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ORIGINAL ARTICLE

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Phase I trial of continuous infusion 5-aza-2′-deoxycytidine

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Abstract *Purpose*: To identify a dose of the demethylating agent 5-aza-2′-deoxycytidine (DAC) with acceptable side effects, and to study its effect on the methylation patterns of relevant genes in tumor biopsies before and after treatment with a novel methylation assay using real-time PCR. *Methods*: A group of 19 patients with metastatic solid tumors were treated with DAC by continuous intravenous infusion over 72 h, days 1–3 of a 28-day cycle. Tumor biopsies were taken before and 7 days after starting DAC. *Results*: The dose levels studied were 20, 30 and 40 mg/m^2. Grade 4 neutropenia was found in two of five patients at 40 mg/m^2 and one of six patients at 30 mg/m^2. No objective responses were seen in this study. Steady-state DAC levels of 0.1 to 0.2 μ*M* were achieved in the 30 and 40 mg/m^2 cohorts. Changes in methylation were observed, but no single gene consistently demonstrated evidence of demethylation. *Conclusions*: DAC was tolerated at a dose of 30 mg/m^2 per day for a 72-h intravenous infusion. Changes in gene methylation were observed.

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Introduction

DNA methylation is known to regulate the expression of mammalian genes and there is strong evidence that it contributes to tumorigenesis through silencing of tumor suppressor genes [38]. Methylation occurs at the C-5 position of cytosine residues in a CpG sequence. CpG dinucleotides are found at a lower than expected frequency in mammalian DNA but are clustered in CpG-rich areas called ''CpG islands''. These islands are often found close to the promoters of widely expressed genes and are usually unmethylated in normal somatic tissues, whether or not the gene is transcriptionally active. This is in contrast to tumor cells, in which aberrant region-specific hypermethylation of CpG islands located in or near gene promoters is associated with their transcriptional inactivation [18, 33, 40].

The list of genes associated with promoter region CpG island hypermethylation in human tumors has grown significantly over the past 2 years and its potential role as a therapeutic target is being actively studied. For example, loss of expression of the DNA mismatch repair (MMR) genes (*hMLH1* amongst others) results in a microsatellite instability phenotype (MSI) and is thought to contribute to neoplastic transformation in colon carcinomas. In patients with hereditary nonpolyposis colorectal cancer (HNPCC), the loss of expression is due to inherited mutations in these genes [42]. However, MSI is also seen in up to 15% of sporadic colorectal carcinomas and in these cases, structural changes of the DNA MMR genes are much less common [46]. Hypermethylation of the MLH-1 promoter has been found to correlate with loss of gene expression in a large proportion of the sporadic colorectal carcinomas with MSI [16, 22], and has also been found in gastric and endometrial carcinomas that

display a MSI phenotype [13, 24, 39]. Demethylation with 5-azacytidine or 5-aza-2′-deoxycytidine (DAC) results in reexpression of the gene in vitro and restoration of mismatch repair activity in these cancers [16, 44].

The p16 gene is another whose function is frequently lost in solid tumors, such as brain, breast, colon and head and neck cancers. This gene encodes a cyclin-dependent kinase inhibitor that plays an important role in cell cycle control. Its loss occurs mostly by deletion of the coding region and/or by hypermethylation of a CpG island in its promoter. Demethylation of this promoter with DAC has been shown to decrease the rate of tumor growth in animal models [4]. E-cadherin is a cell adhesion molecule that plays an important role in maintaining the epithelial phenotype. Downregulation of this molecule has been reported in various carcinomas and is associated with invasion and metastasis. Methylation of its 5′ CpG promoter has been shown in several carcinoma cell lines to silence its expression [47]. Interestingly, one group found that the patterns of promoter region methylation of the E-cadherin gene are heterogeneous within individual lesions, vary along the stages of malignant progression and change according to the cellular microenvironment [15].

