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## **Application for Y2 Leukemia SPORE Developmental Project**

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**Project title:** Mitochondrial Defects and Therapeutic Implications in Leukemia

**Abstract:** During the first year of our research (Developmental Project), we made a significant progress in our studies on the biochemical and molecular basis for ROS stress in leukemia cells and its therapeutic implications. Specifically, we demonstrated that primary CLL cells contain significantly higher basal levels of ROS than normal lymphocytes. Our studies showed that mitochondrial DNA mutations and malfunction of mitochondrial respiration and biogenesis may contribute to increased ROS stress and altered drug sensitivity in leukemia cells. There is also a significant correlation between mitochondrial abnormality and reduced apoptotic response to drug treatment (publications 1-6). Based on these findings, we now propose in our Y2 Developmental Project to further develop and test novel therapeutic strategies to effectively kill leukemia cells and overcome drug resistance associated with mitochondrial abnormality, with particular emphasis on the intrinsic apoptotic pathway and alteration in energy metabolism in leukemia cells. We have developed several proprietary compounds with promising anti-leukemia activity for our studies. These compounds either directly damage mitochondria or interfere with altered energy metabolism associated with mitochondrial defect in cancer cells. We will use our established experimental model systems with clones of leukemia cells containing defective mitochondria to test the activity of these compounds and validated the biochemical and molecular end points relevant to the underlying mechanisms of the drug action. The therapeutic activity of these novel compounds, either used alone or in combinations with other anti-leukemia agents, will be evaluated in leukemia cell lines and in primary leukemia cells. The compounds and combination protocols with most promising activity *in vitro* will then be tested in leukemia animal models. This research project is likely to have high potential for translational application, and is directly relevant to the goal of the Leukemia SPORE.

### **Hypothesis:**

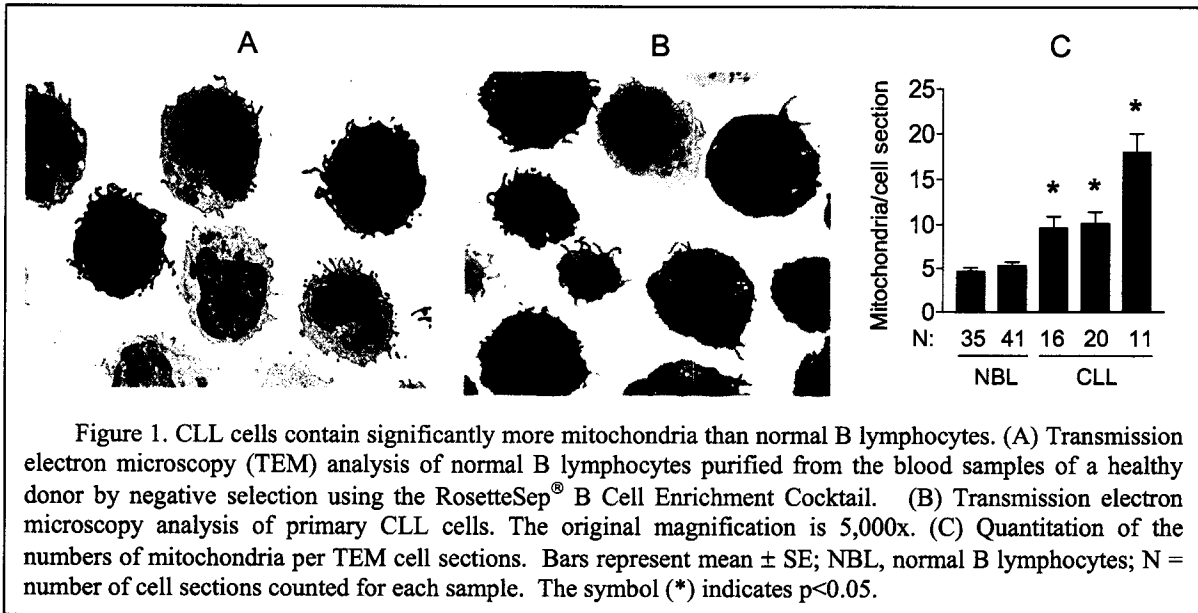
Mitochondrial malfunction in leukemia cells lead to alteration in drug sensitivity, and serve as a biological basis for the development of novel therapeutic strategies to effectively kill leukemia cells and overcome drug resistance.

