Meeting Report

State of the Translational Science: Summary of Baltimore Workshop on Gene Re-expression As a Therapeutic Target in Cancer January 2003

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ABSTRACT

A workshop was held in Baltimore, Maryland in January 2003 to discuss translational aspects of cancer therapies targeted at impacting aberrant gene transcription due to epigenetic changes. The mission of the meeting was the development of strategies for scientifically sound, clinically feasible applications targeting epigenetics in cancer therapy. Sessions included preclinical discussions of DNA methylation, the histone code, chromatin remodeling, and transcriptional control. Data on the histone deacetylase and DNA methyltransferase inhibitors under preclinical and clinical investigation were presented and discussed. The optimal correlative laboratory studies for monitoring clinical trials with these agents remain controversial. DNA methyltransferase and histone deacetylase inhibitors will be combined with each other to maximally re-express genes silenced through promoter methylation. Other classes of agents that may be rationally combined with these classes of drugs include retinoids, steroid hormones, and cytotoxic drugs.

INTRODUCTION

Specific patterns of gene expression determine the cellular characteristics of normal cells, such as position in the cell cycle or differentiation along a distinct developmental program. In a similar fashion, the pattern of gene expression in a particular tumor cell reflects underlying oncogenic lesions and represents the molecular signature that determines its biological and physiological properties. Recent progress in understanding the regulation of gene expression by chromatin organization, both in normal and cancer cells, has stirred much interest in the development of anticancer drugs that directly target these processes. Although genetic changes such as mutations and deletions may be difficult to correct, epigenetic changes which direct chromatin organization are potentially reversible. By altering the transcriptional output of a cancer cell, one may be able to change its molecular signature and achieve selective differentiation and/or tumor cell death.

Targets for drug development directed at reversing epigenetic changes include enzymes such as histone acetyltransferases, histone deacetylases (HDACs), DNA methyltransferases (DNMTs), and histone methyltransferases. These enzymes modify chromatin structure and set the patterns of gene expression via the imposition of a combinatorial array of epigenetic modifications. Modification of DNA by methylation of cytosine at CpG dinucleotide represents one example of such epigenetic mark. Although DNA methylation has been recognized for decades, the mechanisms through which it represses gene expression and its relationship to the variety of epigenetic modifications targeting nucleosomal histones (H3, H4, H2A, and H2B), such as acetylation, phosphorylation, methylation, and ubiquitination, are only now beginning to emerge. It is also becoming increasingly clear that gene silencing through DNA methylation and other epigenetic changes plays a major role in the pathogenesis of human cancer. Although the specificities of these aberrant silencing processes are poorly understood, it is likely that given a very large number of combinatorial possibilities for regulation of gene expression, combinations of chromatin-modifying enzymes that are specifically associated with a carcinogenic process can be identified and targeted therapeutically. The workshop “Clinical Translation of Gene-Re-Expression in Cancer” hosted in Baltimore, MD (17–19 January 2003) by The Sidney Kimmel Cancer Center at Johns Hopkins and The Cancer Therapy Evaluation Program of The National Cancer Institute, was organized to address the progress in the use and development of potential anticancer drugs with abilities to modify chromatin structure and gene expression.

DNA METHYLATION AND THE HISTONE CODE

In humans, DNA methylation can occur in a fifth carbon position at a cytosine base located immediately 5’ to a guanosine (the CpG dinucleotide). Approximately 50% of RNA polymerase II-dependent promoters possess clusters of CpG dinucleotides called CpG islands. In contrast to normal dividing cells, CpG island methylation is commonly observed in cancer cells and leads to transcriptional repression of adjacent genes. Very recently, the relationship between DNA methylation and the histone code has begun to unravel. Changes in DNA methylation and a variety of posttranslational modification of histones interact to dictate, or at least mark, the transcriptional activity of the local chromatin. In mammalian cells for example, methylation of lysine 9 on histone H3 and hypoacetylation of histones are usually associated with methylated DNA, heterochromatin, and gene silencing. Histone hyperacytlation,
pecific acetylation of lysine 9, and methylation of lysine 4 on histone H3, on the other hand, are associated with unmethylated DNA, euchromatin, and gene expression. It remains to be seen whether in analogy to acetylation, modification of histones through methylation is a dynamic process and whether enzymes that can demethylate histones exist. Given that changes in chromatin states can be very rapid, it is likely that some active mechanism exists for histone demethylation.

