

## Progress in Differentiation Induction as a Treatment for Acute Promyelocytic Leukemia and Beyond

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### Abstract

The Joint International Conference on Acute Promyelocytic Leukemia and Differentiation Therapy held from October 4–7, 2001 in Rome, Italy was part of a series of biannual conferences, which had its beginnings in Sardinia in 1985, with the goal of establishing differentiation induction and programmed cell death as cancer cell-selective therapies. As in the past, the organizers of this meeting joined basic and clinical investigators in workshops to establish collaboration and information exchange. Because only a portion of the conference is summarized, additional information can be obtained from the abstracts published in *Journal of Biological Regulators and Homeostatic Agents*, Volume 15, 2001. The next International Conference on Differentiation Therapy will be held in Shanghai from October 24–27, 2003.

### Introduction

APL<sup>2</sup> is the paradigm for clinically successful differentiation therapy. Consequently, the majority of the basic or clinical aspects of this conference focused on agents or mechanisms directly or indirectly stemming from experiences with this disease or its pathogenesis. The main areas of focus were molecular mechanisms underlying the processes of gene transcription, rational drug design for overcoming transcriptional repression, differentiation therapy, cellular differentiation, terminal cell division, and apoptosis. Updates of major multicenter clinical trials for treatment of APL were also presented at the meeting.

### Transcriptional Regulation of Differentiation and Apoptosis

Oncogenic processes, particularly in AML, often result from single mechanisms that regulate gene transcription. Mechanisms of normal and aberrant transcriptional regulation through modification of chromatin structure due to changes in core histone methylation and acetylation were discussed. HMTs, characterized by a conserved SET domain, can be recruited by transcription factors to DNA target sites. MeCP2 recruits complexes containing HDACs to methylated DNA and also recruits HMTs, therefore directly linking DNA and histone methylation. This is demonstrated by the role of the Suv39H1-HPI complex in the transcriptional corepressor function of Rb (Fig. 1). Tony Kouzarides (Wellcome CRC Institute, Cambridge, United Kingdom) reported that deacetylation and methylation of histone H3 at lysine 9 were followed by recruitment of heterochromatin-binding proteins and general gene silencing. In contrast, methylation of lysine 4 by the newly characterized SET-1 HMT

prevented recruitment of HDAC-containing NuRD complex and led to activation of gene transcription. An arginine-specific HMT, CARM-1, has also been discovered. These enzymes have a high substrate specificity and may be targets for cancer therapy.

KAP-1, a transcriptional corepressor protein, has the capability to both bind to sequence-specific DNA-binding subunits (KRAB domain-contacting zinc finger proteins) and recruit NuRD complexes containing HDAC and heterochromatin proteins (HP1s; Frank Rauscher III; Wistar Institute, Philadelphia, PA). KAP-1 associates with KIS, a novel protein containing a SET domain that has histone methylase activity specific for lysine 9 of histone H3.

Miguel Beato (Centre de Regulacion Genomica, Barcelona, Spain) discussed studies on hormonal induction of the mouse mammary tumor virus promoter and its regulation by chromatin remodeling using positioned nucleosomes. Positioned nucleosomes account for constitutive repression and participate in hormonal induction by mediating the reciprocal synergism between hormone steroid receptors and the transcription factor NF1 by ATP-dependent nucleosome remodeling.

### Strategies for Induction of Terminal Cell Division and Selective Apoptosis in Malignancies

Various kinase pathways for inducing differentiation and selective apoptosis were presented. DAP-k is required for apoptosis induction in a number of tissues (Adi Kimchi; Weizmann Institute of Science, Rehovot, Israel). DAP-k dominant negative mutants block oncogene-induced cell death, and expression of DAP-k is inactivated by DNA methylation in a number of human tumors. Understanding which DMT and HMT may be involved in silencing of DAP-k gene will be important for targeted reactivation of its expression in tumor cells and selective apoptosis induction.