### **Specific Aims:**

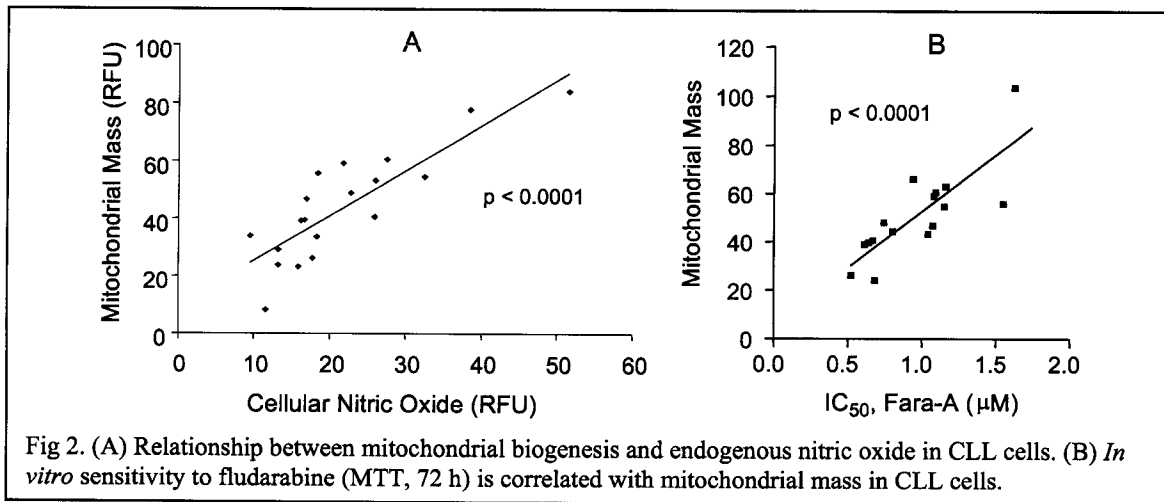
1. Examine the relationship between mitochondrial alterations and changes in drug sensitivity, and investigate the underlying mechanisms.
2. Test the therapeutic activity of a novel compound OSW-1 against leukemia cells *in vitro* and *in vivo*, and evaluate its mechanisms of action.
3. Develop mechanism-based combination strategies to effectively kill leukemia cells and overcome drug resistance associated with mitochondrial malfunction.

### **Background & Significance:**

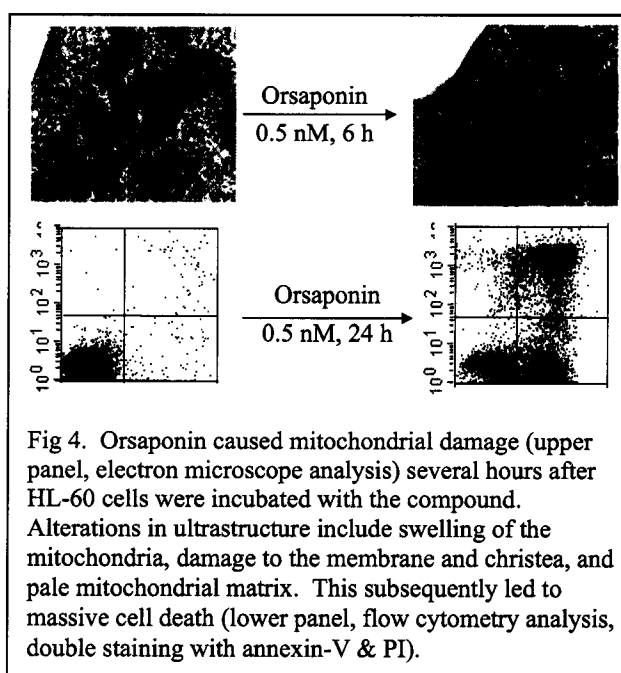
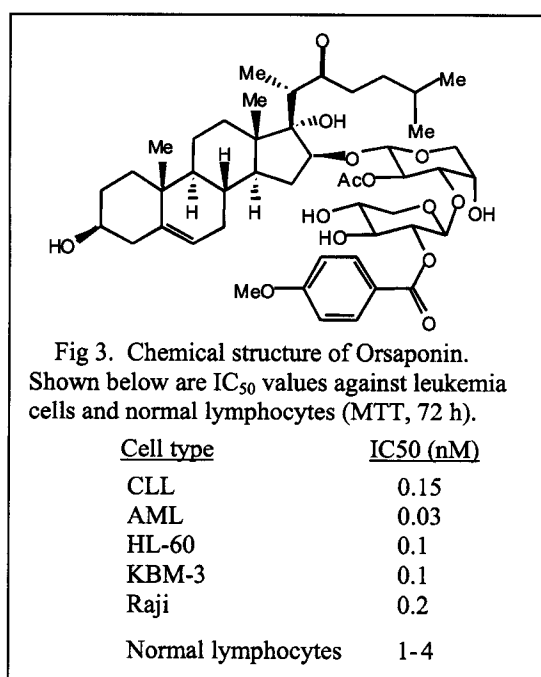
Significant progress has been made in the recent years in our understanding of the biology of human leukemia and its clinical treatment. However, development of drug resistance to currently available therapeutic agents remains a significant problem in CLL treatment. Thus, investigating new mechanisms of drug resistance and identification of new anticancer agents with potent therapeutic activity and without cross-resistance with current anti-leukemia agents are very important areas of research.