In the workshop’s opening session, Wolfgang Fischle (University of Virginia, Charlottesville, VA) described a wide array of posttranslational modifications that can be found on core histones, particularly in their NH₂-terminal tails. These modifications are thought to make up a combinatorial histone code that represents chromatin conformation and, therefore, its transcriptional state. He discussed how such a combinatorial code can be established, propagated, and read, as well as how various histone modifications can influence each other. The complexity of cross-talk between various histone modifications was illustrated with an example from yeast genetics indicating that rad6 ubiquitin E2 ligase and H2B lysine 123 ubiquitination are required for H3 lysine 4 methylation. This also illustrated that cross-talk between different histone modifications can occur in trans. Fischle described the histone code as a central element in a basic input/output circuit in which specific input signals (physiological, environmental, and so forth) lead to the writing of a code that defines a particular state of chromatin by a collection of histone modifying enzymes. The resultant histone code can then be interpreted by transcription factors or other proteins (e.g., heterochromatin protein 1 that binds to methylated lysine 9 of histone H3) and translated into chromatin conformation and gene expression patterns that are required for a particular output process (e.g., apoptosis).

In the context of such a model, Fischle then discussed the data from his laboratory (under the leadership of David Allis), which indicate that phosphorylation of histone H2B on serine 14 is characteristic of apoptotic cells. By a biochemical approach, they have identified the mammalian Ste20-like kinase 1 as a candidate enzyme that may induce apoptosis by acting downstream of caspase 3 on a substrate such as H2B serine 14. Fischle speculated that such a modification plays a role in DNA fragmentation characteristic of apoptotic cells. He also discussed recently published work that identified the leukemia-associated MLL protein, encoded by a gene related to the Drosophila trithorax (trx) and yeast histone methyltransferase SET1, as a histone H3 lysine 4-specific methyltransferase. MLL prefers to methylate acetylated histones indicating that methylation by MLL can be downstream of an acetylation step. It is interesting that despite its important functional role, the MLL SET domain is lost from every MLL leukemogenic fusion protein. Whether this loss is important for leukemogenesis remains to be established.

Recent studies have shown that CpG island methylation can often result in the loss of tumor suppressor gene expression, such as APAF in melanoma, and thus play as important a role (often complementary) in the pathogenesis of cancer as mutations do in DNA sequence. At the present time, the molecular basis for the establishment of abnormal DNA methylation patterns in cancer is not understood. Peter Jones (University of Southern California, Los Angeles, CA) discussed some of his early and recent studies addressing DNA methylation in cancer and the use of pharmacological DNA methyltransferase inhibitors such as 5-azacytidine and 5-aza-2’-deoxycytidine (Fig. 1) to reactivate the expression of silenced tumor suppressor genes. Using the T24 bladder carcinoma cell line as a model and techniques that detect methylated cytosine, such as bisulfite sequencing, Dr. Jones showed that in comparison with normal fibroblast cells the promoter region of p16INK4A was highly methylated in T24 cells. The enhanced DNA methylation correlated with the presence of methyl-CpG-binding protein 2 (MeCP2) and decreased acetylation of histone H3 and H3 lysine 9 methylation (epigenetic characteristics of inactive chromatin). Treatment of T24 cells with 5-azacytidine or 5-aza-2’-deoxycytidine cytosine analogs, which incorporate into DNA, cannot be methylated, and irreversibly bind DNA methyltransferase, completely reversed the changes associated with repressed chromatin (leading to histone H3 lysine 9 acetylation and lysine 4 methylation). This resulted in reactivation of p16INK4A expression. These changes occurred within 24 h and required an optimal concentration of 5-aza-2’-deoxycytidine, above or below which there was decrease in activity. Additionally, Peter Jones described work with a novel cytosine analog zebularine (Fig. 1). Zebularine has a potential advantage over 5-azacytidine and 5-aza-2’-deoxycytidine in that it is more stable and may be orally bioavailable. Assayed in vitro, 30 μM zebularine had the same effects on p16INK4A expression as did 5-aza-2’-deoxycytidine (~ 1 μM). At 100 μM, administered orally or s.c. to nude mice, zebularine was able to re-induce p16INK4A expression in vivo in grafted T24 cells and inhibited tumor growth by more than 50% without adverse effects on animals. Zebularine could thus be a useful clinical inhibitor of DNA methyltransferase; however, optimization of its pharmacokinetic/pharmacodynamic properties is likely to be required.

