行政院國家科學委員會專題研究計畫　成果報告

合併使用 superoxide dimutase 抑制劑及 proteasome 抑制劑對急性髓性白血病治療之體外及動物研究

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計畫主持人：劉興璟

計畫參與人員：林書帆

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中　華　民　國 94 年 10 月 28 日

行政院國家科學委員會補助專題研究計畫

■成果報告

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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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□國際合作研究計畫國外研究報告書一份

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執行單位：

中　華　民　國　94　年　10　月　25　日

I. 中文摘要

本研究在於測試兩種新藥,ＭＧ132 及 2-methoxyestradiol(2ME)對急性白血病治療的可行性.我們先以不同的白血病白血病細胞株,HL-60, THP1, NB4, K562 測試對此二種藥物的敏感度,發現ＭＧ132 可以抑制這四種細胞的生長.並造成細胞週期停止於 G0/G1 期,相對於 MG132, 2-ME 則對ＮＢ４之抑制效果較對其他細胞突出.其對細胞週期之抑制則集中於 G2/M 期.我們進一步研究 2-ME 對 NB4 細胞的影響及分子機轉.研究顯示 2-ME 可以增加細胞內氧化游離基的量,降低 glutathione 的量.并引發細胞彫亡,而且可以降低 PML/RARalpha 並且造成中性細胞球的分化.

我們同時發現 MG132 及 2-ME 均可抑制 NF-kappaB 的活化.但合併使用此兩種並不會有 synergistic 效果. 但是我們發現 2-ME 與三氧化二砷合用則有加成作用.更令人驚訝的是 2-ME 對 all-trans retinoic acid 或三氧化二砷產生抗藥之 NB4 仍舊有效.

我們更進一步進行動物實驗發現,2-ME 雖無法增進存活,卻可以減少體重減輕的情形.因此我們認為 2-ME 未來可能可以用以特別治療急性前髓性白血病.(本研究成果將於 2005 年美國血液學學會年會發表)

關鍵詞: MG-132, 2-methoxyestradiol, 急性前髓性白血病

II. English abstract

In this study, we intended to explore the novel therapy for acute myeloid leukemia (AML) especially for acute promyelocytic leukemia especially in patients who are refractory to standard all-trans retinoic acid (ATRA) and arsenic trioixde (ATO) therapy. We proposed that the APL which is refractory to either or both ATRA/ATO might be sensitive to proteasome inhibitor, MG-132 and/or superoxide dismutase inhibitor, 2-methoxyesteraidiol (2-ME), an agent that is currently evaluated in early phase of clinical trials for solid tumors.

We showed that MG132 was effective in inhibiting the growth of multiple AML cells. MG132 significantly induced the cell cycle arrest at G0/G1 phase. Of all the AML cells we tested, THP1 (acute monocytic leukemia cell line), NB4 (APL cell line), HL-60 (bipotent AML), and K562 (erythroleukemia) were equally sensitive to MG132. There was no preferential sensitivity of MG132 to APL cells. 2-ME was also tested on different types of AML cells. In contrast to MG-132, NB4 cells were exclusively sensitive to 2-ME, compared with other types of AML cells. Differentfrom MG-132, 2-ME induced cell cycle arrest at G2/M phase. Both agents inhibited the activation of NF-kappaB. We then went on to test whether a combination of MG-132 and 2-ME was more effective than either agent alone. A combination of MG-132 and 2-ME has additive but not synergistic effect. We next studied the mechanisms underlying the sensitivity of NB4 to 2-ME. 2-ME induced apoptosis of NB4 cells through mitochondrial pathway, depleted the cellular glutathione, increased the intracellular reactive oxygen species, and degraded PML/RARalpha. Furthermore, 2-ME induced NB4 to undergo neutrophilic differentiation. We then tested whether 2-ME can be useful in ATRA or ATO resistant NB4 cells. We found that 2-ME was still active in both ATRA and ATO resistant APL cells. We then tested the in vivo effect of 2-ME. NOD/SCID mice injected with NB4 cells were treated with 2-ME for 6 weeks. Although we did not notice a difference in survival in mice treated with 2-ME. Mice treated with 2-ME had less body weight loss, suggesting that 2-ME could be effective in vivo.