Our recent studies showed that primary leukemia cells isolated from CLL patients contained significantly more mitochondria than normal B lymphocytes isolated from healthy donors (Figs 1A-1C). The mitochondrial mass in CLL cells was significantly correlated with the endogenous nitric oxide radical (NO) levels (Fig 2A). Expression of the mitochondrial biogenesis factors nuclear respiratory factor-1 and mitochondrial transcription factor A were elevated in most CLL specimens examined. Treatment of B-cells with exogenous NO caused a substantial increase in mitochondrial mass. Significantly, *in vitro* sensitivity of CLL cells to fludarabine was highly related to mitochondrial mass in that cells with greater mitochondrial mass were less sensitive to the drug (Fig 2B). Consistent with these observations, a clone of respiration-defective (C6F) cells derived from myeloid leukemia cells (HL-60) also exhibited mitochondrial respiration defects, increased mitochondrial mass, and reduced sensitivity to arsenic trioxide (3). These observations suggest that abnormal mitochondrial biogenesis may be associated with mitochondrial defects and free radical stress, and may lead to change in cellular apoptotic responses and reduced sensitivity to anti-leukemia agents such as fludarabine and arsenic trioxide. This also suggests that it is important to identify novel compounds and new therapeutic strategies to overcome such drug resistance mechanisms and effectively kill leukemia cells with mitochondrial defects.



We recently discovered that the steroid disaccharide compound Orsaponin possesses an extremely potent cytotoxic activity against a wide spectrum of human cancer cells including various leukemia cell lines and primary leukemia cells from CLL and AML (Fig 3). The effective concentrations of this compound against primary AML and CLL cells are less than 0.3 nM in most cases, with the IC<sub>50</sub> values in the range of 0.1-0.3 nM. Normal lymphocytes isolated from healthy donors are relatively less sensitive to Orsaponin, with the IC<sub>50</sub> value range of 1-5 nM, approximately 10-15 times more resistant than leukemia cells. These data suggest that Orsaponin may provide a reasonable therapeutic selectivity. Importantly, CLL that became resistant to fludarabine remained very sensitive to Orsaponin, indicating that this compound might be effective in treating drug resistant leukemia. Further studies suggest that Orsaponin seems to have a unique mechanism of action, causing early mitochondrial damage and subsequently massive cell death. The potent activity, relative selectivity, and apparent unique mechanism of action of Orsaponin seem promising, and it is important to further evaluate this compound as potential agents for effective treatment of leukemia.



## Research plans:

**Specific Aim 1.** Examine the relationship between mitochondrial alterations and changes in drug sensitivity, and investigate the underlying mechanisms. Several experimental approaches will be used to investigate how mitochondrial alterations in leukemia cells lead to change in drug sensitivity.

(1) Because our preliminary studies showed that the abnormal increase of mitochondrial biogenesis and malfunction appeared to be associated with increase of endogenous nitric oxide (NO) radical in CLL cells and was correlated with reduced sensitivity to fludarabine, we will first test if such sensitivity decrease is unique for fludarabine, or holds true for other anti-leukemia drug such as ara-C, arsenic trioxide, and doxorubicin. We reason that structural and functional alterations in mitochondria may lead to change in apoptosis response and thus cause decrease in cellular sensitivity to multiple agents. This will be tested both in primary leukemia cells as well as clones of leukemia cell lines with mitochondrial respiration defects and increased mitochondrial mass. These experiments will provide important information on if mitochondria alterations could be a general mechanism of drug resistance in leukemia cells, and thus have direct clinical implications.