Inhibition of DNA methylation with cytidine analogues such as 5-azacytidine and DAC has been shown to reactivate the expression of genes silenced by hypermethylation in tumor cells in vitro and in rats in vivo [4, 35, 38]. DAC is incorporated into replicating DNA and forms a covalent complex with active sites of the methyltransferase enzyme. This depletes cells of functional methyltransferase activity and results in progressive loss of DNA methylation with each round of cell division. Both demethylation and damage induced by DNA-protein complexes are thought to mediate DAC-induced toxicity [19, 27, 36]. Demethylation has been used to treat acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS) and chronic myelogenous leukemia, and to induce fetal hemoglobin in patients with sickle cell anemia and thalassemia. However, preliminary experience with hypomethylating agents in solid tumors has been disappointing because response rates have been low [26, 31, 40].

The primary endpoint of this trial was to find a dose of DAC with acceptable side effects for clinical use. A secondary objective was to study the effects of DAC on the methylation patterns of a set of 19 genes chosen because they are known to be hypermethylated in certain solid tumors, or because they are known to be involved in tumorigenesis (Table 1) [3, 11]. The MethyLight methylation assay used in this trial has been recently described by Eads et al. [10] and consists of a high-throughput quantitative methylation assay that uses fluorescence-based real-time PCR. Compared with prior methylation assays, this one has the advantage of being rapid, sensitive and specific [14, 31].

Materials and methods

Patient selection and evaluation

This trial was designed and conducted as part of the NCI-funded California Cancer Consortium. Patients with incurable advanced or metastatic solid tumors who had failed standard therapy or for whom no standard therapy existed were entered. They were required to have cutaneous, mucosal or lymph nodal lesions sufficient in number or size that two large-bore needle or excisional biopsies could be obtained. They had to be older than 18 years, have an ECOG performance status of 0 or 1 and normal hematologic, renal and liver function. All patients signed a written informed consent approved by the Institutional Review Boards of LAC/USC Medical Center and City of Hope National Medical Center.

Pretreatment evaluations included a complete history and physical examination, ECOG performance status, a complete blood count, a comprehensive metabolic panel, coagulation studies, a urinalysis and appropriate tumor markers. A chest radiograph, electrocardiogram, radiologic studies to assess the extent of measurable disease and a biopsy were also obtained prior to treatment.

Study design

A standard 3 + 3 phase I design was used in which three patients were treated at each new dose level, and all patients in a cohort were observed for at least 2 weeks after completion of their first course before escalating to a higher dose. If one of the first three patients suffered a dose-limiting toxicity (DLT), another three patients were treated at that dose level. DLT was defined as grade III nonhematologic toxicity not reversible to grade II or less within 96 h, or any grade IV toxicity related to treatment. The dose was to be doubled in each cohort of patients until an optimum biologic effect or the maximum tolerated dose (MTD) was reached. MTD was defined as the highest dose tested in which none or only one patient experienced DLT attributable to the study drug, when at least six patients were treated at that dose and were evaluable for toxicity.

Starting dose

Preclinical animal studies demonstrated induction of p16 and tumor antigen gene expression at DAC plasma levels of 0.1 to 1 μM [4, 5, 45]. In clinical studies of patients with AML, acute lymphoblastic leukemia and MDS, plasma levels in this range were achieved with DAC doses in the range 20–200 mg/m^2 [26]. In vitro experiments had shown that cytotoxicity is dependent on concentration and time of exposure, with prolonged exposure leading to greater antitumor activity [29] but prior clinical experience [26] had shown that 72 h is the longest that doses of DAC can be administered to achieve serum levels in the 0.1 to 1 μM range without severe hematologic toxicity. Based on this, we chose 20 mg/m^2 as the starting dose.

Drug administration

DAC was produced by Pharmachemie (Haarlem, The Netherlands) and was provided by the National Cancer Institute. It was supplied as a freeze-dried powder and was diluted in a total volume of 50 ml sterile water immediately prior to administration. The treatment was given to outpatients for three consecutive days through a central venous catheter using 12-h infusion cassettes that were supplied twice a day by the pharmacist and refrigerated at 4–8°C until used. DAC is unstable in aqueous solution at 25–37°C, with loss of 10–20% of activity over 24 h, accounting for the frequency of preparation. Cycles of therapy were repeated every 4 weeks in responding patients at tolerated doses.

