Materials and Methods

Cell line and cell culture

The HL-60 cell line, derived from a patient with AML (20), was maintained in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 80 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air. The other human leukemia cells were also cultured under the same conditions (65).

Assay of cell growth and properties of differentiated cells

Suspensions of cells ($0.5x10^5$ cells/ml) in 2 ml of culture medium were incubated with or without the test compounds in multidishes (Costar, Cambridge, MA, USA). All of the cytokinins, other adenine analogs and ATRA were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and, VD_3 was from Wako Chemicals (Osaka). The inhibitors of reactive oxygen species; N-acetyl cysteine and ambroxol (Sigma Chemical Co., St. Louis, MO, USA), the inhibitors of mitogen-activated protein kinase; PD98059 (Calbiochem, La Jolla, CA, USA), the inhibitors of caspase-3; BOC-Asp(OMe)-CH2F (FK-011) (Enzyme System Products, Livermore, CA, USA), and the inhibitors of signal transduction; were pre-treated 3 hours before treatment with differentiation inducers. Cell numbers were counted with a model Z1 Coulter Counter (Beckman-Coulter Electronics, Miami, FL, USA). Superoxide-generating oxidase was determined by the ability of the cells to reduce nitroblue tetrazolium (NBT) upon exposure to 12-*O*-tetradecanoyl phorbol-13-acetate (64). Cells were incubated in 1 ml of RPMI 1640 medium containing 1 mg/ml of NBT (Sigma Chemical Co., St. Louis, MO., USA), and 12-*O*-tetradecanoyl phorbol-13-acetate (100 μg/ml) at 37°C

for 50 minutes. The reaction was stopped by adding 5 M HCl (1 M, final concentration). The suspension was kept at room temperature for 20 minutes and then centrifuged. Formazan deposits were solubilized in dimethyl sulfoxide, and the absorption of the formasan solution at 560 nm/10^7 cells was measured in a spectrophotometer. Lysozyme activities were determined by a lysoplate method with lysoplates containing 1% agar, 1/15 M sodium phosphate buffer (pH 6.6), 50 mM NaCl and heat-killed *Micrococcus lysodeikticus* (0.5 mg/ml) (53). Expression of cell surface differentiation-associated antigens (CD markers) was determined by monoclonal antibody labeling and flow cytometry. Briefly, cells ($2x10^6$) were washed with phosphate-buffered saline (PBS) and incubated with fluorescein isothiocyanate (FITC)-labeled antibodies for CD11b (DAKO Japan , Tokyo), in 50μl of PBS containing 0.1% bovine serum albumin (BSA) at 4 °C for 30 minutes. The cells were washed with PBS and then analyzed in an Epics XL flow cytometer (Coulter Electronics, Hialeah, FL, USA) (65). Morphological changes were examined in cell smears using light microscopy of cytospin preparation stained with May-Grünwald-Giemsa solution (Merck, Darmstadt, Germany).

Annexin V binding

Cells were harvested and washed with ice-cold PBS, then incubated in PBS containing 2% BSA, 0.01% NaN_3, and 10% casein for 20 minutes on ice to block nonspecific binding sites. Subsequently, the cells were washed with stainig buffer [1% BSA in 50 mM HEPES buffer (pH 7.4)] and fixed with 4% paraformaldehyde in staining buffer for 10 minutes. Fixed cells were labeled with FITC-labeled annexin V (Genzyme Co., Cambridge, MA, USA) (2.5 μg/ml in HEPES buffer containig 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$, pH 7.4) for 15 minutes on ice to determine

phophatidylserine exposure to the outer face of the cell membrane. FITC-conjugated murine IgG monoclonal antibodies of unrelated specificity were always used as controls. After staining, cells were washed and mean fluorescence intensity percentages of positive and negative cells were analyzed by flow cytometry.

