

# Clinically Potential Subclasses of Retinoid Synergists Revealed by Gene Expression Profiling<sup>1</sup>

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## Abstract

**Retinoids have chemopreventive and therapeutic potency in oncology and dermatology, although their application is restricted by many undesirable side effects. For the development of more effective and less toxic retinoids, gene expression analyses using DNA microarrays have the potential to supplement conventional screening methods, which are based on the changes in cell morphology and/or function. In this study, we applied the class prediction algorithm, which was used in the molecular phenotyping of tumors, for the classification of synthetic retinoids (Am80 and Tp80) and retinoid synergists (HX630, TZ335, and PA024) as all-*trans* retinoic acid-like, 9-*cis* retinoic acid-like, and control-like classes. By analyzing the effects of all-*trans* retinoic acid and 9-*cis* retinoic acid on the gene expressions in a human promyelocytic leukemia cell line, HL60, we successfully selected 50 marker genes whose expression pattern could distinguish these classes. Moreover, the classification revealed the existence of two subclasses among the retinoid synergists used with Am80. Close inspection of the DNA microarray analyses indicated that these two subclasses had different effects on the apoptosis of HL60 cells, and this was confirmed by *in vivo* experiments. These results indicate that the retinoid activity of Am80, which has already been used in clinical trials, could be modulated differently by the two classes of retinoid synergists. Thus, these two subclasses of retinoid synergists have the potency to widen the usage of Am80. Our analyses demonstrated that the gene expression profiling could provide important information for developing useful retinoid**

**synergists by compensating conventional screening methods.**

## Introduction

Retinoids are natural or synthetic analogues of all-*trans* retinoic acid (Fig. 1) and have potential chemopreventive and therapeutic applications in the fields of dermatology and oncology (1), e.g., all-*trans* retinoic acid and synthetic retinoid, Am80, have a remarkable remedial effect on acute promyelocytic leukemia treatment (2, 3). As the therapeutic applications of retinoids have become wider, a number of synthetic retinoids has been developed. However, because of the pleiotropic biological activities of retinoids, such as their effects on cell differentiation, proliferation, apoptosis, and embryonic development, retinoid therapy is still restricted by many undesirable side effects. Retinoid activities are mainly achieved through the transcriptional regulations of specific genes via two kinds of nuclear hormone receptors: (a) RAR<sup>3</sup>  $\alpha$ ,  $\beta$ , and  $\gamma$ ; and (b) RXR  $\alpha$ ,  $\beta$ , and  $\gamma$ . All-*trans* retinoic acid preferentially binds RARs, and its 9-*cis* isomer, 9-*cis* retinoic acid, binds both RARs and RXRs. From this fact came the idea to develop compounds that are able to modulate RARs and RXRs selectively, because such compounds could be expected to control gene expression differently and cause fewer side effects.

HL60 cells, which were established from an acute promyelocytic leukemia patient, are a line of leukemic myeloid precursor cells (4). When HL60 cells are cultured with all-*trans* retinoic acid, they terminally differentiate into granulocytes (5). This differentiation system has been used for the functional screening of chemically synthesized compounds, because it is a sensitive assay to detect retinoid activities. A variety of compounds has been synthesized and screened by this system. Some of them are able to differentiate HL60 cells alone (synthetic retinoid; Fig. 1). There is another class of compounds that is not able to differentiate HL60 cells by itself but is able to enhance the differentiation induced by synthetic retinoids. This class of retinoids is called "retinoid synergists" (Fig. 1; Refs. 6–8). Synthetic retinoids, such as Am80, bind to and activate RARs selectively, whereas retinoid synergists bind to and activate RXRs selectively. The different effects of synthetic retinoids and retinoid synergists are thought to be explained by their selectivity to RARs and RXRs. However, the transactivation process in the physiological situation is not so simple (Ref. 9 and reviewed in Ref. 10) because they form heterodimers that also recruit auxiliary proteins. The HL60 cell differentiation assay is sensitive and simple to perform; still, it depends on the morphological or functional changes of the cells. Considering

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<sup>3</sup> The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; PML, promyelocytic leukemia; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; NBT, nitro-blue tetrazolium; POD, PML oncogenic domain.