Table 1 Genes analyzed in the tumor samples. Roles in tumorigenesis and evidenceof hypermethylation in human cancers

Gene	Tumors with hypermethylation[a]
Angiogenesis	
THBS1 (thrombospondin 1)	Colon (1)(83), stomach (32), glioblastomas (42)
Invasion and metastasis	
TIMP3 (tissue inhibitor of metalloproteinase)	Colon(2), pancreas (74), esophagus (17) stomach (32), kidney (2), lung (2) (85),breast (2), brain (2)
CDH1 (E-cadherin)	Prostate(41) (47), bladder (46) (8) (11), kidney (55), uterine cervix (15), colon(80) (83), stomach (70) (40), lung (85), breast (53), oral (52) (12), thyroid(61)
APC (adenomatouspolyposis coli)	Prostate(47), bladder (46), uterine cervix (15), stomach (22), pancreas (22), liver(22), colon (22), esophagus (17), lung (75) (77), breast (77)
CTTNB1 (β-catenin)	–
Growth regulation	
ESR1 (estrogenreceptor α)	Prostate(43) (65), breast (53), esophagus (17)
TGFBR2 (transforming growth factor receptor β II)	–
Replication regulation	
ARF (p14)	Endometrium(19), kidney (19), colon (19) (83), stomach (19), esophagus (82) (19), liver(71), nasopharynx (37), oligodendrogliomas (78)
CDKN2B (p15)	Stomach (40), esophagus (82), nasopharynx (37)
CDKN2A (p16)	Uterinecervix (15), bladder (11), colon (1) (83), stomach (40) (32), esophagus (82)(17), pancreas (74), lung (85), nasopharynx (37)
RB1 (retinoblastoma)	Retinoblastoma(62) (69)
DNA repair	
MGMT (O^6-methylguanine-DNA-methyltransferase)	Esophagus (17), colon (83), lung (85), nasopharynx (37), gliomas (21)
MLH1 (mutL homolog 1)	Endometrium (63), colon (36) (83), stomach (40) (32)
Tissue differentiation	
MYOD 1 (myogenic determinant 1)	Ovary (13), esophagus (17), breast (26)
Carcinogen detoxification	
GSTP1 (glutathioneS-transferase π)	Prostate (47), kidney (20), stomach (40), liver (72), breast (20)
Cell metabolism	
MTHFR (methylenetetrahydrofolate reductase)	–
TYMS (thymidylate synthetase)	–
CALCA (calcitonin)	Esophagus (17), breast (27)
PTGS2 (cyclooxygenase 2)	Colon (73), stomach (34)

[a]Hypermethylation found in $>10\%$ of each series of primary tumors

Pharmacokinetics

To determine the average DAC plasma concentration during the 72-h infusion, blood samples were obtained prior to and 60–90 min following the start of cassettes 1, 3, and 5. Heparinized peripheral blood (5 ml) was collected from a site distal to the site of drug infusion and kept on ice until centrifuged at 600 *g* for 10 min and at 4°C. Plasma was then transferred in 0.5-ml aliquots into vials containing 0.5 mmol (125 mg) tetrahydrouridine (THU) and stored frozen at <-70°C until analysis.

Plasma DAC levels were determined using a validated LC-MS/MS assay method described elsewhere [48]. Briefly, following the addition of an internal standard, a plasma protein precipitation step was used to clean up the sample prior to injection onto the LC system. Separation of DAC and internal standard from potentially interfering substances was achieved using reverse-phase liquid chromatography with gradient elution. Detection of DAC was performed by multiple reaction monitoring, in a Micromass Quattro tandem mass spectrometer running in positive ion mode. This assay had a limit of quantitation for DAC in plasma of 0.04 μM (10 ng/ml).

Toxicity assessment

Toxicity using the National Cancer Institute Common Toxicity Criteria was assessed on days 1 through 7, then days 15, 21 and 28. A complete blood count with differential and platelets and a comprehensive metabolic panel were repeated on days 1, 7, 15, 21 and 28. A second biopsy was obtained on day 7 and the extent of disease was reassessed 4 weeks after initiation of therapy. Objective responses were assessed using standard WHO definitions [28].