Because DNA hypermethylation is a common mechanism for the loss of tumor suppressor genes in cancer, identification of genes silenced by DNA methylation in specific cancers can be of high therapeutic, diagnostic, and prognostic value. Stephen Baylin [Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine, Baltimore, MD (SKCCC)] described a microarray approach to identifying loci silenced by DNA methylation in selected cancer cell lines. The workers at that Center have treated cells with 5-aza-2’-deoxycytidine and surveyed for re-expression of silenced gene by cDNA microarrays. A tumor-specific pattern of silenced genes could be identified. Reactivation of silenced genes has also been demonstrated in colon cancer cells by deactivation of DNMT1 and DNMT3b by gene targeting, indicating cooperativity between these DNMTs in gene silencing in cancer. Although DNA methylation invariably leads to deacetylation of histones in the corresponding chromatin regions, treatment with deacetylation inhibitors alone does not reactivate most of the genes. The HDAC inhibitor trichostatin A (TSA) potentiated the effects of DNMT inhibitors, indicating the rationale for use of 5-azacytidine or 5-aza-2’-deoxycytidine followed by an HDAC inhibitor in anticancer therapy. The results from Dr. Baylin’s laboratory on the effects of DNA demethylating agents on the expression of the MLH1 gene (encoding a DNA repair protein) in the HCT166 colon cancer cells paralleled those described by Peter Jones for the p16INK4A locus. The reversal of silencing and
negatively acting modifications around the MLH1 promoter was completed within 48 h. DNA demethylation was very rapid and was first detected by 12 h and was followed by transcript appearance within 24 h. These results suggest that gene reactivation may drive histone code reversal. However, the methods used to assay different modifications may vary in sensitivities, and one would need to look at the single cell level to definitively establish the hierarchy of events.

HISTONE DEACETYLASE INHIBITORS

The discovery of HDACs and demonstration of direct involvement of these chromatin-modifying enzymes in cancer pathogenesis, as in acute promyelocytic leukemia (APL), for example, has stimulated new interests in compounds that inhibit HDACs as potential anticancer agents. As a result, new clinical trials have been initiated using a variety of HDAC inhibitors (HDACs). The structures of the HDAC inhibitors discussed are illustrated in Fig. 2. Michael Carducci (SKCCC) discussed the results of Phase I clinical trials with sodium phenylbutyrate (PB) in refractory solid tumors and hematological malignancies. Enhancement of acetylation was not consistently seen until a dose of 400 mg of PB per kg per day was reached when given as a continuous i.v. infusion for 120 h (repeated every 21 days). This dose was well tolerated, and no major toxicities were seen. Using an oral preparation of PB in a standard dose escalation trial Carducci demonstrated that the drug was tolerated well at doses that achieved plasma concentrations shown to have biological activity in vitro. The recommended Phase II dose was 27 g per day. Although 30–35% of patients showed disease stabilization for more than 6 months, no clear antitumor activities were observed. Dose-limiting toxicity was neurocortical in nature and hypocalcemia was seen with solid formulation of PB. Other toxicities were mild and included nausea and fatigue. Continuous i.v. infusion of PB in patients with high risk and with relapsed and refractory myeloid malignancies achieved submillimolar plasma concentrations of PB that induce histone hyperacetylation in vitro. Dose-limiting toxicity was similar in these studies; suggestions of clinical activity were seen in the form of lineage responses and decreases in blast percentage. Another small fatty acid HDAC inhibitor discussed by Carducci was valproic acid (VPA), a drug used to treat certain types of seizure disorders. In laboratory studies, increasing doses of VPA
decreased colony formation of the acute myeloid leukemia (AML) ML-1 cell line and increased histone acetylation and expression of the p21<sup>Cip1/Waf1</sup> cyclin-dependent kinase inhibitor. VPA could also induce morphological changes in prostate cancer cells. Published studies have suggested that VPA may effectively combine with retinoic acid (RA) to induce terminal differentiation in primary AML cells. Because plasma concentrations targeted for the neuropsychiatric use of VPA are in the 0.5–0.75 mM range, VPA may be a convenient orally bioavailable short-chain fatty acid HDAC inhibitor. However, current studies have not investigated the relationship between the free VPA concentration in tissue culture preparations in comparison to free VPA plasma concentrations in patients with seizure and affective disorders. In the latter indications, free VPA plasma concentrations are in the 50–μM range. It will be important to establish that under the conditions in which VPA effectively