(2) We will further examine how mitochondrial respiration defect may cause reduced drug sensitivity. This will first be investigated by evaluating the respiration-defective clones for possible changes in transmembrane potential, alterations in drug-induced mitochondrial membrane permeability transition (MPT), cytochrome c release, and translocation of AIF. Any significant findings in the model system will be further tested in primary leukemia cells that exhibit compromised mitochondrial respiration (as indicated by decreased oxygen consumption and increased lactate production). Our laboratory has expertise in these assays.

(3) It is possible that nitric oxide (NO) may induce mitochondrial biogenesis as well as enhance cell survival. The role of NO in causing increased mitochondrial mass and reduced drug sensitivity in leukemia cells will be further evaluated using exogenous NO donors and inhibitors of nitric oxide synthase (NOS). Because it takes 3-4 days for NO to stimulate mitochondrial biogenesis, this makes it possible to use time-course experiments to test if NO promotes cell survival through altering mitochondrial mass or by its direct effect on cell survival pathways.

**Specific Aim 2. Test the therapeutic activity of a novel compound OSW-1 against leukemia cells in vitro and in vivo, and evaluate its mechanisms of action.**

(1) Test the ability of orsaponin to kill leukemia cells, especially those cells that are resistant to common therapeutic agents. Cell lines with different drug sensitivity as well as primary leukemia cells isolated from patients at various disease stages and with different clinical treatment states (previously treated or untreated) and outcomes (responsive or refractory) will be tested. The primary leukemia cells will be tested for their sensitivity to orsaponin in comparison with standard anticancer agents commonly used in clinic. For instance, if a CLL patient has previously been treated with fludarabine + cyclophosphamide and become refractory, we will use these two drugs for comparison with orsaponin in the *in vitro* cytotoxicity assay with the patient's CLL cells, which are likely resistant to fludarabine and cyclophosphamide. Thus it is important to test if the FC-resistant CLL cells are still sensitive to orsaponin. Similar approach will be used to test the activity of Orsaponin in AML cells that are resistant to ara-C, or CML cells that are resistant to gleevec. A demonstration that orsaponin could effectively kill drug resistant cells would provide important rationale to develop this novel compound.

(2) Determine the *in vitro* therapeutic selectivity of orsaponin. In parallel with the cytotoxic assays described above, we will further test the effect of Orsaponin on various types of normal human cells, including normal lymphocytes, normal epithelial cells, normal fibroblasts, and normal endothelial cells. Most of the normal control cells are commercially available (Clonetics, San Diego). Normal lymphocytes will be isolated from peripheral blood samples from healthy donors, and will be used as the key control for comparison with leukemia cells in parallel experiments. MTT assay will be used to determine the IC<sub>50</sub> values. These IC<sub>50</sub> values will be compared. The *In vitro* therapeutic index can then be calculated using the IC<sub>50</sub> values for the normal cells divided by the IC<sub>50</sub> values of the leukemia cells.

(3) Investigate the mechanisms of action of Orsaponin. The mechanism by which Orsaponin exerts its cytotoxic activity is still largely unknown. Our microarray analyses revealed an altered expression of genes involved in mitochondrial respiration. Transmission electron microscopic analysis also showed mitochondrial damage after Orsaponin treatment. Future studies will focus on the intrinsic (mitochondrial) apoptosis pathway induced by Orsaponin, with particular emphasis on the drug effect on the ability of mitochondria to regulate calcium flux and trigger apoptosis.

(4) Test the therapeutic activity of Orsaponin *in vivo*. We have two mouse models available in the laboratory for testing the *in vivo* activity of Orsaponin. The first animal model is the TCL-1 transgenic CLL mouse model provided to us by Dr. Carlo Croce. The second model is a syngenic CML leukemia model containing Bcr-Abl oncogene. We will test the therapeutic activity of Orsaponin in both leukemia models, using mouse survival as the major end point. Key hematologic parameters such as blood cell counts, surface antigens, molecular markers (TCL-1, Bcr-Abl, etc), leukemia infiltration to bone marrow and spleen will also be analyzed as appropriate.