Measurement of biologic activity

Biologic activity was determined using the MethyLight assay (Fig. 1) to measure changes in methylation in tumor biopsies. Genomic DNA was isolated from tumor biopsies by proteinase K digestion as previously described [23], and sodium bisulfite conversion was performed using the agarose bead method [34]. Bisulfite-converted genomic DNA was then analyzed by the MethyLight technique [10, 11] in which the DNA is amplified by PCR, using three oligonucleotides: a probe with a 5′ fluorescent reporter dye and a 3′ quencher dye, and two locus-specific PCR primers that flank the probe. Sets of primers and probes were designed for each gene of interest to amplify completely methylated molecules. During PCR amplification, the nuclease activity of the *Taq* DNA polymerase cleaves the probe and the reporter is released. This results in a fluorescent signal that is proportional to the amount of PCR product generated and is detected by the ABI Prism 7700 sequence detection system. A reference set of primers and probes designed for a region of the β-actin gene (ACTB) lacking any CpG dinucleotides, is run in parallel with the reaction for each specific gene to normalize for DNA input. The ratio between the values obtained for these two sets of primers and probes is used as a measure of the degree of methylation at a specific locus. The specificity of the reactions for methylated DNA is confirmed

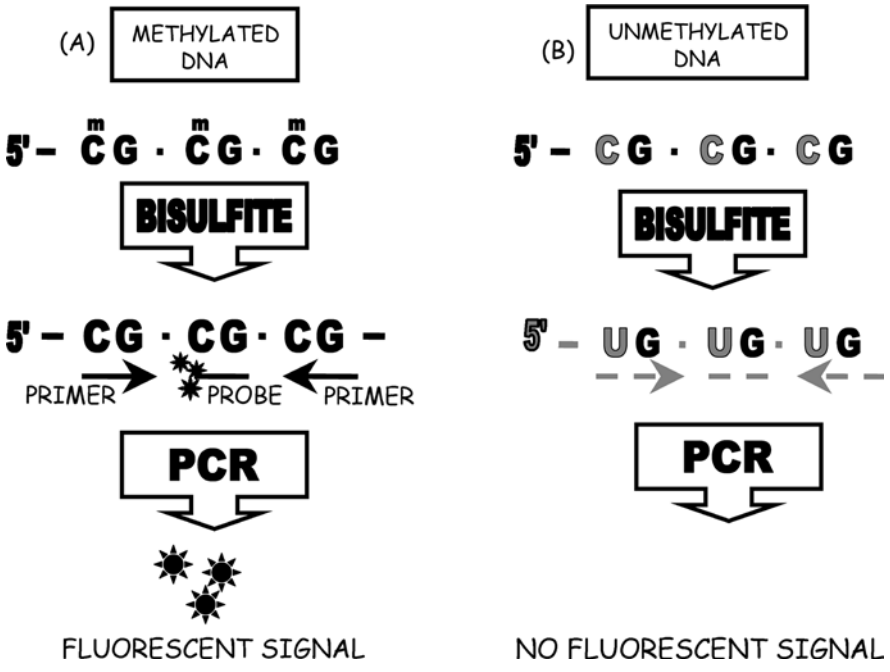

Fig. 1A, B MethyLight reaction. Treatment of DNA with bisulfite leaves methylated residues intact (**A**) but converts unmethylated cytosines to uracil (**B**). Primers and a probe linked to a fluorescein reporter are designed to amplify a fully methylated strand (**A**). These oligonucleotides will not be complementary to the unmethylated DNA because of the sequence change induced by the bisulfite treatment (**B**). The fluorophore in the probe is released during PCR amplification, resulting in a signal that is proportional to the concentration of the DNA template. A region of the β-actin gene (ACTB) that lacks CpG dinucleotides (i.e. unmethylated) is processed in the same manner as a control for DNA input. The ratio between the value obtained for the gene of interest (GENE) and the value obtained for the actin gene (ACTB) is used as a measure of methylation

separately using sperm DNA (unmethylated) and sperm DNA treated with the DNA methyltransferase SssI (fully methylated). Primer and probe sequences have been previously published [11].