In conclusion, we found that 2-ME, not MG-132 were exclusively effective on acute promyelocytic leukemia, and could be used in both ATRA and ATO resistant situation. Further in vivo study shows that 2-ME could reduce the tumor burden. Therefore, further investigation

of 2-ME on APL will be needed to demonstrate its efficacy in vivo. **(The results will be presented in 2005 American Society of Hematology, Atlanta)**

Keywords: MG-132, 2-methoxyestradiol, acute promyelocytic leukemia

I.前言

Acute myeloid leukemia (AML) is a devastating hematologic malignancy, mainly affecting old patients. Chemotherapy is the main therapy for AML. However, the 5-yearsurvival rates of AML using conventional chemotherapy are no more than 30%. Although hematopoietic stem cell transplantation (HSCT) can produce cure in a subset of patients but most of patients are too old to receive HSCT. Therefore, it is imperative to develop new treatment modalities for AML. Arsenic trioxide (ATO) has emerged as a novel agent for acute promyelocytic leukemia (APL), a subtype of AML. ATO can induce both apoptosis and cytodifferentiation of APL cells. The arsenic-induced apoptosis is partly mediated by inhibition of NF-kB activation (Mathas et al., 2003) Although ATO can effectively achieve complete remission in 80% of patients with APL, 20% of the patients will relapse. We have been trying to develop novel therapy for APL. Previously we have tested arsenic trioxide in non-APL leukemia in vitro(Huang et al., 2002). Data from our laboratory and other investigators show that intracellular levels of glutathione and bone marrow microenvironment modulate the resistance to ATO. Intracellular glutathione levels affect the catabolism of reactive oxygen species (ROS).ROS can be metabolized to hydrogen peroxide by superoxide dimutase (SOD). Hydrogen peroxide is further catabolized by glutathione peroxidase and catalase. ATO inhibits glutathione peroxide, leading to the accumulation of hydrogen peroxide. Accumulation of hydrogen peroxide then perturbs the mitochondrial membrane potential, and releases the cytochrome C, causing apoptosis of the leukemic cells. Increased cellular glutathione can metabolize the ATO to glutathione arsenite, which abrogate the apoptotic effects of ATO. Recently 2-methoxyestradiol (2-ME), a specific SOD inhibitor has been used to induce apoptosis of malignant lymphoma cells by blocking the generation of hydrogen peroxide from superoxide. Addition of ATO in combination with 2-ME has synergistic effects in the cells with high content of ROS. As SOD is the upstream enzyme in the metabolism of superoxide in the cells, 2-ME mightbe effective in the ATO-resistant leukemia cells. As the ATO-induced apoptosis is partly mediated by inhibition of NF-kB, cells resistant to ATO is likely to have reactivated NF-kB(Mathas et al., 2003). Recently anovel proteasome inhibitor, MG-132, has been shown to inhibit the activation of NF-kB by blocking the degradation of NF-kB regulator, IκB. Therefore MG-132 might be alsoeffective in the ATO-naive and ATO-resistant leukemic cells. In this project, we attempted to elucidate the role of 2-ME and MG-132 in the setting of ATO-resistance.

II. Materials and Methods.

Chemicals. 2-ME and MG132 (Sigma) was dissolved in dimethylsulfoxide (DMSO) to 10 mg/mL, respectively, and further diluted in culture media before use. ATO (1 mg/mL) was kindly provided by TTY Pharmaceuticals (Taipei, Taiwan). A working solution was prepared by diluting the stock solution to 100 mM in phosphate-buffered saline (PBS) before use. Buthione sulfoxime (BSO), and N-acetylcystein (NAC)(Sigma) was prepared by dissolved in the culture medium to 1 mM and further diluted to the indicated concentrations.

Cell culture and assessment of cell growth inhibition and viability. Human myeloid leukemia HL-60, K562, THP-1, and NB4 cells were maintained in suspension in RPMI 1640 medium containing 10% fetal bovine serum (Hyclone) and 100 U/ml penicillin, and 100 mg/ ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 in air. To generate ATO-resistant NB4 cells, cells were selected from cultures with increasing concentration of ATO, and finally maintained at 1 mM. Cell growth and viability were assessed by the MTT assays

Analysis of DNA fragmentation by agarose gel electrophoresis. Cellular DNA was extracted by the phenol/chloroform method. Electrophoresis was performed in a 1.0% agarose gel in Tris-borate-EDTA buffer (pH 8). After electrophoresis, DNA was visualised by ethidium bromide staining.