Another signaling cascade initiated by Ser/Thr kinase(s) critical for phorbol myristate acetate-induced macrophage differentiation was dissected by Eli Huberman (Argonne National Laboratory, IL). This pathway is initiated by protein kinase C $\beta$ , which turns on a novel kinase named PRKX, which is ultimately wired to production, release, and deposition of fibronectin, an end product that is required for macrophage differentiation. Dr. A. Bonati (Institute of Medical Pathology, University of Parma, Parma, Italy) described a third type of kinase cascade whose targeting inhibits the ERK pathway and proliferation of AML blasts. An inhibitor of ERK-1 down-regulates the ERK kinase, resulting in growth inhibition and apoptosis.

Hinrich Gronemeyer (IGBMC, Strasbourg, France) described that ATRA-induced differentiation of leukemia cells and programmed cell death by activation of the proapoptotic death receptor ligand TRAIL. He also described an alternate differentiation pathway of APL cells in response to RXR and protein kinase A agonists. In the absence of protein kinase A agonist, RXR ligands cause leukemia cells to short-circuit the differentiation pathway and undergo apoptosis.

Dr. Dan Lieberman (Fels Institute for Cancer Research and Molecular Biology, Philadelphia, PA) used subtractive cDNA libraries to

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<sup>2</sup> The abbreviations used are: APL, acute promyelocytic leukemia; HMT, histone methyltransferase; HDAC, histone deacetylases; DAP-k, death-associated protein kinase; ERK, extracellular signal-regulated kinase; AML, acute myelogenous leukemia; ATRA, all-*trans*-retinoic acid; RXR, retinoid X receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; RAR, retinoic acid receptor; CHT, chemotherapy; AraC, 1- $\beta$ -D-arabino-furanosylcytosine; PB, sodium phenylbutyrate; TSA, trichostatin A; TPX, trapoxin; 5-AC, 5-azacytidine; DMT, DNA methyltransferase; PML, promyelocytic leukemia.

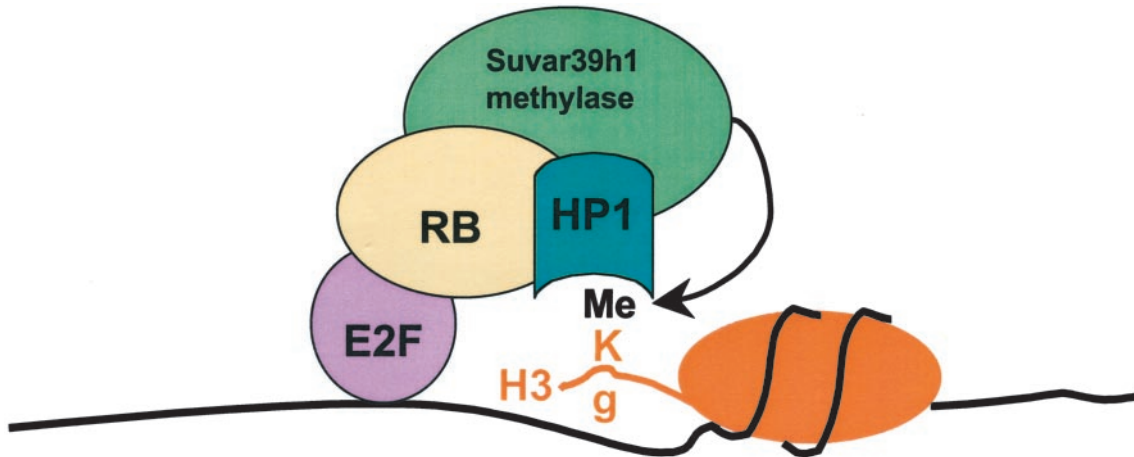


Fig. 1. The role of the Suv39H1-HP1 complex in the transcriptional corepressor function of Rb.

identify three members of the GADD 15 family that are directly induced during myeloid terminal differentiation. Biochemical data, transfection-based experiments, and results from knockout mice suggested that these genes regulate homeostasis of hematopoietic tissues by modulating growth arrest and apoptosis. These genes also have an important role in DNA repair.