![Fig. 2 A, B, short-chain fatty acids. C, D, cyclic tetrapeptides. E, benzamide. F, G, H, hydroxamic acids; F, TSA, trichostatin A; G, SAHA, suberanoylhydroxamic acid. H, pyroxamide; I, LAQ824, LAK974; HDAC, histone deacetylase.](image-url)
targets HDAC in vitro, free VPA are within a clinically achievable range. Clinical trials of VPA, alone and in combination with retinoids and DNA MT inhibitors, are planned or are underway at several centers including Mount Sinai Medical Center in New York, Ohio State Cancer Center, University of Pennsylvania, and Heinrich-Heine-University, Duesseldorf, Germany.

John Byrd and Guido Marcucci (Comprehensive Cancer Center, Ohio State University, Columbus, OH) described their clinical experience in the treatment of refractory chronic lymphocytic leukemia (CLL) and AML with a class I selective HDAC inhibitor depsipeptide (FK228, Fujisawa). Depsipetide has been shown to induce histone hyperacetylation and apoptosis of AML and CLL cells in vitro. The drug was administered i.v. over 4 h at 13 mg/m² at days 1, 8, and 15; and this cycle was repeated every 28 days. The doses were reduced or increased based on biological outcome. Initially, the drug was well tolerated, but it was not possible to continue for more than two cycles because of progressive constitutional symptoms. Transient improvement that correlated with increased histone acetylation was observed in both CLL and AML; this included cases of tumor lysis syndrome. No sustained responses were achieved. These studies have demonstrated activity of single agent FK228, but suggest that alternative dosing schedules or the use of the drug in combination may produce better results, particularly if the profound asthenia can be ameliorated.

Susan Bates (Division of Cancer Therapeutics, National Cancer Institute, NIH, Bethesda, Maryland) described the results from Phase I and Phase II studies of depsipeptide in cutaneous T-cell lymphoma (TCL) and peripheral TCL at a dose of 14 mg/m². Overall, encouraging results were observed in these malignancies. In cutaneous TCL, two patients were in 2-year remissions, three patients achieved partial remission, and three patients were with stable disease. In the peripheral TCL group, two of eight patients achieved partial remission and one patient was with stable disease. Hyperacetylation of histones was observed in Sezary cells. The degree of histone hyperacetylation varied among patients. Enhanced expression of MDR1 and CD25 was observed. Side effects included thrombocytopenia, fatigue, and vomiting.

Ivana Gojo (University of Maryland, Greenebaum Cancer Center, Baltimore, MD) reported the use of another HDAC inhibitor, MS275 (Schering AG), in refractory malignant diseases such as acute lymphoblastic leukemia (ALL), AML, and multiple myeloma, for example. Although no significant toxicities were observed, only three patients were reported to be in stable disease, and no consistent response correlation with histone acetylation was observed. In vitro, 1 µM concentration MS275 had potent HDAC inhibitor activity, increased expression of p21<sup>Waf1/Cip1</sup>, and inhibited tumor cell growth. Optimization of dosing and schedule continue.

Qin Ryan [National Cancer Institute (NCI), Frederick, MD] described the use of MS275 in nonhematological malignancies. In her experience, this compound has shown potent antitumor activity in vitro (NCI cell lines) and in vivo tumor xenografts (such as gastric, pancreatic, and colon cancer, for example). Patients tolerated the drug well, but no clear clinical benefits have been reported. Major problems were nausea-vomiting, hypoalbuminemia, and fatigue. No evidence for WBC count decline was observed. Overall, the above results indicate that in vivo activities of HDAC inhibitors may be tumor specific and may not always correlate with in vitro effects observed. Importantly, MS-275 has been found to have an extremely long half-life, not predicted by animal models, making intermittent dosing (weekly or biweekly) possible.