Results

Patients

Of 19 patients who entered the study (9 women and 10 men; 16 Caucasian, 1 Asian and 2 Hispanic), 12 had melanoma, 4 had breast cancer and 1 each had renal cell cancer, colon and bladder cancer. All patients were extensively pretreated with chemotherapy and/or immunotherapy with a mean of three prior regimens. Their median age was 49 years (Table 2). No objective tumor responses were seen in this study. The median time to progression was 0.9 months. The median survival for the entire group of patients was 1.25 months.

Table 2 Patient characteristics ($n = 19$)

Sex	
Male	10
Female	9
Ethnicity	
Caucasian	16
Asian	1
Hispanic	2
Age(years)	
Median	49
Range	32–71
ECOG	
0	3
1	13
2	3
Diagnosis	
Melanoma of skin	12
Breast adenocarcinoma	4
Urinary bladder transitional cell	1
Colona denocarcinoma	1
Renal cell carcinoma	1

Toxicity and dose escalation

Eight patients (six evaluable for toxicity) were treated at the starting dose of 20 mg/m^2. Five (three evaluable) were given 40 mg/m^2 but unacceptable toxicity was evident at this level so an intermediate dose of 30 mg/m^2 was given to six patients (five evaluable; Table 3). Of these, one patient received two cycles of therapy. The rest progressed after only one cycle and were taken off study. At 20 mg/m^2 per day DLTs included grade 4 nausea and vomiting in one patient. Another two suffered grade 4 vomiting, one patient suffered grade 3 neuromotor toxicity and another suffered grade 3 hyperbilirubinemia and abdominal pain, but these episodes were felt to be unrelated to the treatment. At 40 mg/m^2 per day, two patients suffered grade 4 neutropenia and at 30 mg/m^2 per day one patient suffered grade 4 neutropenia and one other suffered grade 3 fatigue, constipation, nausea and neuromotor symptoms. Other toxicities are detailed in Table 3.

Table 3 Toxicities

Toxicity	Grade											
	20 mg/m^2/day ($n=8$; 6 evaluable)				40 mg/m^2/day ($n=5$; 3 evaluable)				30 mg/m^2/day ($n=6$; 5 evaluable)			
	1	2	3	4	1	2	3	4	1	2	3	4
Fatigue	–	1	–	–	1	2	–	–	–	–	1	–
Fever	1	2	–	–	1	–	–	–	–	–	–	–
Nausea	2	1	2	1[a]	1	–	–	–	–	–	1	–
Vomiting	–	2	–	3[a]	–	–	–	–	–	–	–	–
Constipation	3	2	–	–	–	1	–	–	–	–	1	–
Weakness	–	–	1	–	–	–	–	–	–	–	1	–
Anemia	1	4	–	–	1	–	1	–	1	1	–	–
Granulocytopenia	–	–	3	–	–	2	–	2	–	–	2	1
Leukopenia	1	–	4	–	–	1	3	–	–	1	1	–
Thrombocytopenia	1	–	–	–	2	–	–	–	–	–	1	–
DLT				1				2				1

[a]The grade 4 nausea and two of thegrade 4 vomiting episodes were felt to be unrelated to treatment

Biologic activity

Analysis of the methylation status of loci from a panel of 19 genes (Fig. 2) was performed on tumor biopsies taken before and 7 days after treatment with DAC in seven patients (five with melanoma and two with breast cancer) who had sufficient biopsy material both before and after DAC treatment. A posttreatment decrease in methylation was observed in five of seven patients in the methylenetetrahydrofolate reductase (*MTHFR*), calcitonin (*CALCA*) and O^6-methylguanine-DNA methyltransferase (*MGMT*) genes, in three of four patients in the estrogen receptor and p15 genes, in three of seven patients in the cyclooxygenase 2 gene and in two of four in the tissue inhibitor of metalloproteinase 3 (*TIMP3*) gene. The E-cadherin (*CDH1*) and adenomatous polyposis coli (*APC*) genes showed a decrease in DNA methylation after treatment in two of seven samples, but also a post-treatment increase in DNA methylation in three of the seven samples, as did the myogenic determinant 1 (*MYOD1*) gene. Also, although p15 is usually lost in hematopoietic malignancies and p16 in solid tumors, in our study changes in methylation were more apparent in the p15 gene than in the p16 gene. We were not able to detect a relationship between the dose of DAC and the effect on methylation.

