogenesis* 2001, **22**:1615-1623.
47. Coral S, Sigalotti L, Gasparollo A, Cattarossi I, Visintin A, Cattelan A, Altomonte M, Maio M: **Prolonged upregulation of the expression of HLA class I antigens and costimulatory molecules on melanoma cells treated with 5-aza-2'-deoxycytidine (5-AZA-CdR).** *J Immunother* 1999, **22**:16-24.
48. Serrano A, Tanzarella S, Lionello I, Mendez R, Traversari C, Ruiz-Cabello F, Garrido F: **Rexpression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells following 5-aza-2'-deoxycytidine treatment.** *Int J Cancer* 2001, **94**:243-251.
49. Morimoto Y, Toyota M, Satoh A, Murai M, Mita H, Suzuki H, Takamura Y, Ikeda H, Ishida T, Sato N, Tokino T, Imai K: **Inactivation of class II transactivator by DNA methylation and histone deacetylation associated with absence of HLA-DR induction by interferon-gamma in haematopoietic tumour cells.** *Br J Cancer* 2004, **90**:844-582.
50. Satoh A, Toyota M, Ikeda H, Morimoto Y, Akino K, Mita H, Suzuki H, Sasaki Y, Kanaseki T, Takamura Y, Soejima H, Urano T, Yanagihara K, Endo T, Hinoda Y, Fujita M, Hosokawa M, Sato N, Tokino T, Imai K: **Epigenetic inactivation of class II transactivator (CIITA) is associated with the absence of interferon-gamma-induced HLA-DR expression in colorectal and gastric cancer cells.** *Oncogene* 2004, **23**:8876-8886.
51. Jones PA, Taylor SM: **Cellular differentiation, cytidine analogs and DNA methylation.** *Cell* 1980, **20**:85-93.
52. Abele R, Clavel M, Dodion P, Bruntsch U, Gundersen S, Smyth J, Renard J, van Glabbeke M, Pinedo HM.: **The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas.** *Eur J Cancer Clin Oncol* 1987, **23**:1921-1924.
53. Issa JP: **Decitabine.** *Curr Opin Oncol* 2003, **15**:446-451.
54. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM: **Phase I study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies.** *Blood* 2004, **103**:1635-1640.

55. Chan AT, Tao Q, Robertson KD, Flinn IW, Mann RB, Klencke B, Kwan WH, Leung TW, Johnson PJ, Ambinder RF: **Azacytidine induces demethylation of the Epstein-Barr virus genome in tumors.** *J Clin Oncol* 2004, **22**:1373-1381.

56. Goffin J, Eisenhauer E: **DNA methyltransferase inhibitors- state of the art.** *Ann Oncol* 2002, **13**:1699-2116.

57. Beaulieu N, Fournel M, Macleod A: **Antitumor activity of MG98, an antisense oligonucleotide targeting DNA methyltransferase-1 (DNMT1) [Abstract].** *Clin Cancer Res* 2001, **7**:3800S.

58. Davis AJ, Moore MJ, Gelmon KA: **Phase I and pharmacodynamic study of human DNA methyltransferase (MeTase) antisense oligodeoxynucleotide (ODN), MG98, administered as 21-day infusion q4 weekly [Abstract].** *Clin Cancer Res* 2000, **6**:4517S.

59. Aparicio A, Eads CA, Leong LA, Laird PW, Newman EM, Synold TW, Baker SD, Zhao M, Weber JS: **Phase I trial of continuous infusion 5-aza-2'-deoxycytidine.** *Cancer Chemother Pharmacol* 2003, **51**:231-239.

60. Yoo CB, Cheng JC, Jones PA: **Zebularine: a new drug for epigenetic therapy.** *Biochem Soc Trans* 2004, **32**:910-912.

61. Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W, Yang CS: **Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines.** *Cancer Res* 2003, **63**:7563-7570.