Kevin Kelley (Memorial Sloan-Kettering Cancer Center, New York, NY) described the results of a clinical trial with a HDAC inhibitor suberanoylhydroxamic acid (SAHA; Merck Pharmaceuticals). SAHA is a hexamethylene-bis-acetamide derivative that, at micromolar concentrations, inhibits HDAC activity in vitro and enhances acetylation of histones in vivo. In a variety of tumor cell lines, this agent has been shown to cause growth arrest, p21<sup>Waf1/Cip1</sup> induction, and/or programmed cell death. Cancers treated during this trial include prostate, bladder, breast, and colon. The drug was administered i.v. over 2 h, 3 days a week for 3 weeks, initially at a dose of 300 mg/m². Dose escalation was carried out by increasing frequency of administration (from 3 to 5 days), increasing the number of weeks of drug administration and increasing the dosage of the drug from 300 to 900 mg/m². Dose-limiting toxicity was established at 600 mg/m² in this heavily pretreated group of patients. Toxicities included hyperglycemia, respiratory distress, and cardiac toxicity. Enhanced acetylation was observed in patients’ mononuclear cells as well as in tumor biopsies. Some patients showed a considerable reduction in tumor burden. Oral formulation of SAHA was also investigated in advanced solid tumors and refractory multiple myeloma. Most common side effects were diarrhea, dehydration, and fatigue. One patient with diffuse large-cell lymphoma achieved complete remission, and some partial responses were noted in lung and laryngeal cancer. Histone hyperacetylation was observed in each case.

Boyd Mudenda and Peter Atadja (Novartis Pharmaceuticals) described two novel class-II HDAC inhibitors, LAQ824 and LBHS589. Both compounds showed potent activity as measured by in vivo hyperacetylation of histones, up-regulation of p21<sup>Waf1/Cip1</sup>, and inhibition of tumor cell growth.

The data presented on clinical development of HDAC inhibitors demonstrated that these agents could be administered safely at concentrations that impact the acetylation of histones in tumor cells and mononuclear cells. The fact that objective tumor responses were achieved in response to monotherapy with several of the HDAC inhibitors was encouraging. The mechanism of tumor response by this class of drugs needs clarification. Critical issues in the further development of these drugs include the determination of optimal correlative laboratory studies. Many of these studies have included the qualitative or semi-quantitative measurement of histone hyperacetylation (Western analysis, immunofluorescence, or immunohistochemistry), usually in peripheral blood mononuclear cells. Because these trials have been Phase I trials, insufficient numbers of responses have been recorded to validate these assays as valuable surrogate markers. At this point, these assays are useful to indicate that the drug reaches and affects the proximal target (histone acetylation) in some tissues. Chromatin immunoprecipitation assays (ChIP) have been extensively used in vitro to detect changes in promoter-specific epigenetic chromatin modifications. Such assays have not been applied to clinical trials to date, and experts with experience in this field, including Dr. Victoria Richon.
(Merck Pharmaceuticals, Whitehouse Station, NJ) raised questions about the feasibility of such assays in the context of clinical trials.

Another critical need in this field is the understanding of the (currently) 11 known HDAC enzymes. The specific use of these enzymes alone and in combination to modify chromatin at specific promoter regions is suspected, and such specificity could direct tumor-specific targeting. A complementary need is the understanding of the specificity of currently available HDAC inhibitors as well as of HDAC inhibitors under preclinical development.

**DNA METHYLTRANSFERASE INHIBITORS**

William Nelson (Sidney Kimmel Cancer Center at Johns Hopkins) studied the methylation of the APC and GSTP1 genes during cancer progression. The results from studies of distinct tumor stages indicated that, in prostate cancer, methylation of GSTP1 occurs relatively early in the carcinogenic process and raised the question to what extent DNMT inhibitors would be useful in advanced and metastatic disease. However, during these later stages additional genes may be silenced and reaction of such genes could be of a therapeutic value. His results parallel those of others who showed that deactivation of the retinoic acid receptor β (RARβ2) gene is also an early step in carcinogenesis. Interestingly, the loss of all-trans-retinoic acid (RA) signaling in prostate cancer may also play a role in early down-regulation of GSTP1 because RA has been shown to positively regulate the expression of the latter gene. Nelson also described the need for development of direct inhibitors of DNMTs, which may have higher specificities and lower toxicities than the currently available nucleosides. The anti-arythmic procainamide may represent such a drug because it has been shown to inhibit DNA methylation, reactivate gene expression, and inhibit tumor growth without being incorporated into DNA. However, procainamide also has been associated with cardiac toxicity, which may limit its potential clinical use.