Assay for caspase-3 activity

Caspase-3 activity was assayed with the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-MCA) (Peptide Institute, Inc., Osaka). Briefly, 10^7 cells were extracted with 1 ml of 10 mM Tris-HCl (pH 8.1) containing 9 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 100 μM leupeptin, 1.5 μM pepstatin, 18.4 μmol/ml phosphoramidon, and 5 mM EDTA at 0 °C for 30 minutes, and then centrifuged at 15,000 x g for 2 minutes. Extracts were stored at -80 °C until use. For assay, the extracts were mixed with the substrate (final, 100 μM) and Tris-HCl (final, 10 mM; pH 7.4), and incubated at 37 °C for 90 minutes. An equal volume of 1 mM acetic acid was added and the supernatants were analyzed by fluorescence spectrophotometer (excitation at 370 nm and emission at 460 nm). Enzyme activity was expressed as pmol aminomethylcoumarin/min/mg protein.

DNA fragmentation assay

Cell lysates were prepared in DNA isolation buffer [10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.65 M NaCl, 1% sodium dodecyl sulfate (SDS) and 0.2 mg/ml proteinase K]. DNA was extracted with phenol/chloroform, and then precipitated with ethanol. After treatment with 0.1 mg/ml RNase A (Sigma Chemical Co., St. Louis, MO, USA) for 1 hour at 37°C, equal

amounts of DNA were detected by electrophoresis in a 1.5% agarose gels and visualized by ethidium bromide staining (Sigma Chemical Co., St. Louis, MO, USA) and ultraviolet transillumination.

Determination of intracellular adenosine-5'-triphosphate (ATP) content

Suspensions containing $1x10^7$ cells were washed three times with cold PBS. The cells were centrifuged at 1,000 x g for 5 minutes. The supernatant was discarded, and 1 ml of 10 % (w/v) perchloric acid was added. The mixture was allowed to stand for 20 minutes at 4°C. The cell lysates were centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was neutralized with 1M KOH, and then centrifuged at 10,000g for 10 min at 4°C. The clarified sample (100 μl) was mixed with 100 μl of luciferase-luciferin reagent (Wako Chemicals, Osaka). Luminescence from the reaction at room temperature was measured with a luminometer. The blank was subtracted from the raw data, and the ATP concentration was determined from a log plot of the standard curve data.

Measurements of mitochondrial transmembrane potential and intracellular reactive oxygen species (ROS)

Mitochondrial membrane potential was quantitated by the flow cytometric analysis of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iode)-stained cells, using DePsipher™ kit (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. The cells (1×10^6) were harvested and centrifuged at 5,000 x g for 5 minutes at room temperature. The cell pellets were resuspended with 1ml of reaction buffer, prewarmed at 37 °C and 1 μl of DePsipher™ solution. They were incubated at 37 °C, 5 % CO_2 for 20 minutes. Then, the samples were washed twice in PBS with centrifugation at 5,000 x g between each

wash. The cells were resuspended in 1 ml of PBS and analyzed quickly by frow cytometry (488nm argon laser).

For measurement of ROS, cells were incubated with 20 ng/ml hydroethidine (Molecular Probes Inc., Eugene, OR, USA) in PBS at 37°C, washed twice with PBS and then analyzed as soon as possible by flow cytometry using the red laser channel. This assay is based on the chemical properties of hydroethidine, a weak blue fluorescent dye which is selectively converted by O_2^- to ethidium with bright a red fluorescence (8).

Uptake of BA and assay of its metabolites

Cells were preincubated at 37°C for 10 minutes, and 37 kBq of [8-^{14}C]-BA (1,961 MBq/mmol; Moravek Biochemicals, Inc., Brea, CA, USA) was added to the cell suspension (2 x 10^7 cells/10 ml). Incubation was carried out at 37 °C for various durations up to 180 minutes, and stopped by adding 5 volumes of cold PBS. The cells were then washed three times with cold PBS and homogenized in 1 ml cold 10% (w/v) perchloric acid. After standing on ice for 20 minutes, the sample was centrifuged and the supernatant was set aside as the acid-soluble fraction. The perchloric acid-insoluble pellet was washed twice in 1 ml 5% perchloric acid and once in 1 ml of ethanol. RNA was extracted from the acid-insoluble pellet by hydrolysis for 20 hours at 37°C in 0.5 ml of 1 M KOH. After alkaline hydrolysis, the sample was cooled and neutralized with 0.1 ml of 6 M HCl-5% perchloric acid. The resultant supernatant and pellet were considered RNA and DNA/protein fractions, respectively. Aliquots were then taken for the assay of radioactivity. Radioactivity was determined by liquid scintillation counting. To further analyze the labeled RNA, DNA and protein,

I isolated them by the procedure of Chomczynski (18) using Isogen (Nippon Gene, Toyama), a reagent that contains phenol and guanidine thiocyanate.