62. Lin X, Asgari K, Putzi JM, Gage WR, Yu X, Cornblatt BS, Kumar A, Piantadosi S, DeWeese TL, De Marzo AM, Nelson WG: **Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide.** *Cancer Res* 2003, **63**:7563-7570.

63. Villar-Garea A, Fraga MF, Espada J, Esteller M: **Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells.** *Cancer Res* 2003, **63**:4984-4989.

64. Rubin RL: **Drug-induced lupus.** *Toxicology* 2005, **209**:135-147.

65. Sarzi-Puttini P, Atzeni F, Capsoni F, Lubrano E, Doria A: **Drug-induced lupus erythematosus.** *Autoimmunity* 2005, **38**:507-518.

66. Alarcón-Segovia D, Worthington JW, Ward LE, Wakim KG: **Lupus diathesis and the hydralazine síndrome.** *New Engl J Med* 1965, **272**:462-466.

67. Price EJ, Venables PJ: **Drug-induced lupus.** *Drug Saf* 1995, **12**:283-290.

68. Litwin A, Adams LE, Zimmer H, Foad B, Loggie JH, Hess EV: **Prospective study of immunologic effects of hydralazine in hypertensive patients.** *Clin Pharmacol Ther* 1981, **29**:447-56.

69. Dubroff LM, Reid RJ Jr: **Hydralazine-pyrimidine interactions may explain hydralazine-induced lupus erythematosus.** *Science* 1980, **208**:404-406.

70. Yamauchi Y, Litwin A, Adams L, Zimmer H, Hess EV: **Induction of antibodies to nuclear antigens in rabbits by immunization with hydralazine-human serum albumin conjugates.** *J Clin Invest* 1975, **56**:958-969.

71. Richardson B, Kahn L, Lovett EJ, Hudson J: **Effect of an inhibitor of DNA methylation on T cells. I. 5-Azacytidine induces T4 expression on T8+ T cells.** *J Immunol* 1986, **137**:35-39.

72. Richardson B: **Effect of an inhibitor of DNA methylation on T cells. II. 5-Azacytidine induces self-reactivity in antigen-specific T4+ cells.** *Hum Immunol* 1986, **17**:456-470.

73. Richardson B, Powers D, Hooper F, Yung RL, O'Rourke K: **Lymphocyte function-associated antigen 1 overexpression and T cell autoreactivity.** *Arthritis Rheum* 1994, **37**:1363-1372.

74. Yung R, Powers D, Johnson K, Amento E, Carr D, Laing T, Yang J, Chang S, Hemati N, Richardson B: **Mechanisms of drug-induced lupus. II. T cells overexpressing lymphocyte function-associated antigen 1 become autoreactive and cause a lupus-like disease in syngeneic mice.** *J Clin Investig* 1996, **97**:2866-2871.

75. Quddus J, Johnson KJ, Gavalchin J, Amento EP, Chrisp CE, Yung RL, Richardson BC: **Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice.** *J Clin Invest* 1993, **92**:38-53.

76. Yung RL, Quddus J, Chrisp CE, Johnson KJ, Richardson BC: **Mechanism of drug-induced lupus. I. Cloned Th2 cells modified with DNA methylation inhibitors in vitro cause autoimmunity in vivo.** *J Immunol* 1995, **154**:3025-3035.

77. Kaplan MJ, Deng C, Yang J, Richardson BC: **DNA methylation in the regulation of T cell LFA-1 expression.** *Immunol Invest* 2000, **29**:411-425.

78. Richardson B, Scheinbart L, Strahler J, Gross L, Hanash S, Johnson M: **Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis.** *Arthritis Rheum* 1990, **33**:1665-1673.

79. Kaplan MJ, Deng C, Yang J, Richardson BC: **DNA methylation in the regulation of T cell LFA-1 expression.** *Immunol Invest* 2000, **29**:411-425.