**Specific Aim 3. Develop mechanism-based combination strategies to effectively kill leukemia cells and overcome drug resistance associated with mitochondrial malfunction.**

(1) **Combination of ROS-generating agents (arsenic trioxide + 2-methoxyestradiol).** The basic principle of this approach is to use arsenic trioxide that generate ROS in combination with 2-ME to further impair the elimination of ROS, leading to a severe accumulation of ROS in leukemia cells and cell death (Fig 5A). This design is based on our previous observations that primary leukemia cells, especially those from patients in late disease stage or with prior chemotherapy history, have high frequency of mitochondrial mutations and increased endogenous ROS stress, possibly due to malfunction of the mitochondrial respiratory chain. We hypothesize that leukemia cells with mtDNA mutations and increased intrinsic ROS stress will be more vulnerable to additional free radical insults imposed by ROS-generating agents such as combination of arsenic trioxide (ATO) and 2-methoxyestradiol (2-ME). Indeed, we have shown that this combination has synergistic effect in CLL cells (Fig 5B) and in HL-60 cells (3). We will further test if the ATO/2-ME combination is effective in leukemia cells that are resistant to standard anti-leukemia agents.

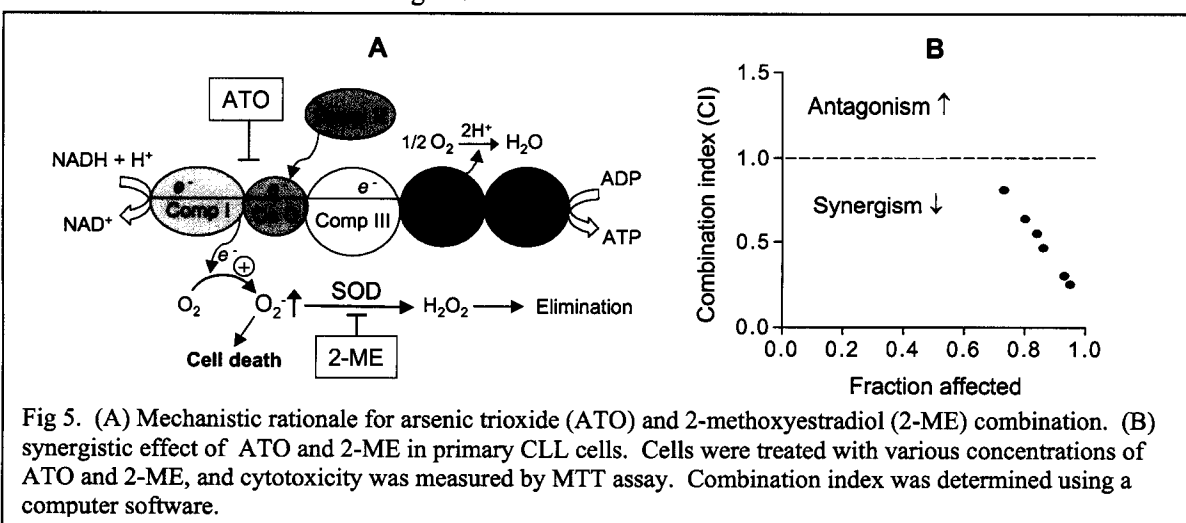


Fig 5. (A) Mechanistic rationale for arsenic trioxide (ATO) and 2-methoxyestradiol (2-ME) combination. (B) synergistic effect of ATO and 2-ME in primary CLL cells. Cells were treated with various concentrations of ATO and 2-ME, and cytotoxicity was measured by MTT assay. Combination index was determined using a computer software.

(2) **Combination of anti-leukemia agents with ER stressor.** The rationale is that mitochondrial defects may blunt apoptotic response, such defects may also compromise the ability of the leukemia cells to uptake calcium release from ER induced by an ER stressor, thus lead to abnormal increase of cytosolic Ca<sup>++</sup> and trigger apoptosis. Brefeldin A (BFA), a naturally occurring compound that is able to cause ER stress by interfering with ER protein processing, will be used in our studies. Our preliminary studies showed that this compound is very effective in killing CLL cells, which seem to have high content of ER network. We will test the effect of fludarabine + BFA combination in CLL cells, and ara-C + BFA combination in AML cells, using various drug incubation sequences. The best combination protocols will be selected to test *in vivo* therapeutic activity in the animal leukemia model describe above.