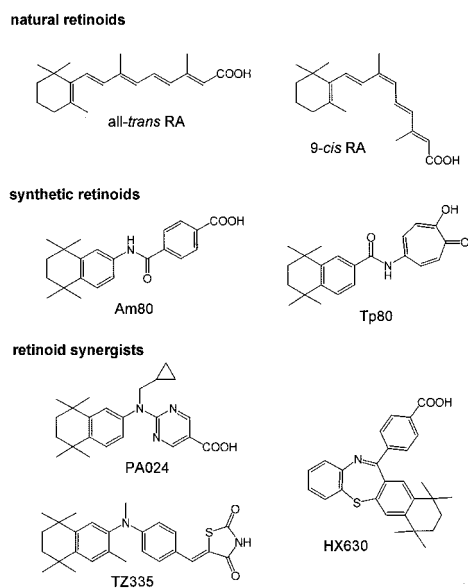


Fig. 1. Structures of retinoids and retinoid synergists used in this study.

the complexity of the RAR-RXR heterodimer activation process, the conventional cell differentiation assay has a limitation in its usefulness for more precise classifications of chemical compounds.

According to Nagy *et al.* (11), ligand activation of the RAR moiety in the RAR-RXR heterodimer is sufficient to induce the differentiation of HL60 cells. Ligand activation of the RXR moiety is not sufficient to induce differentiation, although it may potentiate the differentiation-inducing activity of RAR-binding ligand. These results indicated that gene expressions during the differentiation of HL60 cells were controlled differently by synthetic retinoids, retinoid synergists, or their combinations. So, if one could identify (a) group(s) of genes whose expressions are differently regulated by RARs and RXRs, a precise classification of compounds would be possible. Such a classification method provides a useful supplement to the conventional HL60 cell differentiation assay.

Recently, DNA microarray analysis has been used for the molecular phenotyping of human tumors. In this application, genes specifically expressed in tumor samples are selected based on their expression information from DNA microarray analyses, and the profile of the selected genes is used as a marker for tumor diagnosis. Several groups have reported that they were able to select such marker genes and predict the clinical status of patients (e.g., Refs. 12 and 13). These successful results indicated that two or more different statuses are distinguishable based on the expression profiles of genes selected by DNA microarray analyses. In this study, we applied this gene expression profiling approach for the prediction of the classification of synthetic retinoids and retinoid synergists. Comparison of gene expression pattern in *all-trans* retinoic acid- and *9-cis* retinoic acid-treated HL60 cells by DNA microarray successfully selected 50 marker genes. Classification of several synthetic retinoid and retinoid synergists was predicted based on the expression pat-

tern of these 50 marker genes. The results of the prediction were consistent with previous observations on synthetic retinoids. Moreover, the classification of retinoid synergists unexpectedly revealed two subclasses. The results described in this study indicate that gene expression profiling by DNA microarray is as useful for the classification of chemicals as for the classification of tumor status.

## Materials and Methods

**Chemicals.** *All-trans* retinoic acid, *9-cis* retinoic acid, and TPA were purchased from Sigma Chemical Co. (St. Louis, MO). NBT was purchased from Wako Pure Chemicals (Osaka, Japan). Am80 (14), Tp80 (15), PA024 (6), TZ335 (7), and HX630 (8) were synthesized at The University of Tokyo. Retinoids and retinoid synergists were dissolved in ethanol.

**Cells, Cell Culture, and Induction of Differentiation.** The human promyelocytic leukemia cells, HL60, were provided by Dr. F. Takaku (Faculty of Medicine, The University of Tokyo). The cells were cultured in suspension in RPMI 1640 (Biomedicals, Inc., Aurora, OH) supplemented with 5% fetal bovine serum (BioWhittaker, Walkersville, MD) and penicillin-streptomycin (Invitrogen, Grand Island, NY) under 5% CO<sub>2</sub> at 37°C. Retinoids, retinoid synergists, or equivalent concentrations of ethanol (0.5% of culture medium) were added to cells for the hours indicated, and then cells were harvested and assayed.