Measurement of cellular glutathione. Cellular glutathione was measured using Glutathione Assay Kit (Caymen Chemical). Briefly $5x10^6$ cells were homogenized in MES buffer and the supernatant was deproteinated in 5% metaphosphoric acid. Particulate matter was separated by centrifugation at 4,000g. Supernatant was used for GSH measurement according to the manufacturer's instructions, while the pellet was dissolved in 1 mol/L NaOH and analyzed for protein concentrations by colorimetric protein assay (Amresco). The GSH content was calculated using the glutathione standards provided by the manufacturer, and expressed as mmoles per milligram protein.

Measurement of cellular superoxide. Intracellular O_2^- contents were measured by flow cytometry analysis using a hydroethidine method. Briefly, leukemic cells ($1x10^6$ cells in 1 mL) were incubated with hydroethidine (50 ng/mL) for 1 hour, washed once with 2 mL PBS, and resuspended in 1 mL PBS on ice before analysis. The samples were analyzed by flow cytometry using a FACSCalibur and the data were analyzed using the CellQuest software package.

Differentiation and phenotyping. Phenotypic changes was evaluated by flow cytometry. For staining of cell surface markers, aliquots of 10^6 cells were harvested, washed twice in ice-cold PBS then incubated in 100 mL final volumes with luorescein isothiocyanate (FITC)-conjugated CD11b, CD11c, CD13, CD14 or CD95 to defined surface markers. After incubating on ice for 30 minutes, the cells were washed twice in cold PBS, resuspended in 0.5 mL of the same buffer and propidium iodide (PI) added to a final concentration of 2 mg/mL immediately before data acquisition. Data were collected on a FACSCalibur (BD) and analyzed by the CellQuest software

package.

Cell-cycle analysis. The cell cycle was analyzed using PI staining. Briefly, cells were fixed by the addition of cold ethanol, suspended with 250 mg /mL RNase A in 1.12% sodium citrate at 37 °C for 30 minutes, and stained with 50 mg/mL PI(Sigma) on ice for more than 30 minutes. The cell-cycle status of the cells was analyzed by flow cytometry.

Western blot for apoptosis-related proteins. Total cellular protein or cytoplasmic protein for cytochrome C detection was extracted and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred from the gel onto a PVDF-Immobilon membrane. After blocking with 5% nonfat milk/TBS/Tween buffer, the membrane was immunoblotted with individual antibodies according to manufacturers' instructions. Antibodies for Bcl-xL, Bcl-2, Bak, Bax, IkappaB and caspase 3, 6, 7, 8 and 9 were purchased from Cell Signaling Technologies. Antibody for β-actin was purchased from US Biologicals. Antibodies for cytochrome C and PML/RARa were obtained from Santa Cruz Biotechnology. After probing with primary antibody, the membrane was blotted secondary antibody conjugated with horseradish peroxidase. Immunoblot was revealed by using enhanced chemiluminescence detection kit (NEN) by autoradiography.

Statistical analysis. The results were reported as mean± SEM and the statistical significance ($P<0.05$) was determined by two-sided Student's t-tests.

III. Results

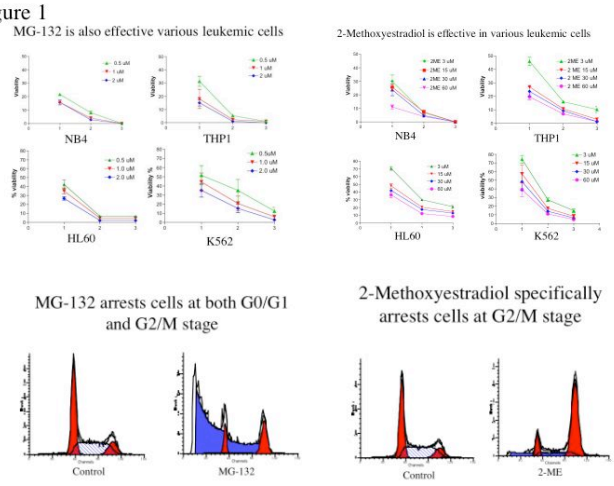
Both MG-132 and 2-ME inhibit the growth of AML cells, but 2-ME is exclusively effective on AML cells

To test our hypothesis that MG-132 and 2-ME might effective on AML cells, we treated different leukemic cell lines and measured their viability at 24 hours using the MTT assay. Both MG-132 and 2-ME were effective on 4 different types of leukemic cells (Figure 1A and B)

The growth inhibition of NB4 cells by 2-ME was more evident compared with other cell lines (Figure 1B)