**Differentiation Induction of APL and Other Acute Leukemias**

Understanding the molecular mechanisms involved in the differentiation block causing APL (Fig. 2) could have broad application in cancer therapeutics. Pier Giuseppe Pelicci (European Institute of Oncology, Milan, Italy) observed that HDACs may serve as common molecular targets in AML. Additionally, he described the involvement of DMT in the function of APL-associated oncoproteins, thus pointing out that HDAC inhibitors and demethylating agents may be a useful combination in antileukemic therapy.

Anne Dejean (Institut Pasteur, Paris, France) described that some HDACs, particularly HDAC1, HDAC4, and a related protein called MITR, can be modified *in vivo* by small ubiquitin-like protein SUMO-1.

This modification appeared to stimulate HDAC activity, thus identifying an additional level of potential therapeutic intervention.

Arthur Zelent (Institute of Cancer Research, London, United Kingdom) described cloning of a novel class II HDAC (HDAC9) with predominant expression in the B-cell lineage with abnormal activities in B-cell malignancies.

Zhu Chen (Shanghai Institute of Hematology, Shanghai, China) described the clinical experience of using arsenic trioxide ( $As_2O_3$ ) to overcome ATRA-resistant APL.  $As_2O_3$  degrades PML RAR $\alpha$  protein and induces partial APL cell differentiation. He also discussed the effectiveness of  $As_2O_3$  in the treatment of chronic myelogenous leukemia, multiple myeloma, and non-Hodgkin's lymphoma where partial responses, perhaps due to induction of apoptosis, have been observed.  $As_2O_3$  displays minimal cross-resistance with other chemotherapeutic agents, making it a useful drug. Dr. A. Kakizuka (Kyoto University, Kyoto, Japan) observed that  $As_2O_3$ -induced apoptosis of APL cells is associated with ASK-1 kinase recruitment to the reorganized PML bodies, and this activates a death kinase cascade consisting of SEK-1 and c-Jun NH<sub>2</sub>-terminal kinase.