Pharmacokinetic studies

Pharmacokinetic data were available from patients at all three dose levels, but the only detectable values were obtained from patients in the 30 and 40 mg/m^2 dose cohorts. In our study, the average plasma concentrations during the infusions in two patients at 30 mg/m^2 were 0.12 and 0.16 μM, which are at the lower end of values associated with a biologic effect (Fig. 3).

Discussion

Hypermethylation of CpG islands in cancer is associated with transcriptional silencing of genes. DAC has been shown to reverse DNA methylation and reactivate gene expression. In this trial, although doses of 20 to 40 mg/m^2 given for 72 h were relatively well tolerated (with myelosuppression being the DLT), no objective tumor responses were seen and DNA methylation did not appear to change in a consistent manner. Several observations might explain these findings.

Tumor biopsies were obtained 7 days apart, which may not be the optimal time interval to assess methylation changes. When cells in culture are treated with DAC, a decrease in methylation is apparent at 36 h and maximum demethylation is obtained at 48 to 72 h [5], after at least two to three doublings. In vivo, tumors do not grow exponentially, but follow a Gompertzian model, so that it is difficult to predict at what point sufficient cell doublings have occurred to permit detection of significant changes in methylation. Tumor biopsies were obtained from different metastatic sites in this trial, and it is possible that their methylation patterns differed at baseline making a comparison after treatment difficult to interpret. In this regard, it is interesting to note that in a study by Nass et al. [32], methylation of the estrogen receptor and E-cadherin CpG islands was shown to be heterogeneous within the same tissue in a set of breast cancer samples, such that the proportion of methylated to unmethylated alleles varied among different specimens from the same patient.

It is unclear how changes in methylation influence tumor behavior. In studies performed in acute leukemias, hypomethylation was seen after 7 days of treatment, but did not correlate with tumor response [37]. In cell cultures remethylation is observed 96 h after