Ascending chromatography on Silica Gel 60F254 (Merck, Darmstadt, Germany) was used to separate BA and its metabolites. The cell suspension (0.4 ml) was mixed with tetrahydrofuran (1.2 ml). Authentic compounds were then added to the supernatant and an aliquot (50 μl) was spotted on a chromatographic sheet, which was developed in a solvent system of chloroform/ methanol/water (80/20/1, v/v/v) or *n*-butanol/water (86/14, v/v). The zones corresponding to authentic compounds were evaluated by autoradiography with a Fuji Bio-Image Analyzer BAS2000 (Fuji Film, Co. Ltd., Tokyo). Digestion of RNA and DNA into nucleosides was accomplished using *Micrococcus* nuclease (Takara Bio Inc., Tokyo) and alkaline phosphatase (Takara Bio Inc., Tokyo). DNA or RNA (10 μg) was incubated with 50 U of *Micrococcus* nuclease in 50 μl of reaction buffer at 37°C for 60 minutes, and then 5 μl of 10 x buffer and 10 U of calf intestine alkaline phosphatase were added and incubation was continued for an additional 60 minutes. After incubation, the digested sample was mixed with 0.15 ml of tetrahydrofuran and used to identify radioactive BA in RNA and DNA. Nucleotide monophosphates in the acid-soluble fraction were digested to nucleosides by this phosphatase treatment.

Separation of nucleotides by column chromatography

A cell suspension (0.4 ml) was mixed with tetrahydrofuran (1.2 ml). The supernatant fluids were dried up, dissolved in distilled water and put on a column (Dowex 1- X 8, 200-400 mesh, formate form; Muromachi Technos Co, Ltd., Tokyo). Elution was performed with distilled water and then 0.1 M formic acid to remove BA, and the nucleosides and monophosphates were

removed by 1 M and 4 M formic acid, respectively. The nucleotide diphosphates, triphosphates and others were removed by 0.2 M-ammonium formate-4M-formic acid, 0.8 M-ammonium formate-4 M-formic acid and 88% formic acid (12).

Western blot analysis

Cells were packed after washing with cold PBS, and then, lysed at a concentration of 2 x 10^7 cells/ml in sample buffer [60mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.003% bromophenol blue]. Then the lysates were resolved on 10% SDS-polyaclilamide gels. The proteins were transferred electrophoretically from gel to an Immobilon-P membrane (Millipore, Bedford, MA, USA) and immunoblotted with anti-C/EBPα protein (sc-61), anti-C/EBPβ protein (sa-150), anti-C/EBPδ protein (sc-636), anti-C/EBPε protein (sc-158), and anti-C/EBPγ protein (1:1000 dilution; Santa Cruz Biotechnnnology, Inc., Santa Cruz, CA, USA). Alkaline phosphatase-conjugated IgG (Bio-Rad Laboratories, Hercules, CA, USA) was used as a secondary antibody (1: 2000 dilution). The bands were developed by treatment with the Immune-Star™ AP chemiluminescent (Bio-Rad Laboratories, Hercules, CA, USA) for 3 minutes at room temperature, and detected using a Fuji Lumino Image Analyzer LAS-1000 system (Fuji Film, Co. Ltd., Tokyo).