We then measured the differences in the cell cycle distribution by MG-132 and 2-ME (Figure 1C and 1D). MG-132 were both effective in G0/G1 and G2/M but more on G0/G1, but 2-ME more on G2/M (Figure 1C and D). A combination of 2-ME and MG-132 does not significantly enhance the effect of either agent (data not shown)

Figure 1



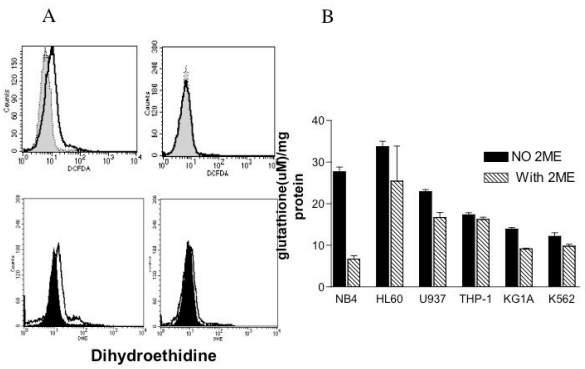
2-ME increases cellular reactive oxygen species and superoxide, causing apoptosis of leukemic cells

As 2-ME has been shown to inhibit SOD activity, we next studied whether inhibition of SOD is correlated with the drug sensitivity (Figure 2A). 2-ME treatment

significantly reduced the SOD activity in NB4 cells, but not in other types of AML cells.

If 2-ME significantly inhibits SOD activity, we then asked whether 2-ME increased the cellular ROS levels. AML cells treated with 0.5 μM 2-ME were stained with carboxy-H2DCFCA, a fluorescent indicator of cellular ROS. The pretreatment levels of ROS did not correlate with the drug sensitivity (data not shown). Instead, 2-ME treated increased the ROS in NB4 cells but not in HL-60 cells.

Figure 2



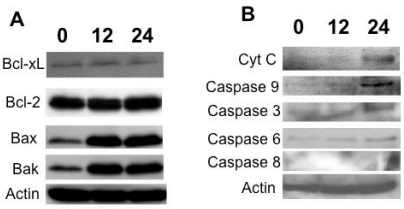
Previous studies have demonstrated that the sensitivity to ATO in leukemic cells correlates with the levels of endogenous glutathione. Glutathione, an cellular antioxidant, protects cells from the damage by ROS. To determine the role of glutathione in the sensitivity to 2-ME, we measured the changes of glutathione upon 2-ME treatment. The pretreatment level of glutathione did not predict the sensitivity to 2-ME (Figure 2B). In contrast, the levels of post-treatment glutathione correlated with the sensitivity to 2-ME. The level of post-treatment glutathione in NB4 cells was the lowest one in NB4 among all cells treated.

To investigate the role of antioxidants in the sensitivity to 2-ME in detail, we measured the 2-ME activity on NB4 and HL-6 cells after depleting their glutathione by pretreating the cells with BSO for 24 hours. Consistent with prior report, BSO treatment depleted the cellular glutathione (data not shown). The addition of BSO significantly enhanced the cytotoxic effects of 2-ME in HL-60 cells, but not in NB4 cells. To further elucidate the role of superoxide in the 2-ME-induced apoptosis, we next investigated whether the anti-oxidant, NAC, blocks the cytotoxicity of 2-ME. NAC significantly reduced, but could not completely prevent, the cytotoxicity of 2-ME in NB4 cells, suggesting that multiple mechanisms might be responsible for its sensitivity. The effect of NAC was less evident in HL-60 cells(data not shown).

2-ME induces apoptosis through mitochondrial pathway

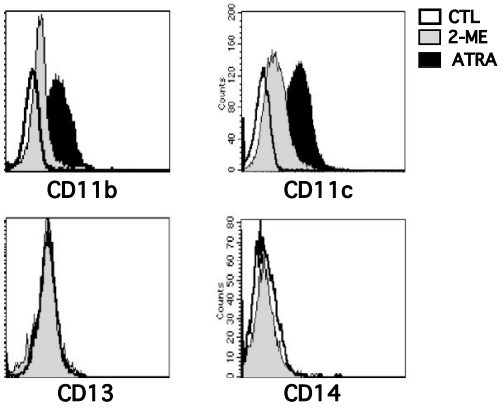
Increased ROS damages the mitochondrial membrane, causing release of cytochrome C, and inducing apoptosis through the activation of pro-apoptotic Bcl-2-related proteins. Consistent with prior reports, 2-ME treatment significantly upregulated pro-apoptotic Bak and Bax. But the levels of Bcl-2 and Bcl-xL were not affected (Figure 3). The damaged mitochondria released cytochrome C, and activated the cleavage of pro-caspase 9, 3 and 7. 2-ME did not activate extrinsic apoptotic pathway as the levels of cleaved caspase 8, or the CD95 expression remained unchanged upon treatment (data not shown).