**NBT Reduction Assay.** After harvesting the cells, they were incubated for 20 min at 37°C in RPMI 1640 (5% fetal bovine serum) and an equal volume of PBS containing NBT (0.2%) and TPA (200 ng/ml). The percentage of cells containing blue-black formazan was determined with a minimum of 200 cells under the microscope (14).

**Total RNA Preparation.** After washing the cells twice with PBS, total RNA was prepared with RNeasy Mini total RNA Preparation Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

**Detection of Apoptotic Cells.** Apoptotic cells were stained by Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol. The percentage of annexin-V-positive cells was determined with a minimum of 200 cells under a confocal fluorescence microscope in triplicate.

**DNA Microarray Analysis.** Converting total RNA to the targets for Affymetrix GeneChip DNA microarray hybridization was done according to the manufacturer's instructions. The targets were hybridized to human genome U95A GeneChip DNA microarray (Affymetrix, Santa Clara, CA) for 16–24 h at 45°C. After the hybridization, the DNA microarrays were washed and stained on Fluidics Station (Affymetrix) according to the protocol provided by Affymetrix. Then, the DNA microarrays were scanned, and the images obtained were analyzed by Microarray Suite Expression Analysis Software (version 4.0; Affymetrix). The results of DNA microarray analyses are available on request.<sup>4</sup>

**Experimental Design of the Class Prediction of Retinoids and Retinoid Synergists.** HL60 cells were incubated with retinoids and/or retinoid synergists for 72 or 96 h. HL60

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cells cultured with the same amount of ethanol (0.5% of culture medium) were used as a control. Total RNA was prepared at each time point and converted to the target for GeneChip hybridization. The gene expression of each time point was analyzed in duplicate by Human Genome U95A GeneChip using these targets. The induction of differentiation was done twice independently for some retinoids and retinoid synergists and designated as experiments 1 and 2.

#### Selection of “Predictor Genes” to Classify Samples into Control Class, All-*trans* Retinoic Acid-treated Class, and 9-*cis* Retinoic Acid-treated Class.

The selection of predictor genes was done in three steps based on the GeneChip measurements obtained from control, all-*trans* retinoic acid-treated, and 9-*cis* retinoic acid-treated samples (“reference set”). The periods of the retinoid treatments were 72 and 96 h. The first step was selecting genes whose GeneChip measurements were reproducible. To do this, the difference in duplicate measurements (“Average Difference” value) of each gene for each treatment was calculated. If the difference of a given gene in a given treatment was  $>2$ -fold, that gene was excluded from additional analyses. A group of 3,035 genes of 12,626 genes on the U95A GeneChip was left at this step. The second step was picking up genes whose expressions were affected by the all-*trans* retinoic acid and/or 9-*cis* retinoic acid exposures. The induction or suppression of a given gene expression in a given treatment was judged by applying the following criteria on the parameters provided by “Comparison Analysis” of Affymetrix Analytical Suite software: (a) “Difference Call” was “Induced,” “Marginally Induced,” “Decreased,” or “Marginally Decreased”; (b) the induction ratio (“Fold Change”) was  $\geq 2.5$  (in induced cases) or  $\leq -2.5$  (in suppressed cases); and (c) the Average Difference value was  $\geq 50$  in the retinoid-treated samples (in induced cases) or controls (in suppressed cases). Genes that met these criteria in at least one of the measurements of the reference set were picked up as affected genes. A group of 333 genes was left after the second selection. The third step was identifying predictor genes that classified control, all-*trans* retinoic acid-treated, and 9-*cis* retinoic acid-treated samples most efficiently. For this purpose, Average Difference values of all measurements of these 333 genes were analyzed by the “Predict Parameter Value” function in GeneSpring Expression Analysis software (Silicon Genetics, Redwood City, CA). Fifty predictor genes were selected, whose expression levels were clearly different among the three treatments with high reproducibility.

