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Hexamethylene bisacetamide inhibits malignant phenotype in T-ALL cell lines

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Abstract

T acute lymphoblastic leukemia cell lines treated with hexamethylene bisacetamide (HMBA) undergo a delay in cell cycle progression and increase susceptibility to apoptosis, although they never overcome the differentiation block.

In accordance with changes in cell cycle and apoptosis, transitory p53 pathway activation commonly occurs. Bcl-2 inhibition further favours the pro-apoptotic effect of HMBA. Notch1 expression is down regulated by reduction of its transcription level. Accordingly, Notch1 protein and transcriptional activity were affected. Even if HMBA generally reduces Notch1 level in T acute lymphoblastic leukemia (T-ALL) cell lines, this does not commonly influence the biological response; in fact all the analysed cell lines, except CEM cells, display no biological effect following DAPT-induced Notch inhibition.

Keywords: T-ALL; HMBA; Notch1; p53; Bcl-2; Bax; Apoptosis; Cell cycle

1. Introduction

Hybrid polar compounds are potent inducers of differentiation in hematopoietic and not hematopoietic neoplastic cells [1–3]. The prototype of this class, hexamethylene bisacetamide (HMBA) was been extensively studied *in vitro* in MEL murine erythroleukemia cell line. HMBA alters factors which control G1 to S phase transition therefore leading to arrest in G1/G0. This is associated with: (1) changes affecting the pRB family [1–3]; (2) changes in the pattern of expression of cyclins and CDKs, the most relevant variation is related to the CDK4 decrease which is critical for the suppression of cell cycle entry [1,3]; (3) changes in p53 protein levels [4].

HMBA also influences apoptosis in many cell lines [1]. This effect is induced by the modulation of different genes involved in apoptosis control, i.e. in human myeloma cells the anti-apoptotic function of Bcl-2 gene is suppressed [5]. Another anti-apoptotic gene, *Notch1*, is downregulated following HMBA administration in murine MEL cell line [6].

Hybrid polar compounds are promising anti-neoplastic drugs. Although HMBA displays important side effects, Marks et al. [3] have synthesized an improved "second generation" compounds. Among these, suberoanylanilide hydroxamic acid (SAHA) was proven to be effective on a variety of tumors in preclinical studies and is currently in clinical trials.

T acute lymphoblastic leukemia (T-ALL) is characterized by a blockage at early differentiation stages. Many genes are involved in T-ALL leukemogenesis and, in the recent years, *Notch1* has taken the front seat since different gain-of-function variants have been found: *t*(7;9)(q34; q34.3) translocation is present in approximately 1% T-ALL patients

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and generates the over expression of a truncated constitutively active Notch1 receptor [7]; *Notch1* mutations in HD and/or PEST domains have been identified in approximately 60% of T-ALL and influences either ligand-independent signalling and/or Notch1 half-life in the nucleus [8]. In addition, this laboratory has shown that high levels of *Notch1* activity represents a general feature of T-ALLs both *in vitro* [9] and *in vivo* [10]. The oncogenic role of deregulated Notch1 in T-ALL must be related to its ability to affect both cell cycle and apoptosis rate during thymocytes maturation [11].

Here we report the biological and molecular effects of HMBA treatment on different T-ALL cell lines. HMBA induces a delay in cell cycle progression and increases susceptibility to apoptosis, although it is not able to overcome the T-ALL block of differentiation. The expression analysis on Molt4 cell line indicates that activation of the p53 pathway and the inhibition of Bcl-2 are molecular events consistent with HMBA biological effect; by contrast this last seems to occur independently from the observed Notch1 modulation, in fact Notch pathway inhibition obtained by treatment with γ-secretase inhibitors does not affect neither cell cycle nor apoptosis.

2. Materials and methods

2.1. Materials

HMBA (22,423-5); antibodies to p53 (P5813), Bax (B3428), Bcl-2 (B3170) and CDK4 (C8218) were from Sigma–Aldrich. DAPT (565770) was purchased from Calbiochem; it was used within 3 months from reconstitution. Antibodies to Notch intracellular domain (NOTCH-IC; sc-6014) and to Actin (sc-10731) were from Santa Cruz Biotechnologies Inc. and phospho-specific (Ser15) anti-p53 (PC461) was from Oncogene Research Products.

2.2. Cell cultures and treatment

Human T-ALL cell lines were maintained as previously reported [9] in 5% CO_2 atmosphere in complete RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (Euroclone). All treatments were performed with exponentially growing cells at the concentration of 4×10^5 cells/ml. Cells were treated with HMBA 5 mM replacing the medium each 2 days. Following reconstitution in DMSO, DAPT was administered to Molt4 cells after medium replacement (each 2 days) at a final concentration of 5 μM, control cells were treated with an equal concentration of DMSO.

2.3. RT-PCR

Total RNA samples were extracted [12] and retrotranscribed by M-MLV Reverse Transcriptase (Gibco BRL-Life Technologies). PCR was performed in a 20 μl reaction mixture containing 2 μl of cDNA, 1× PCR buffer II, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.25 units of Ampli-Taq DNA polymerase (Perkin-Elmer Corporation). Reactions amplified in a GeneAmp PCR System 9700 thermal cycler were performed as previously reported [10] with the exception of the reaction addressed to amplify p21 cDNA: the following set of primers was used, GGAAGACCATGTGGACCTGT (Fwd) and GGATTAGGGCTTCCTCTTGG (Rev), through 26 cycles of amplification at a 56 °C melting temperature. All amplified cDNAs were separated by agarose gel electrophoresis and images were acquired by the Image Acquisition System EDAS 2400 (Kodak) and following analyzed by Kodak 1D Image Analysis System.

2.4. Western blot assay

Total proteins (60 μg/lane) were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were probed with the indicated antibodies and signal was detected with the enhanced chemiluminescence (ECL) kit (Amersham Biosciences). Signal quantification was performed following image acquisition by means of EDAS 2400 System (Kodak) and subsequent analysis by Kodak 1D Image Analysis System.

2.5. Apoptosis analysis by annexin V and PI staining

Cells undergoing apoptosis were identified by Annexin-V/FITCH and Propidium Iodide staining (Immunotech). Briefly, 5×10^5 cells were washed once in PBS and resuspended in 500 μl of binding buffer 1× (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Propidium iodide (PI, final concentration 2.5 μg/ml) and FITC-conjugated Annexin-V (final concentration 0.05 μg/ml) were added; cell suspension was incubated on ice for 10 min in the dark and analyzed by flow cytometry using FACScalibur with CellQuest software.

2.6. Cell cycle analysis by PI staining

For cell cycle analysis, 2×10^6 cells were washed once in PBS, resuspended in 0.5 ml of ice-cold GM solution (6 mM glucose, 137 mM NaCl, 5 mM KCl, 1.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.1 mM KH_2PO_4, 0.5 mM EDTA), fixed by adding 1.5 ml of 96% ethanol and stored at 4 °C. Cells were then washed once in PBS and incubated overnight at 4 °C in PI solution (25 μg/ml PI, 25 μg/ml RNAse A). DNA analysis was performed on at east 20,000 cells for each sample by using FACSCalibur instrument (Becton Dickinson) and the percentage of the cells in the different cell cycle phases was analyzed using ModFit program [13].