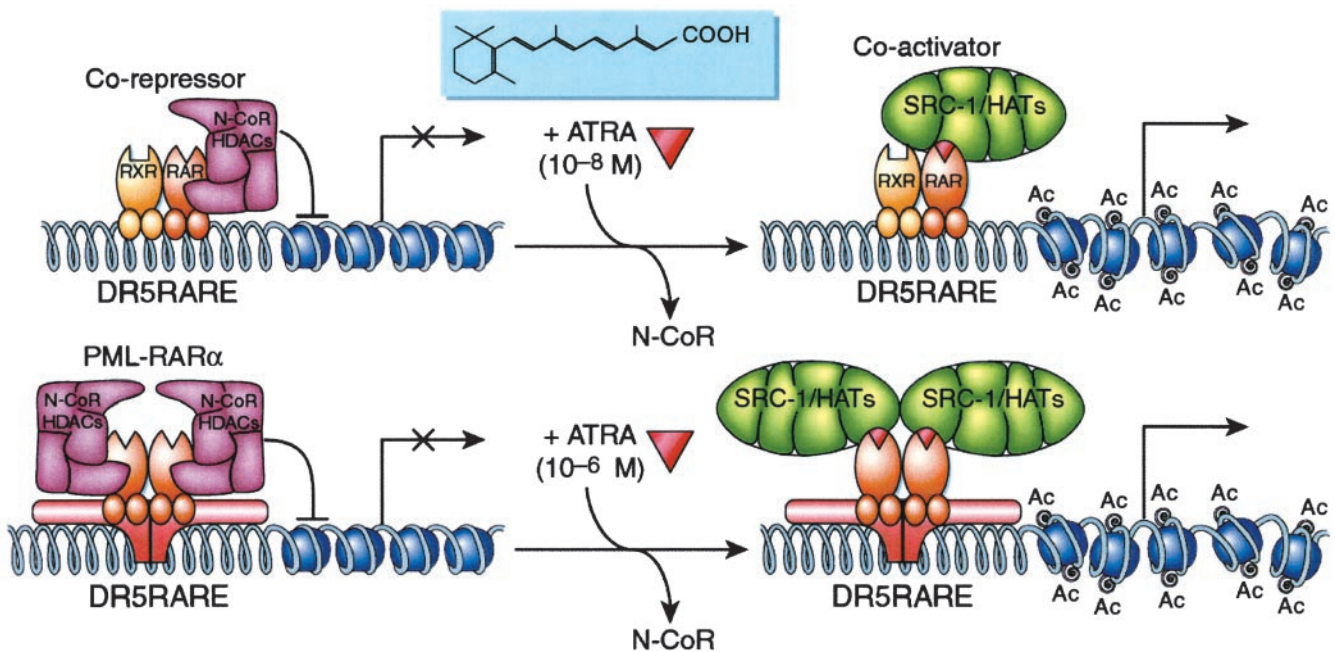


Fig. 2. Mechanism underlying the molecular pathogenesis of APL and its response to ATRA.

Dr. E. Garattini (Istituto di Ricerche Farmacologiche "Mario Negri," Bergamo, Italy) examined the ability of the tyrosine kinase inhibitor imatinib mesylate (STI-571) to enhance differentiation therapy. Imatinib mesylate, which is itself inactive, enhanced differentiation and apoptosis of HL-60, U937, NB4, and partially retinoic acid-resistant NB4.R1 cell lines treated with suboptimal concentrations of ATRA. Differentiation enhancement required RAR $\alpha$ - but not RXR-specific retinoids. Degradation of RAR $\alpha$  and PML RAR $\alpha$  was slower when imatinib mesylate was added, suggesting inhibition of the proteasome complex. Phosphorylation of c-abl, but not c-kit, was inhibited in NB4 cells after treatment with imatinib mesylate. Such inhibition of phosphorylation was seen in the presence or absence of retinoic acid and thus is not sufficient for induction of differentiation.

Pier Paolo Pandolfi (Memorial Sloan-Kettering Cancer Center, New York, New York) described the function of the *POKEMON* gene, which was disrupted by homologous recombination. *POKEMON*, which encodes a PLZF-related POZ domain zinc finger transcriptional repressor, appears to be required for terminal differentiation of multiple cell lineages and may be a target in tumorigenesis.

### Answered Questions and Investigational Issues in the Treatment of APL

Updated results on front-line therapy of APL obtained in several large multicenter studies were presented at the meeting. These included trials conducted by the French-European (Dr. H. Dombret), MRC (United Kingdom; Dr. Alan K. Burnett), GIMEMA (Italy; Dr. G. Avvisati), PETHEMA (Spain; Dr. M. A. Sanz), University of Texas M. D. Anderson Cancer Center (Houston, TX; Dr. E. Estey), JALSG (Japan; Dr. R. Ohno), and ALLG (Australia and New Zealand; Dr. H. Iland) groups. Based on these studies (which, taken together, recruited more than 2000 patients), a number of therapeutic advances were established where a consensus does exist. Meanwhile, several issues that remain controversial were pointed out.

Combined anthracycline-containing CHT and ATRA provide the best results in terms of complete remission and long-term disease-free survival (Fig. 3). The simultaneous use of ATRA and CHT is superior to the sequential ATRA plus CHT scheme and probably allows better control of the ATRA syndrome. The inclusion of AraC in the CHT backbone does not seem to add significantly in terms of antileukemic effect, and this drug is indeed omitted from the induction phase in several trials. The PETHEMA, University of Texas M. D. Anderson Cancer Center, and Australasian studies also omitted AraC from consolidation. The role of AraC is being tested in a randomized fashion in the new ongoing trial of the French group.

*Molecular remission* (absence of PML RAR $\alpha$  transcript) is now widely accepted as a more advanced therapeutic goal in this disease because it predicts for increased probability of long-term disease-free survival. Patients with *molecular relapse* who receive early salvage therapy seem to survive longer than those who are treated when in overt relapse, based on preliminary data of the GIMEMA.