**Class Prediction of Synthetic Retinoid and/or Retinoid Synergist-treated Samples.** GeneChip measurements (Average Difference) of synthetic retinoids (Am80 and Tp80) and/or retinoid synergists (HX630, TZ335, and PA024; “test set”) were analyzed by the GeneSpring software. Classes of “test set” samples were predicted by applying the “k-nearest neighbors” algorithm in the “Predict Parameter Value” function. Predict Parameter Value function is a supervised learning method where the algorithm learns from samples with known class membership (reference set) and establishes a prediction rule to classify new samples (test set). In this case, k-nearest neighbors algorithm was used as the prediction rule, which classifies each sample from the test set by finding

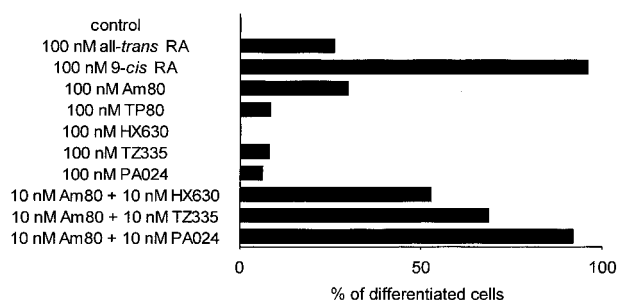


Fig. 2. Effects of retinoid synergists on HL60 cell differentiation. HL60 cells were incubated with retinoids and/or retinoid synergists for 48 h, and the NBT reduction assay was performed to determine the percentage of differentiated cells, which is the conventional screening method for discrimination of retinoidal activity of a drug. A representative result is shown.

the k-nearest neighboring reference sample based on the Euclidean distance of expression intensity of predictor genes. The number of nearest neighbors, k, was set at 5, and the *P* cutoff was set at 0.2.

## Results

**Characteristics of Retinoids.** The retinoids used in this study are shown in Fig. 1. All-*trans* retinoic acid and 9-*cis* retinoic acid are the endogenous retinoids. The others were chemically synthesized and characterized previously (6–8, 14, 15). In brief, Am80 and Tp80 showed retinoidal activities as strong as or in some instances much stronger than natural retinoids. PA024, TZ335, and HX630 showed no retinoidal activities when they were used alone, but they could enhance the potency of Am80 in the HL60 differentiation assay. Because of this activity, they were considered to be retinoid synergists (6–8). To illustrate these activities, HL60 cells were cultured with these retinoids alone or in combination with Am80 for 48 h, and the progress of the differentiation was measured by an NBT reduction assay. A representative result is shown in Fig. 2. All-*trans* retinoic acid (100 nM) induced differentiation in 26% of the entire population at this time point. The same concentration of 9-*cis* retinoic acid differentiated HL60 more rapidly (~100%). Am80 differentiated a little bit more (30%) than all-*trans* retinoic acid, and Tp80 did ~10%. All-*trans* retinoic acid, Am80, and Tp80 differentiated 91, 88, and 76% of HL60 cells after 72-h exposure. The retinoid synergists HX630, TZ335, and PA024 by themselves differentiated  $<10\%$  of HL60 cells at the concentration of 100 nM. The percentage of differentiated HL60 cells by the retinoid synergists was  $<10\%$  even after 72-h exposure (data not shown). When these retinoid synergists, HX630, TZ335, and PA024, were used with Am80 simultaneously, the percentages of differentiated HL60 cells were enhanced to 53, 69, and 92%, respectively. The magnitude of differentiation was almost equal to or a little less than that of 9-*cis* retinoic acid but more than all-*trans* retinoic acid, Am80, or Tp80 alone. This enhancement was striking because the concentration of the synthetic retinoid and retinoid synergists was one-tenth (10 nM) of the concentration of the chemicals used individually (100 nM).