Lewis Silverman (Mount Sinai School of Medicine, New York, NY) discussed the results from Phases II and III clinical trials with 5-azacytidine (Pharmion) in myelodysplastic syndromes. In Phase II studies, excellent overall response rates of 49% and 53% for i.v. and s.c. preparations was observed with the same dosage and schedule (75 mg/m² per day for 7 days every 4 weeks). In a Phase III randomized trial, myelodysplastic syndrome patients were treated with s.c. 5-azacytidine (75 mg/m²/day for 7 days every 28 days), and their responses were compared with those of patients randomly assigned to supportive care. Compared with a 5% response rate in patients on the supportive care arm, the overall response rate in patients treated with 5-azacytidine was 60%. The relationship between clinical response, methyltransferase inhibition, and gene re-expression was not examined in that trial. Responses were durable (median, 14 months). Azacytidine was well tolerated, with mild transient myelosuppression being the most common toxicity. Dr. Silverman commented on the possibility that the use of 5-azacytidine followed by an HDAC inhibitor may lead to even better overall results.

Jean-Pierre Issa and Hagop Kantarjian (M. D. Anderson Cancer Center, Houston, TX) used low doses of i.v. 5-aza-2'-deoxycytidine (decitabine; SuperGen) in elderly patients with refractory relapsed leukemia. Summing results across doses in a Phase I trial, a 60% response rate was observed. However, responses appeared dose and schedule dependent, with the best apparent response rate seen at 15 mg/m² given as daily infusion for 10 days. These results were consistent with parabolic in vitro dose-response curve discussed by Dr. Jones (see above). Dose- and schedule-dependent decrease in p15 methylation was also observed but was not necessarily correlated with clinical response. Interestingly, the H19 imprinted locus showed no changes in methylation pattern, indicating potential specificity of therapeutic effect for aberrantly methylated promoters. This may also reflect higher order of control that exists in imprinted genes versus genes abnormally silenced in cancer cells.

James Herman (Sidney Kimmel Cancer Center at Johns Hopkins) described methods for the qualitative and quantitative detection of gene-specific promoter methylation in patient samples. Such methods are needed for close evaluation of biological response to therapeutic agent(s) and potential correlation with the clinical course. Enzymatic regional-methylation analysis is based on bisulfite sequencing and on PCR amplification using primers that are based on the expected bisulfite product and that quantitatively tag the PCR product. The resulting PCR product is then subjected to labeling by a bacterial methyltransferase and by a bacterial enzyme that detects the tag. The dual labeling enables the determination of promoter in a highly quantitative manner. Other methods of determination of gene methylation, including methylation-specific PCR, were discussed. The relative merits of Herman’s approach, studying the methylation status of a small set of genes in a specific cancer versus the use of expression microarrays to detect gene re-expression, was also the subject of active debate. The latter method has limitations including the need to study uniform populations of cells, such as isolated CD34+ cells in the case of myelodysplastic syndromes. Such studies may be limited by the amount of RNA available. The difficulty in interpreting the large amounts of data obtained is a second potential problem; however, Dr. Baylin’s laboratory (and others) has demonstrated that the application of DNMT and HDAC inhibitors to cell lines in vitro leads to alterations in genes, numbering in the low hundreds, not thousands.

The data discussed demonstrated the clinical activity of the two currently available DNMT inhibitors in myeloid malignancies. The question of whether clinical activity is mediated through DNMT inhibition, reversal of methylation, and resultant gene re-expression, remains open. The addition of new agents that could reverse methylation would be welcome. As with HDAC inhibitors, the optimal biological correlative assays are not clear; however, wide consensus existed that such assays were absolutely critical in future Phase II and III trials to ultimately establish the relationship between the clinical activity of these drugs and their molecular activity as DNMT inhibitors.

**COMBINATORIAL APPROACHES**

Guido Marcucci (Comprehensive Cancer Center, Ohio State University Medical Center, Columbus, OH) described plans to carry out Phase I studies using a combination of DNMT and HDAC inhibitors in AML. As discussed by Drs. Jones and Baylin, the rationale for such an approach is provided by the
observation that 5-aza-2'-deoxycytidine (decitabine) synergizes with HDAC inhibitors in inducing gene expression in a variety of cancer cell systems. Dr. Marcucci described his own studies with theAML1-ETO-bearing Kasumi-1 cell line in which a combination of decitabine and depsipeptide (or VPA) synergistically enhanced histone acetylation and interleukin 3 expression and inhibited growth.