Novel agents (or revisited old compounds) such as the calicheamicin anti-CD33 conjugate (Mylotarg) and arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) are being used successfully in both untreated and relapsed patients, but long-term results on efficacy and toxicity of these drugs are still awaited. The role of As<sub>2</sub>O<sub>3</sub> in front-line consolidation after an ATRA plus CHT induction is currently being tested in a randomized study by the United States Intergroup.

The inclusion of ATRA in maintenance therapy has been shown to result in better disease-free survival in the United States Intergroup and French studies. The schedule of ATRA administration, its combination with low-dose CHT, and overall duration of maintenance therapy are issues currently under investigation.

Early death and disease relapse still represent the main obstacles to

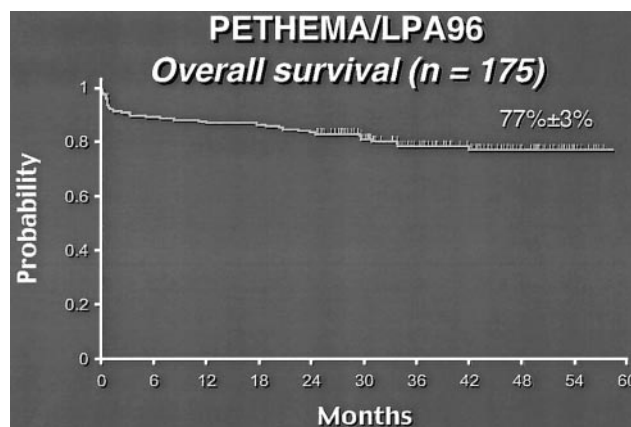


Fig. 3. Combined anthracycline-containing CHT and ATRA provide the best results in terms of complete remission and long-term disease-free survival.

final cure in this disease. Death during induction is most frequently linked to the life-threatening coagulopathy observed at presentation. No consensus exists for criteria for high-risk patients, supportive measures, and therapeutic approaches to counteract the coagulopathy.

Better identification of risk categories at diagnosis is needed for more appropriate targeting of treatment intensity. The 80% long-term survival in APL is also associated with severe late sequelae such as secondary myelodysplasia/AML. This suggests that studies should be designed to spare low-risk APL patients from excessive CHT-induced toxicity. Conversely, high-risk disease should be better defined with tailored investigation. Given the relatively low numbers we are dealing with, it appears that cooperation at the national and international level is urgently needed to better address most of the above-mentioned clinical research issues.

### Clinical Trials: Differentiation Therapy in Non-APL Hematological Malignancies

Alan K. Burnett (University of Wales College of Medicine, Cardiff, United Kingdom) described the role of antibody-targeted therapy (anti-CD33 antibody linked to calicheamicin toxin) in combination with different chemotherapeutic protocols (daunorubicin/AraC/thioguanine or fludarabine/AraC/granulocyte colony-stimulating factor/idarubicin) for induction and consolidation in AML with monitoring of minimal residual disease using quantitative PCR.

Dr. S. D. Gore (Johns Hopkins Oncology Center, Baltimore, MD) presented preliminary data from a Phase I trial examining the feasibility of treating patients with myelodysplastic syndrome and AML sequentially with 5-AC, a DMT inhibitor, followed by PB, a HDAC inhibitor. Administration of PB led to increased acetylation of histones H3 and H4 in peripheral blood mononuclear cells and bone marrow mononuclear cells in many patients. Administration of 5-AC led to modest inhibition of DMT activity. Reversal of methylation in the p15<sup>INK4B</sup> promoter was demonstrated in patients. There is interest in adding other drugs that may effect more complete differentiation (for example, hematopoietic growth factors). This approach may be particularly interesting to study in patients with leukemias with specific fusion genes that recruit the transcriptional repression complex (such as AML-ETO), potentially in the postremission minimal residual disease state.