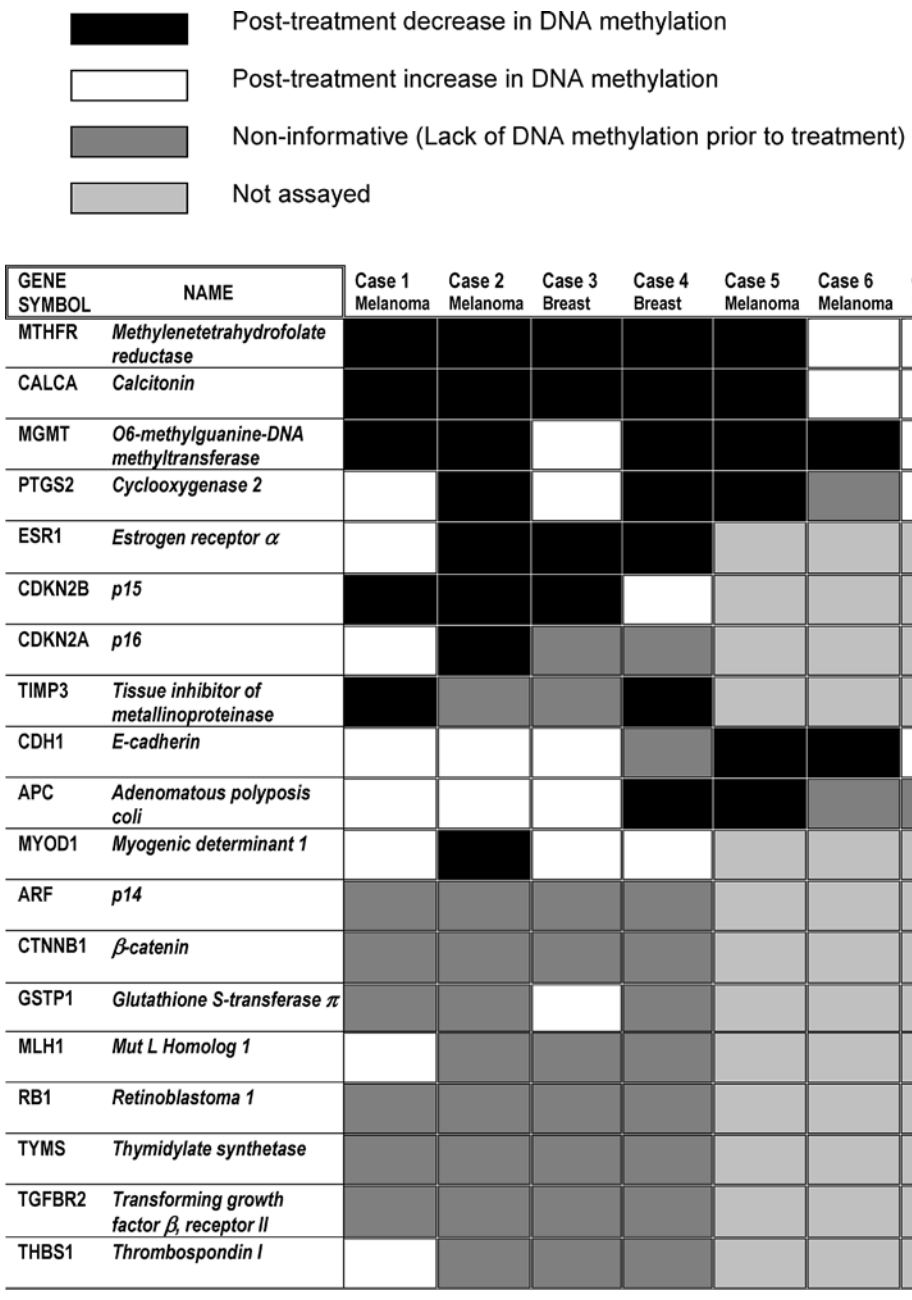

Fig. 2 Methylation changes after DAC treatment. Increases and decreases in methylation of the indicated genes are shown as indicated using the MethyLight quantitative PCR assay as described in Patients and methods. For each of the genes studied, the pretreatment GENE:ACTB ratio was divided by the posttreatment GENE:ACTB ratio. If the value obtained was greater than 1, the methylation was said to have increased. If the value obtained was less than 1, the methylation was said to have decreased. If the pretreatment GENE:ACTB value was less than 1, the DNA methylation was undetectable pretreatment and the result was reported as non-informative

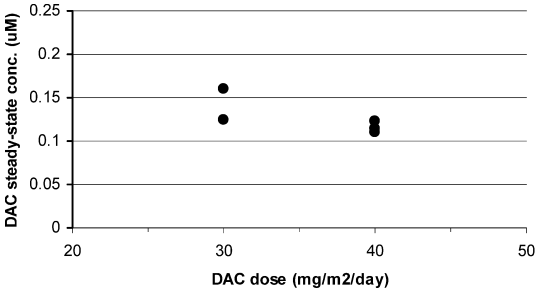

Fig. 3 Steady-state pharmacokinetic levels of DAC. Steady-state DAC levels were determined during continuous infusion of the drug as outlined in Patients and methods. The levels of DAC in the 20 mg/m^2 dose cohort were below the limit of quantitation

treatment with DAC and is a time-dependent but not a cell division-dependent process [5]. Methylation of 30–40% of CpG sites in the CpG island of p15 correlate with a state of complete gene silencing in leukemia cells but lower levels of hypermethylation do not [8]. It is known that methylation of CpG islands located downstream of a promoter in the coding regions is not associated with transcription blockade and may even be associated with increased levels of gene expression [17]. Finally, Soengas et al. [43] have recently demonstrated that the *Apaf-1* gene, a mediator of p53-dependent apoptosis, can be reactivated in melanoma cell lines after treatment with DAC, but that there are no changes in the methylation of the CpG islands of its promoter,

suggesting that a regulatory element downstream of or outside the Apaf-1 core promoter is affected. These findings imply that the site and extent of changes in methylation may be clinically relevant and, in this regard, the MethyLight assay may have limited utility, since it only registers fully methylated molecules but disregards alleles that have heterogeneous patterns of DNA methylation.