Richard Momparler (Centre de Recherche, Hôpital Sainte-Justine, Quebec, Canada) described plans to study the combination of HDAC inhibitors (such as depsipeptide) and 5-aza-cytidine. Animal studies with such drug combinations indicated correlation between in vitro antitumor activity and gene re-expression (RARβ2 for example).

Steven Gore (Sidney Kimmel Cancer Center at Johns Hopkins) presented data from a dose-finding trial combining 5-azacytidine with PB in patients with high-risk myeloid malignancies. On the basis of Baylin’s model, patients are treated with 5-azacytidine s.c. before exposure to PB (administered as an i.v. continuous infusion for 7 days by ambulatory infusion device). Beginning with Silverman’s dose of 75 mg/m2/day azacytidine, plans were to decrease the azacytidine dose based on completeness of methyltransferase inhibition. However, many patients were not evaluated for that end point, and the investigators have, therefore, used enzymatic regional-methylation analysis and methylation-specific PCR to investigate changes in promoter methylation. Decreases in methylation in p15INK4B and E-cadherin have been measured, including in some patients who responded to the drug combination. Increases in histone acetylation in tumor tissue and in peripheral blood mononuclear cells have been measured as quickly as 4 h after exposure to PB. Surprisingly, many patients have demonstrated induction of histone hyperacetylation in response to 5-azacytidine before exposure to the known HDAC inhibitor. A parallel trial in solid tumor run by Dr. Carducci at SKCCC has not shown any clinical responses to date.

Roberto Pili (SKCCC) discussed RA resistance in prostate and breast tumor cell lines due to reduction of the RARβ gene expression levels. His studies showed that re-expression of RARβ2 can be achieved using HDAC inhibitors in a transgenic model of murine spontaneous prostate cancer. Subsequent treatment with RA decreased the number and volume of tumors. Observed decrease in the VGF gene expression suggests that combining antiangiogenic agents may further enhance therapeutic response.

Nancy Davidson and Richard Ambinder (SKCCC) described the use of DNMT inhibitors to reactivate genes encoding proteins that could provide additional therapeutic targets. Dr. Ambinder’s work indicated that such agents could be used to induce EBV-infected tumor cells to express viral thymidine kinase, thus selectively sensitizing these cells to gancyclovir cytoxicity. In a pilot trial, although no clinical responses were observed, some viral gene expression was reactivated. As expected, synergy was observed between 5-azacytidine and butyrate. A potential problem to overcome in this approach is the cytostatic effect of the above agents, which may stop gancyclovir from having the desired cell-killing effect. Nancy Davidson described strategies to reactivate estrogen receptor expression in hormone-resistant cancer cell types, thus rendering them responsive to further hormone therapy. Again, synergism between HDAC inhibitors and demethylating agents was observed.

Samuel Waxman (Mount Sinai School of Medicine) discussed cancer cell-specific differentiation induction and programmed cell death as therapeutic goals of epigenetic therapy (Fig. 3). Drugs designed to induce these changes can be synergistic with cytotoxic chemotherapeutic agents to induce differentiation, terminal cell division, apoptosis, diminish cancer cell recovery, and prevent drug resistance (see diagram). Synergism is schedule dependent because there is antagonism due to the induction of antiapoptotic genes if differentiation agents are used before treatment with cytotoxic agents. This has been demonstrated in the treatment of acute promyelocytic leukemia because all trans-RA induces Bf1–1 expression that inhibits anthracycline-induced apoptosis. Human colon carcinoma cells treated with fluoropyrimidines and butyrate demonstrate similar responses in vitro, and clinical trials are in progress to determine the efficacy of such combinations. Cancer cells induced to differentiate may become more responsive to receptor-specific monoclonal antibodies and hormone antagonists.