### Strategies for Differentiation Induction in Solid Tumors

Studies were described to extend the successful use of differentiation therapy to solid tumors. Reuben Lotan (The University of Texas M. D. Anderson Cancer Center) reported selective killing of non-small cell lung cancer cells by adamantyl retinoid CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid). This selectivity was

achieved through activation of TRAIL receptor DR-5 in tumor cells, but not in normal lung epithelial cells. Normal epithelial cells display elevated levels of decoy receptor DcR-2, which protects cells from TRAIL-induced apoptosis. Use of recombinant TRAIL and CD437 produced synergistic results. CD437 induced apoptosis in a number of other cancer cell lines and in human tumor xenografts.

Michel Sporn (Dartmouth Medical School, Hanover, NH) described a synergistic effect between peroxisome proliferator-activated receptor- $\gamma$  agonist CDDO (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) and rexinoids LG1069 and LG100268 in inducing adipocytic differentiation, suggesting therapeutic opportunities in liposarcoma.

Samuel Waxman (Mount Sinai School of Medicine, New York, NY) furthered the concept of combinatorial therapy in cancer, such as the use of proapoptotic agents and differentiation inducers. In a screen for drugs that stimulate induction of differentiation by ATRA, a compound of the dithiophene family was identified that enhanced differentiation of NB4 cells at nanomolar concentrations through a HDAC-independent mechanism. At higher concentrations (100 nM), this compound had strong proapoptotic activities in several cancer cell lines including melanoma.

Dr. C. P. Reynolds (Children's Hospital of Los Angeles, Los Angeles, CA) described the use of 13-*cis*-retinoic acid for the treatment of stage IV neuroblastoma, in which the N-myc gene is frequently amplified. Retinoic acid therapy down-regulated myc expression. In Children's Cancer Group Study 3891, children with advanced-stage neuroblastoma were randomized to receive CHT at conventional doses or at high doses with stem cell support. Both groups underwent a second randomization to receive 13-*cis*-retinoic acid (165 mg/m<sup>2</sup> daily for 2 weeks/month) or observation for 6 months after therapy. The only group that had statistically superior survival was the group treated with high-dose therapy followed by 13-*cis*-retinoic acid (50% versus 30%). 13-*cis*-Retinoic acid has now been incorporated as a standard treatment after high-dose therapy in subsequent studies.

Development of compounds that inhibit specific HDACs was described by Minoru Yoshida (University of Tokyo, Tokyo, Japan). TSA and TPX are prototypic HDAC inhibitors that induce cell differentiation, cell cycle arrest, and reversal of transformed phenotype but are not specific to a given HDAC. Newer compounds such as FK228, MS-275, or SAHA also possess nonspecific HDAC-inhibiting activities, can inhibit *in vivo* solid tumor growth, and are now in clinical trials. By molecular design, the Yoshida group has created a wide range of compounds (called CHAP for cyclic hydroxyamic acid-containing peptide) that are structural hybrids between TSA and TPX. They have replaced the epoxyketone group of TPX with hydroxyamic acid of TSA. These compounds show 10–100-fold higher activities toward class I than class II HDACs, lack activity against HDAC6, and exhibit antitumor activities *in vivo*. HDAC6 localizes in cytoplasm and specifically deacetylates  $\alpha$ -tubulin, which can be effectively inhibited by TSA. These studies demonstrated the feasibility of developing chemical agents that specifically inhibit a given HDAC.

George Atweh (Mount Sinai School of Medicine) described the schedule-dependent use of a HDAC inhibitor (arginine butyrate) in the treatment of hemaglobinopathies by increasing levels of fetal  $\gamma$ -globin synthesis. Continuous use of butyrate alone was associated with transient responses, whereas intermittent cycles of this drug led to more sustained clinical responses. The use of hydroxyurea combined with butyrate resulted in more lasting effects and overcame butyrate resistance. Butyrate action did not involve changes of  $\gamma$ -globin mRNA levels, suggesting effects on translation of  $\gamma$ -globin mRNAs. These results demonstrate the nonspecificities of weak HDAC inhibitors such as butyrate and the need to develop HDAC-specific inhibitors.