Figure 3



2-ME induces neutrophilic differentiation of NB4 cells

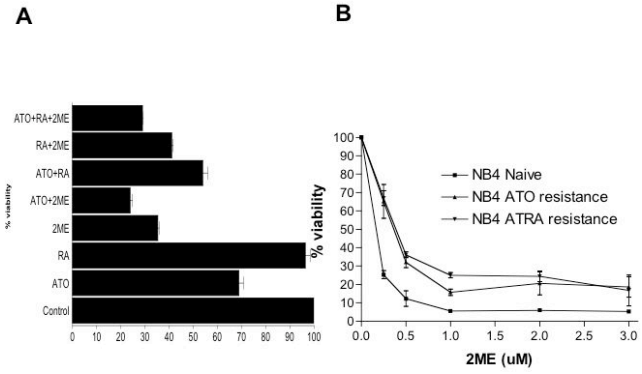
The increased levels of ROS are related to not only apoptosis but also granulocytic differentiation. All-trans retinoic acid, a well-known agent to induce granulocytic differentiation in APL, activates NADPH oxidase, causing increase of cellular ROS. We next investigated whether 2-ME induces granulocytic differentiation of NB4 cells. Cells treated with 2-ME for 24 hours were examined for granulocytic differentiation by NBT reduction assays and phenotypic analysis. 2-ME significantly increased the levels of NBT formazan in a dose-dependent manner, reaching plateau at 3 mM(Fig 4). The granulocytic differentiation was further confirmed by the upregulation of CD11b and CD11c. The 2-ME-induced differentiation was exlusively toward neutrophils, because the expression of CD13, and CD14, markers for monocytic lineages was not affected.



Previous studies have demonstrated that a combination of ATRA and ATO is more active against APL cells than either agent alone. As 2-ME did not specifically degrade PML/RARa, we then asked whether a combination of 2-ME with ATRA and/or ATO is more active against APL cells. Consistent with prior reports, a combination of ATO and ATRA was more effective than either agent alone on NB4 cells, and the addition of ATO significantly enhanced the 2-ME activity against NB4 cells; in contrast, the addition of ATRA partly antagonized the 2-ME activity (Figure 5A).

Because 2-ME did not specifically target PML/RARa, we reasoned that 2-ME might be useful for either ATRA- or ATA-resistant APL cells. We established NB4 cell lines resistant to either 0.5 μM of ATRA or 1 μM of ATO and tested whether 2ME is still effective on these cells. NB4 cells either resistant to ATRA or ATO, although less sensitive to the naïve cells, were still sensitive to 0.5 mM 2-ME, suggesting that 2-ME might be a potentially useful agent for resistant APL cells (Figure 5B).

Figure 5



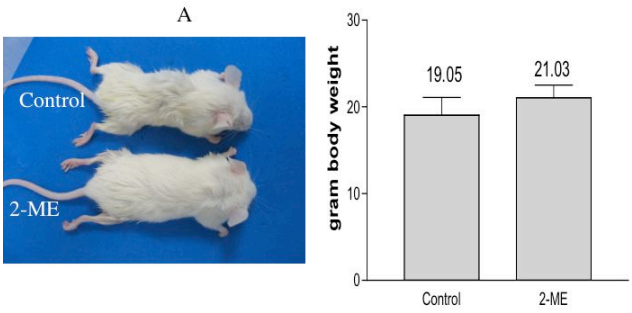
2-ME inhibits the activation of NF-kB

Since previous studies have demonstrated that NF-kB is constitutively activated in leukemic cells, and ATO-induced apoptosis is mediated through the inhibition of NF-kB, we next determined the role of NF-kB in 2-ME-induced apoptosis by the EMSA assay. Treatment with 2-ME or MG-132 significantly inhibited the NF-kB activity in NB4 cells(data not shown). As a result of inhibition of NB-kB, the level of IkBa also decreased after inhibition of NF-κB.