Fig. 3 Schedule for combination cytotoxic-differentiation therapy. Cytotoxic chemotherapeutic agents followed by differentiation inducers result in enhanced differentiation, terminal cell division, programmed cell death, tumor selective apoptosis, and induction of tumor-specific targets. The result is a marked reduction of tumor cell number and prevention of regrowth in between cycles of chemotherapy. (Reprinted with permission from W. H. Miller, Jr. and S. Waxman, Oncogene 21:3496–3506, 2001.)
The final session brought together a number of short presentations, which elaborated further on different combinatorial possibilities for cancer treatment. Ruben Lotan (M. D. Anderson Cancer Center, Houston, TX) discussed briefly the effects of 5-azacytidine together with RA and/or SAHA on some solid tumors. The drug 5-azacytidine was noted to stimulate RA-responsive genes in head and neck cancers and to enhanced growth inhibition by SAHA and RA in breast carcinoma. Arthur Zelent (Leukaemia Research Fund Center, Institute of Cancer Research, London, United Kingdom) described the enhancement of growth factor [granulocyte (G)- or granulocyte macrophage colony-stimulating factor (GM-CSF)-] and retinoid-induced differentiation of different AML cell lines (such as U937, KG-1, and HL60) by pretreatment with 5-azacytidine or 5-aza-2’-deoxycytidine. Wilson Miller (McGill University, Montreal, Canada) described laboratory studies that use APL as a model for overcoming resistance to differentiation therapy. He described the results obtained with a molecule involving a fusion between RA and butyric acid (compound RN1). RN1 is a more potent inducer of RA-resistant APL-cell differentiation than is the RA and sodium butyrate combination. He also described synergism between tumor necrosis factor and RA in responsive and RA-resistant APL cell lines. Steven Grant (Virginia Commonwealth University, Richmond, VA) described the role of HDAC inhibitors in cell cycle arrest and argued for combinatorial use of HDAC and other cell cycle inhibitors, such as flavopiridol, to facilitate cell cycle arrest and differentiation induction. Although no clear induction of differentiation was observed, enhanced proapoptotic effects between flavopiridol and HDAC inhibitors were noted. These effects were associated with a block in p21 induction. A Phase I clinical trial using flavopiridol and MS-275 is planned. Finally, Ed Shaw (Bowman Gray School of Medicine, Winston Salem, NC) used a combination of ionizing radiation and HDAC inhibitor in malignant glioma. One complete remission and 25% partial response of 6 months’ duration have been achieved using oral PB in conjunction with radiation.

PERSPECTIVES

There is no doubt that in the relatively short time since the discovery of HDACs and their role in the pathogenesis of APL, great progress has been made both in the understanding of mediators of gene silencing as well as in the translation of this knowledge into potential clinical use. As with any other anti-cancer agent, the drugs that target chromatin structure also face problems with specificities of action. Given the large number of combinatorial possibilities with which specific genes can be regulated, one may be optimistic that specificities in gene regulation exist and could be exploited as targets with the use of drugs developed to match such specificities.

Clearly the most challenging family of compounds are HDAC inhibitors. When used as single agents, their general therapeutic effects are relatively minor, and the potential for cardiac toxicity is high, given that class II HDACs have been shown to protect the heart from stress-induced damage. Furthermore, it is not clear whether the therapeutic effects of these agents relate to their known biochemical activities as HDAC inhibitors. Many agents exist, some related structurally to HDAC inhibitors (hexamethylene-bis-acetamide, for example), which have a potent antitumor effect and which induce gene expression without inducing histone hyperacetylation. Clearly, much work has to be done before these drugs can be fully exploited for cancer therapy. The most encouraging findings are that HDAC inhibitors can lead to synergistic effects when given in combination with demethylating agents. Good results with demethylating agents in hematological tumors suggest that they may be the best choice for drugs as first-line therapy followed by HDAC inhibitors to potentiate their effects. Encouraging is the fact that demethylating agents have limited side effects, are not profoundly myelotoxic, and do not seem to effect the expression of imprinted genes, which indicates that they may have a relatively high specificity toward genes that have been silenced during carcinogenesis. In addition, 5-aza-2’-deoxycytidine may offer antitumor immunity. This notion has been supported by results from animal models that develop auto-immune complications after 5-aza-2’-deoxycytidine administration and long-lasting remission induction in some cancer patients.

Overall, this field is in its early stages and carries great promise for future therapeutic intervention. Clearly, therapies using a combination of various agents (demethylating agents, HDAC inhibitors, drugs inducing differentiation such as RA) have the best chance for a positive outcome. Because these agents often act in synergy, their individual doses can be reduced to minimize toxic effects and optimize the therapeutic benefit of a given drug combination. A goal for the future is to understand precisely the specificities underlying the silencing of genes involved in cancer pathogenesis and to develop the means to reverse these processes with surgical precision.
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