Alternate dosing schedules might be more effective in inducing demethylation and promoting an antitumor effect. The mechanism of toxicity of DAC in mammalian cells is not yet completely understood. It has generally been attributed to its inhibitory effect on DNA methylation, but Jüttermann et al. [19] suggest that the principal cause of cytotoxicity may be the covalent binding of the DNA methyltransferase to DAC-substituted DNA. Cytotoxicity of DAC is dose dependent but demethylation can occur with low doses. In our study, the steady-state pharmacokinetic values noted in patients at 30 mg/m^2 were 0.1 μM, which is at the lower end of values associated with in vitro demethylation and a biologic effect. It was not possible to achieve higher sustained levels with this schedule of administration given the significant toxicity that was encountered. However, in vitro studies suggest that a continued treatment over a prolonged period of time can increase DAC-induced gene reactivation [9]. Lower doses of DAC ranging from 0.15 to 0.30 mg/kg given 5 days a week for 2 weeks have been shown to induce hemoglobin F production in patients with sickle cell anemia [21].

In clinical trials in MDS and acute leukemias, the best clinical results have been obtained after an average of three courses of treatment, suggesting that repeated courses might be needed for clinical effectiveness [26]. To our knowledge this is the only trial of DAC in patients with solid tumors in which a continuous intravenous infusion over 72 h has been used. In most of the trials reported in the past, the drug has been administered over 1 h and the dose repeated every 8 h up to three times. The doses ranged from 25 to 120 mg/m^2. Of a total of 198 patients in eight trials reviewed, none had a complete response but 3 had partial responses, 5 had minor responses and 17 had stabilization of disease (in some cases prolonged) [1]. In one phase I-II trial, DAC was administered as an 8-h infusion to patients with non-small-cell lung cancer at doses ranging from 200 to 660 mg/m^2 every 5 to 7 weeks. Four patients showed stabilization of disease but there was a slight increase in tumor size after treatment, followed by an arrest in tumor growth, and the authors concluded that DAC has a delayed action on tumor growth, such that its antitumor activity might need to be assessed by a different endpoint other than conventional tumor response [30].

DNA methylation inhibitors may be most effective in the treatment of solid tumors when used to target specific genes in combination with other agents. Hypermethylated DNA is associated with hypoacetylated histones that are characteristic of transcriptionally silent chromatin. DNA methylation in promoter regions suppresses gene transcription by binding a multiprotein suppression complex with histone deacetylase activity, which changes the chromatin structure and limits promoter accessibility [2, 6, 12, 35, 41]. When low-dose DAC is used with the histone deacetylase inhibitor trichostatin A to treat cancer cell lines, a significant increase in gene expression is observed compared to that obtained with either agent alone [7]. 4-Phenylbutyrate and sodium butyrate, two other reversible inhibitors of histone deacetylases that have been used in vivo to increase γ-globin expression in patients with sickle cell anemia or β-thalassemia, have been also shown to have a synergistic effect with DAC in the in vitro reactivation of the *FMR1* gene responsible for the Fragile X syndrome [9]. When Soengas et al. restored Apaf-1 expression with DAC treatment in highly chemoresistant melanoma cell lines, their sensitivity to Adriamycin was significantly enhanced and the apoptotic defects associated with Apaf-1 were rescued [43]. It has also been shown that treatment with DAC results in activation of IFN pathways in cell lines [25] and leads to their sensitization to subsequent IFN treatment [20]. These observations open new clinical pathways for the use of DAC in conjunction with histone deacetylase inhibitors, chemotherapy and biotherapy agents.

We showed that methylation status is altered in different genes by treatment with continuous infusion DAC over 72 h, and provided additional clinical information regarding dosing of DAC for future assessment in clinical trials. Our plans include a phase I clinical trial of daily bolus DAC over a 4-week period starting at a low dose to address the hypothesis that prolonged exposure of tumor cells will result in more effective demethylation and upregulation of gene expression.

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