80. Richardson BC, Strahler JR, Pivirotto TS, Quddus J, Bayliss GE, Gross LA, O'Rourke KS, Powers D, Hanash SM, Johnson MA: **Phenotypic and functional similarities between 5-azacytidine-treated T cells and a T cell subset in patients with active systemic lupus erythematosus.** *Arthritis Rheum* 1992, **35**:647-662.

81. Lu Q, Wu A, Richardson BC: **Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs.** *J Immunol* 2005, **174**:6212-6219.

82. Cornacchia E, Golbus J, Maybaum J, Strahler J, Hanash S, Richardson B: **Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity.** *J Immunol* 1988, **140**:2197-2000.

83. Segura-Pacheco B, Trejo-Becerril C, Pérez-Cárdenas E, Taja-Chayeb L, Mariscal I, Chavez A, Acuna C, Salazar AM, Lizano M, Duenas-Gonzalez A: **Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy.** *Clin Cancer Res* 2003, **9**:1596-1603.

84. Angeles E, Vázquez-Valadez VH, Vázquez-Valadez O, Velzquez-Sanchez AM, Ramirez A, Martinez L, Diaz-Barriga S, Romero-Rojas A, Cabrera G, Lopez-Castañares R, Dueñas-Gonzalez A: **Computational studies of 1-Hydrazinophtalazine (Hydralazine) as antineoplastic agent. Docking studies on methyltransferase.** *Lett Drug Design Discov* 2005, **2**:282-286.

85. Deng C, Lu Q, Zhang Z, Rao T, Attwood J, Yung R, Richardson B: **Hydralazine may induce autoimmunity by inhibiting extracellular signal-regulated kinase pathway signaling.** *Arthritis Rheum* 2003, **48**:746-56.

86. Brueckner B, Boy RG, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, Suhai S, Wiessler M, Lyko F: **Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases.** *Cancer Res* 2005, **65**:6305-6311.

87. Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, Jones PA: **Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine.** *Mol Cancer Ther* 2005, **4**:1515-1520.

88. Tang B, Jiang J: **[Study of the CpG methylation status of ER alpha gene in estrogen receptor alpha-negative breast cancer cell lines and the role of hydralazine demethylation].** *Zhonghua Bing Li Xue Za Zhi* 2005, **34**:283-287.

89. Chavez-Blanco A, Perez-Plasencia C, Perez-Cardenas E, Carrasco-Legleu C, Rangel-Lopez E, Segura-Pacheco B, Taja-Chayeb L, Trejo-Becerril C, Gonzalez-Fierro A, Candelaria M, Cabrera G, Duenas-Gonzalez A: **Antineoplastic effects of the DNA methylation inhibitor hydralazine and the histone deacetylase inhibitor valproic acid in cancer cell lines.** *Cancer Cell Int* 2006, **6**:2.

90. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB: **Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer.** *Nature Genet* 1999, **21**:103-107.

91. Primeau M, Gagnon J, Momparler RL: **Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells.** *Int J Cancer* 2003, **103**:177-184.

92. Zhu WG, Lakshmanan RR, Beal MD, Otterson GA: **DNA methyltransferase inhibition enhances apoptosis induced by histone deacetylase inhibitors.** *Cancer Res* 2001, **61**:1327-1333.

93. Shi H, Wei SH, Leu YW, Rahmatpanah F, Liu JC, Yan PS, Nephew KP, Huang TH: **Triple analysis of the cancer epigenome: an integrated microarray system for assessing gene expression, DNA methylation, and histone acetylation.** *Cancer Res* 2003, **63**:2164-2171.

94. Li L, Shi H, Yiannoutsos C, Huang TH, Nephew KP: **Epigenetic hypothesis tests for methylation and acetylation in a triple microarray system.** *J Comput Biol* 2005, **12**:370-390.