Determination of messenger RNA (mRNA) levels by reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed using a GeneAmp RNA PCR kit (Takara Bio Inc., Tokyo). Total RNA (1 μg) was isolated using Isogen, and reverse-transcribed to synthesize cDNA using random hexamers at 4°C. Subsequently, cDNA were amplified by means of PCR using specific primers

in 20 μl mixtures consisting of 10 mM Tris-HCl (pH 8.3), 50 nM KCl, 1.2 mM $MgCl_2$, and 0.2 mM dNTPs. The oligonucleotides used in PCR amplification were as followes:

sense strand, 5'-AATCTAGCACCATGACGGAA-3';

antisense strand, 5'-CAACAAACACTTTTGGGAAG-3' for S100P;

sense strand, 5'-AGAAAGCCTTGAACTCTATC-3';

antisense strand, 5'-TACTCTTTGTGGCTTTCTTCAT-3' for S100A8;

sense strand, 5'-ATGACTTGCAAAATGTCGCA-3';

antisense strand, 5'-ATCTTGGCCACTGTGGTCTTA-3' for S100A9;

sense strand, 5'-ACTGCTGGCTTTTTGCTGTA-3';

antisense strand, 5'-AGTGTGTTTATTAACTCTTA-3' for S100A12;

sense strand, 5'-GGTCGGAGTCAACGGATTTG-3';

antisense strand, 5'-ATGAGCCCCAGCCTTCTCCAT-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR comprised 25 cycles for S100P, 30 cycles for S100A8, 21 cycles for S100A9, 28 cycles for S100A12 and 18cycles for GAPDH, with denaturing at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 1.5 minutes. The reaction was performed in a GeneAmp PCR system 9700 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). After amplification, the PCR products were detected by electrophoresis in a 2 % agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination.

Assay of mitogen-activated protein kinase (MAPK) activity

The kinase assay was performed using a p44/42 MAP Kinase Assay Kit (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. Briefly, HL-60 cells treated with IPA or ATRA were collected and lysed. Immobilized phospho MAPK antibody was added

to cell lysate, and incubated with gentle rocking over night at 4 °C. The immune complexes were microcentrifuged and washed with lysis buffer. Immuno-precipitated pellets were incubated in kinase buffer containing Elk-1 fusion protein and cold ATP. Then, Elk-1 phosphorylation at Ser383, one of the in vivo targets of MAPK was analyzed using phospho-antibodies by western blotting and chemiluminescent detection.

Microarray analysis

Total RNA (150μg) was isolated using Isogen from HL-60 cells treated with or without inducers for 24 hours. Then, all of the following analysis was commissioned (TaKaRa Bio.Inc., Tokyo). Briefly, the probes were labeled with Cy3 and Cy5 fluorescence dyes individually, hybridized with cDNA microarray representing about 1000 different human genes (TaKaRa, human 1K chip), and their fluorescent intensities were scanned. The genes were screened through the analysis of the difference in two gene expression profiles.

Antisense Oligonucleotides and Cell treatment

Oligomers (18mers) corresponding to the antisense sequences flanking the translation initiation region of the human mRNA for C/EBPα, C/EBPβ, C/EBPδ and S100P were prepared. Oligomers for missense-C/EBPδ was also prepared. The sequence were as follows; C/EBPα, GAAGTCGGCCGACTCCAT; C/EBPβ, GGCCACCAGGCGTTGCAT; C/EBPδ, GAAGAGCGCGGCGCTCAT; missense-C/EBPδ, GCAGAGTGCAGCGCTCAG; S100P, TGTCTCTAGTTCCGTCAT. Assay was performed using Tfx™ reagent (Promega, madison, WI, USA) according to the manufacturer's instructions. Briefly, the Tfx™ reagents

were suspended with distilled water the day before transfection and stored at -20 °C. On the day of transfection, the Tfx™ Reagent/DNA mixture was prepared. To a sterile tube, 0-20 μl of DNA from 500μM stock solution and 0-40μl of Tfx™ reagent were added to a total volume of 0.85ml serum-free medium (prewarmed to 37 °C). The ratio of Tfx™ reagent to DNA was 2 to 1. Tfx™reagent/DNA mixtures were incubated 10-15 minutes at room temperature. While mixtures are incubating, HL-60 cells were washed twice with serum-free medium. An aliquot 0.5ml of cells ($4x10^5$ cells in serum-free medium) was put into each well of a 24-well plate. Tfx™ reagent /DNA mixture were mixed and added to the well (0.2 ml/well). The cells were returned to an incubator. After 12 hours, 0.2ml of serum free medium, 0.1ml of serum, a differentiation inducer and antisense oligomers were added together to each well. The cells were returned to an incubator, and the incubation was continued for an appropriate length of time before analysis.