**Relevance to the Leukemia SPORE:** The major objectives of this research project are to investigate the role of mitochondrial alterations in therapeutic response in leukemia cells, to test the novel compound Orsaponin as a potent agent for treatment of leukemia, and to develop novel drug combination strategies to effectively kill leukemia cells that are resistance to standard chemotherapeutics. This research is translational in nature, has high potential for clinical applications, and is directly relevant to the overall goal of the SPORE program. The studies with in primary leukemia cells and evaluation of the *in vitro* data in connection with clinical parameters will provide close interactions between laboratory and clinical investigators. This project also includes Dr. Pelicano, a junior investigator who has not previously engaged in leukemia translational research. We hope that this project has the potential to develop to a full SPORE project.

## Progress Report and Use of Funds: Y-01 Developmental Project

- (A) Use of the Developmental Funds: The Leukemia SPORE developmental funds has been extremely important in supporting our laboratory research in exploring new ideas and new research areas directly relevant to the overall translational research objectives of Leukemia SPORE. The funds are mainly being used to purchase laboratory reagents and supplies for the research project which is exploratory in nature (specific aims listed below) and not supported by other grants. Because of the support from the developmental research funds, we have been able to effectively move the research forward, and have made significant progress in specific aim.
- (B) Progress highlights: The main objective of the developmental project in Y01 is to explore the difference between leukemia cells and normal cells in their mitochondrial respiration, energy metabolism, and generation of free radicals as a biochemical basis to design novel strategies to enhance anti-leukemia activity and selectivity.
1. Using primary leukemia cells isolated from patients with CLL and human leukemia cell lines in culture, we demonstrated that compared to normal lymphocytes, human leukemia cells are generally under oxidative stress and contain significantly higher basal level of ROS. This intrinsic oxidative stress in leukemia cells provides a biochemical basis for therapeutic sensitivity. We further demonstrated that the increase of ROS generation in primary leukemia cells was associated with increased sensitivity to novel ROS-generating anticancer agents including 2-methoxyestradiol and arsenic trioxide. These findings provide a basis for future design of new therapeutic strategies using ROS-generating to preferentially kill leukemia cells. This work has recently been published (*Blood*, 101:4098-4104, 2003; *Cancer Chemother Pharmacol*, 53:209-219, 2004).
  2. The increased ROS generation in leukemia cells prompted us to explore the underlying mechanism. Since the mitochondrial respiratory chain is a main source of cellular ROS production, we hypothesize that mutation in mtDNA may contribute, at least in part, to the increased ROS in leukemia cells. Direct DNA sequencing of mtDNA from primary CLL cells demonstrated that mutations are indeed frequent in the malignant cells. Furthermore, mtDNA mutations were showed to be associated with prior chemotherapy, and appeared to be associated with increased ROS generation. This work was recently published (*Leukemia*, 17:1437-1447, 2003).
  3. Based on the above findings, we began to design and test novel experimental strategies to take advantage of mtDNA mutations and increased ROS stress in leukemia for therapeutic benefits. The hypothesis is to impose further ROS stress on leukemia cells to trigger apoptosis by using pharmacological agents that generate ROS in the cells. The proof-of-principle strategy using combination of arsenic trioxide and 2-methoxyestradiol has been tested and proven to be effective (*J Biol Chem*, 278:37832-37839, 2003).

### (D) Publications:

1. Zhou Y, Hileman EO, Plunkett W, Keating MJ, Huang P. Free radical stress in chronic lymphocytic leukemia cells and its role in cellular sensitivity to ROS-generating anticancer agents. *Blood*, 101:4098-4104, 2003.
2. Carew JS, Zhou Y, Albitar M, Keating MJ, Huang P. Mitochondrial DNA Mutations in Primary Leukemia Cells after Chemotherapy: Clinical Significance and Therapeutic Implications. *Leukemia*, 17:1437-1447, 2003.
3. Pelicano H, Zhou Y, Hileman EO, Feng L, Plunkett W, Keating MJ, Huang P. Interfering of the mitochondrial electron transport: A novel strategy to enhance drug-induced apoptosis in leukemia cells through a free radical-mediated mechanism, *J Biol Chem*, 278:37832-37839, 2003.
4. Hileman EO, Liu J, Albitar M, Keating MJ, Huang P. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic sensitivity, *Cancer Chemother Pharmacol*, 53:209-219, 2004.
5. Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. *Drug Resistance Update* 7:97-110, 2004.
6. Carew JS, Nawrocki, ST, Xu R, Dunner K Jr., McConkey DJ, Wierda WJ, Keating MJ, Huang P. Increased mitochondrial biogenesis in primary leukemia cells: the role of endogenous nitric oxide and impact on drug sensitivity, *Leukemia*, in press.