J. Gagnen (University of Montreal, Montreal, Canada) reported that human breast cancer cell lines treated *in vitro* with the combination of 5-AC and depsipeptide, a HDAC inhibitor, exhibited additive antitu-

mor effects as measured by clonogenic assay. This was associated with the synergistic induction of the expression of either estrogen receptor  $\alpha$  or E-cadherin.

Dr. M. Carducci (Johns Hopkins Oncology Center) reviewed the roles of DNA methylation and histone acetylation as complementary players in gene silencing in prostate cancer cells. He described preclinical studies that demonstrated that sequential treatment of cells with 5-AC and TSA and/or PB can restore gene expression by reversing the silencing. The Sirchia group from Milan demonstrated reexpression of RAR $\beta$  in breast cancer, and the Johns Hopkins Oncology Center group demonstrated reexpression of RAR $\beta$  in prostate cancer cell lines. Dr. Carducci reviewed *in vitro* studies demonstrating the ability to reexpress glutathione *S*-transferase  $\pi$ , the androgen receptor, and estrogen receptor in prostate cancer cell lines that are silenced by hypermethylation of the promoter regions.

Dr. Carducci and his colleagues initiated a Phase I trial using 5-AC for 7 days followed by 7 days of PB infusions. To date, six of eight patients have had paired tumor biopsies. No change in expression of genes of interest was noted at the first dose levels, although there is an increase in acetylated histone. Baseline tumor DMT levels were low and were not lowered much more after treatment with 5-AC. An elevated DMT level in one patient with hepatocellular carcinoma was reduced after *in vivo* treatment with 5-AC.

Roberto Pili discussed restoring gene expression using a nuclear receptor inducer and a cytotoxic agent, namely, the combination of PB and 13-*cis*-retinoic acid plus paclitaxel. The triple combination given concurrently was most effective against human prostate cancer and colon cancer cells, inducing apoptosis and arrest of cells in both G<sub>1</sub> and G<sub>2</sub>, as compared with the single-agent activities. However, the enhancing effect was not observed if PB and 13-*cis*-retinoic were administered first, followed by paclitaxel. Apparently, the ability of the triple combination to cause simultaneous G<sub>1</sub> and G<sub>2</sub> arrests triggers tumor cell apoptosis.

Candice Johnson (University of Pittsburgh, Pittsburgh, PA) reported that pretreatment of prostate carcinoma cells with vitamin D<sub>3</sub> enhances responsiveness to taxanes and *cis*-platinum. Likewise, glucocorticoids enhance response of cells to vitamin D<sub>3</sub> by increasing the expression of vitamin D<sub>3</sub> receptor. Donald Trump (University of Pittsburgh) reported on the translation of some of Dr. Johnson's preclinical studies to clinical trials. He showed that vitamin D<sub>3</sub> (calcitriol)-induced hypercalcemia was prevented by dexamethasone in patients with androgen-independent prostate cancer. There was a decrease in prostate-specific antigen levels in 13 of 38 patients, and it was concluded that high-dose calcitriol with steroids is feasible on a defined schedule.

Dr. M. J. Campbell (University of Birmingham, Birmingham, United Kingdom) reported that treatment with vitamin D<sub>3</sub> can suppress histone acetylation, whereas TSA increases acetylation. The combination of vitamin D<sub>3</sub> and TSA gave a greater increase in histone acetylation than TSA alone, which was associated with distinct changes in genes that are related to cell growth and death.

## Summary

Although many *in vitro* models suggest that gene reexpression strategies are of potential clinical interest, inadequate preclinical work in animal models has not demonstrated the functionality of silenced genes once restored. Questions continue regarding optimal dose and schedule of these agents *in vitro* and *in vivo*. Currently, the assays of *in vivo* drug effect are still under development and are of unclear significance. Further progress in the clinical development of drugs to induce differentiation by targeting sites of transcriptional repression caused by oncogenic proteins or silenced genes requires more well-defined studies in *in vivo* tumor models and in patients using appropriate surrogate markers for differentiation induction as demonstrated in APL.