2-ME might be effective in vivo

We then evaluated the effect of 2-ME in vivo by using NOD/SCID mice. 4-6 week-old mice were injected with NB4 cells and started treated with 2-ME 2 weeks after feeding 2-ME for 6 weeks. After 6 weeks, the mice were sacrificed and measured the changes of body weight. We did not noticed any significant difference in survival, but we noticed that the body weight of treated mice was on average heavier than the control mice(Figure 6A and B). On dissection, the treated mice appear to have less tumor burden than the treated ones (data not shown).

Figure 6



Conclusion: we think 2-ME is an agent that could be useful for both ATRA/ATO resistant acute promyelocytic leukemia. With regard to the use of MG-132, it might require further study to elucidate its effects on AML.

附件

國科會研究計畫國外研究心得報告

計畫名稱: 合併使用 superoxide dismutase 抑制劑及 proteasome 抑制劑對急性髓性白血病治療之體外及動物研究

計畫編號 NSC 93－2314－B－038－017－

主持人:台北醫學大學醫研所 助理教授 劉興璟

Our lab has been devoted to identify novel agents for treating acute myeloid leukemia. We have been testing several compounds to examine their effects on AML cells. This project is intended to study the effect of novel agents for acute myeloid leukemia. We are supported by National Science Council to present our research result at the American Society of Cancer Research Annual Meeting in California, April. 2005. During this meeting, our research group presented one of our research projects titled "A small-molecule c-Myc inhibitor triggers mitochondria-mediated apoptosis and induces monocytic differentiation of human acute myeloid leukemia".

During this meeting, we also learned the development of novel agents and the identification of new mutations that can be used to diagnose myeloproliferative disorders and develop new therapeutics for these diseases.

In this meeting we did not present our data with regard to the use of superoxide dismutase or proteasome inhibitor, as these results will be presented later in American Society of Hematology.

The detail of our presentation is listed below.

American Association for Cancer Research

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5901 A small-molecule c-Myc inhibitor triggers mitochondria-mediated apoptosis and induces monocytic differentiation of human acute myeloid leukemia

Ming-Jer Huang, Yuenchi Cheng, Shufan Lin, Zhilone Chen, Techeng Su, Gi-Ming Lai, Hsingjin E. Liu, *MacKay Memorial Hospital, Taipei, Taiwan Republic of China, Taipei Medical University, Taipei, Taiwan Republic of China, Dept of Administration, National Health Research Institute, Taipei, Taiwan Republic of China.*

The proto-oncogene c-Myc plays an important role in the control of cell proliferation, apoptosis and differentiation, and its aberrant expression is a common molecular defect in human cancers, including acute myeloid leukemia (AML). The heterodimeric formation of Myc with Max transactivates the multiple downstream genes responsible for leukemogenesis. Recently several small-molecule inhibitors that block the interaction between Myc and Max have shown promise in the treatment of solid tumors. Here we studied whether inhibition of Myc/Max interactions might be useful for AML. We first used MTT assays to screen the sensitivity to a small molecule c-Myc inhibitor (MYCi), 10058-F4, in various leukemic cell lines: KG1a, HL-60, K562, and NB4, and found that HL-60 cells were particularly sensitive to c-Myc inhibition. MYCi arrested HL-60 cells at G0/G1 phase (from 50.9% to 85.0% of cells in G0/G1phase, 48.4% to 11.6% in S phase), and induced apoptosis over prolonged exposure. The MYCi-induced apoptosis was mediated via mitochondrial pathway, by activating caspase 9, caspase 3 and caspase 7 but caspase 6, and caspase 8 were not affected. The expression of CD95 was also not affected by MYCi. MYCi downregulated anti-apoptotic Bcl-2 expression and upregulated pro-apoptotic bax, p21 and p27 expression. But the expression of cyclin D1, cyclin D3, cyclin E and cyclin B was not affected by MYCi. We further examined the effects of MYCi on the differentiation of AML cells. MYCi induced monocytic but not neutrophilic differentiation by upregulating CD11c, and CD14 but not CD11b. We also used real-time PCR to show that MYCi upregulated the expression of c-Jun, Jun-B, C/EBPalpha, EGR-1 and PU.1 but not ICSBP. Therefore, our data suggest that small-molecule c-Myc inhibitors could be useful in myelomonocytic leukemia by inducing cell cycle arrest, apoptosis and monocytic differentiation of through specific pathways. This inhibitor might also provide an alternative approach to identify c-Myc downstream target genes, and to elucidate the role of c-Myc on monocytic differentiation.

Citation format: Proc Amer Assoc Cancer Res 2005;46:5901.

96th Annual Meeting, Anaheim, CA - April 16-20, 2005