95. Busche A, Goldmann T, Naumann U, Steinle A, Brandau S: **Natural killer cell-mediated rejection of experimental human lung cancer by genetic overexpression of major histocompatibil-**

ity complex class I chain-related gene a. *Hum Gene Ther* 2006, **17**:135-146.

96. Alaoui-Jamali MA, Dupre I, Qiang H: **Prediction of drug sensitivity and drug resistance in cancer by transcriptional and proteomic profiling.** *Drug Resist Updat* 2004, **7**:245-255.

97. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F: **Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA.** *Cancer Res* 2003, **63**:7291-7300.

98. Zambrano P, Segura-Pacheco B, Pérez-Cárdenas E, Cetina L, Revilla-Vázquez A, Taja-Chayeb L, Chávez-Blanco A, Angeles E, Cabrera G, Sandoval K, Trejo-Becerril C, Chanona-Vilchis J, Dueñas-González A: **A phase I study of hydralazine to demethylate and reactivate the expression of tumor suppressor genes.** *BMC Cancer* 2005, **5**:44.

99. Sandoval Guerrero K, Revilla Vazquez A, Segura-Pacheco B, Duenas-Gonzalez A: **Determination of 5-methyl-cytosine and cytosine in tumor DNA of cancer patients.** *Electrophoresis* 2005, **26**:1057-1062.

100. Verma M, Maruvada P, Srivastava S: **Epigenetics and cancer.** *Crit Rev Clin Lab Sci* 2004, **41**:585-607.

101. Yoo CB, Jones PA: **Epigenetic therapy of cancer: past, present and future.** *Nat Rev Drug Discov* 2006, **5**:37-50.

102. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS: **Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen.** *J Biol Chem* 2001, **276**:36734-36741.

103. Kramer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA, Brill B, Groner B, Bach I, Heinzel T, Gottlicher M: **The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2.** *EMBO J* 2003, **22**:3411-3420.

104. Chávez-Blanco A, Segura-Pacheco B, Pérez-Cárdenas E, Taja-Chayeb L, Cetina L, Candelaria M, Cantú D, Pérez-Plasencia C, Cabrera G, Trejo-Becerril C, Angeles E, González-Fierro A, García-López P, Zambrano P, Dueñas-González A: **Histone acetylation and histone deacetylase activity of magnesium valproate in tumor and peripheral blood of patients with cervical cancer. A phase I study.** *Molec Cancer* 2005, **4**:22.

105. Asch BB, Barcellos-Hoff MH: **Epigenetics and breast cancer.** *J Mammary Gland Biol Neoplasia* 2001, **6**:151-152.

106. Duenas-Gonzalez A, Lizano M, Candelaria M, Cetina L, Arce C, Cervera E: **Epigenetics of cervical cancer. An overview and therapeutic perspectives.** *Mol Cancer* 2005, **4**:38.

107. Balch C, Montgomery JS, Paik HI, Kim S, Kim S, Huang TH, Nephew KP: **New anti-cancer strategies: epigenetic therapies and biomarkers.** *Front Biosci* 2005, **10**:1897-1931.

108. Maslak P, Chanel S, Camacho LH, Soignet S, Pandolfi PP, Guernah I, Warrell R, Nimer S: **Pilot study of combination transcriptional modulation therapy with sodium phenylbutyrate and 5-aza-cytidine in patients with acute myeloid leukemia or myelodysplastic syndrome.** *Leukemia* 2006, **20**:212-217.

109. Garcia-Manero G, Kantarjian H, Sanchez-Gonzalez B, Verstovsek S, Ravandi F, Ryttling M, Cortes J, Yang H, Fiorentino J, Rosner G, Issa J: **Results of a Phase I/II Study of the combination of 5-aza-2-deoxycytidine and valproic acid in patients with acute myeloid leukemia and myelodysplastic síndrome [abstract].** *Procc ASCO* 2005, **24**:.

110. Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, Jones PA: **The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors.** *Nucleic Acids Res* 1999, **27**:2291-2298.