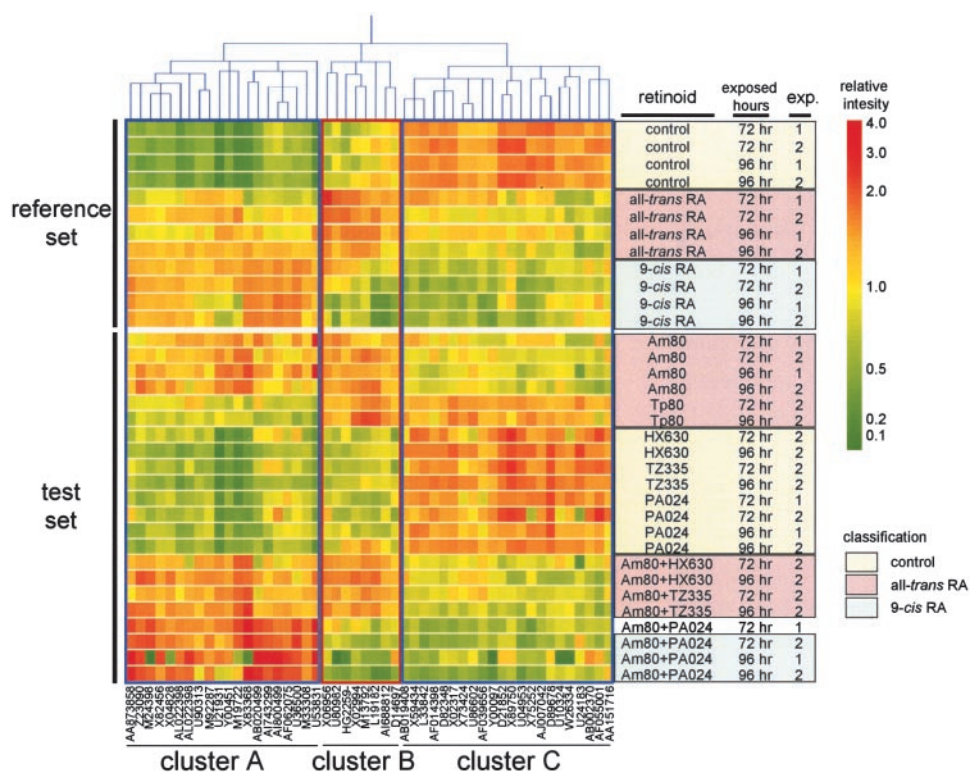


Fig. 3. Classification of synthetic retinoids and retinoid synergists based on the expression pattern of 50 predictor genes. In the top panel, the expression patterns of 50 predictor genes in control, all-trans retinoic acid-treated and 9-cis retinoic acid-treated samples (reference set) are shown. Each row represents an individual sample, and each column represents a single gene whose accession number is shown at the bottom of the panel. The expression level of each gene, the average of duplicate DNA microarray measurements, was normalized across the samples in the reference set, and the relative intensity was depicted in the pseudocolor scale. Fifty predictor genes were arranged according to their similarity of expression patterns across the reference set by hierarchical clustering, and the dendrogram is shown at the top of the panel. Three major nodes of the dendrogram are designated as clusters A–C. In the bottom panel, the expression patterns of predictor genes in synthetic retinoid and/or retinoid synergist-treated samples are shown. The classification of each chemical treatment, which was predicted by k-nearest neighbors algorithm for test set, is shown in color. Two independent experiments were done and designated as 1 or 2 in the exp. column.

**Selection of Genes Differentially Expressed in Control, All-trans Retinoic Acid-treated, and 9-cis Retinoic Acid-treated HL60 Cells.** Next, we analyzed the gene expression pattern of HL60 cells treated with natural and synthetic retinoids and/or retinoid synergists for 72 or 96 h at the same concentration as shown in Fig. 2 by Affymetrix Human Genome U95A GeneChip. Exposure times of 72 and 96 h were chosen rather than 48 h because the gene expression changed more at 72 and 96 h than at 48 h in the preliminary time course experiments (data not shown).

Before starting the precise analyses of the obtained expression data, the number of genes whose expressions were changed by each chemical was counted (data not shown). There were many genes whose expression levels were differently changed by the two natural retinoids, all-trans retinoic acid and 9-cis retinoic acid. Therefore, we chose natural retinoids as references and tried to identify genes that were able to distinguish each natural retinoid (and control) on the basis of the gene expression pattern. For this purpose, the expression data obtained by GeneChip analyses were imported to GeneSpring (ver. 4.1) expression analysis software. Fifty genes were selected as predictor genes because the expression patterns of these genes were reproducibly and clearly different among control, all-trans retinoic acid treated-, and 9-cis retinoic acid-treated samples. The procedure for selecting these 50 genes is described in “Materials and Methods.” The expressions of these genes are shown in the top part of Fig. 3 (designated as reference set) after converting their relative expression levels into a pseudocolor scale. Each row represents an individual sample, and each column represents a single gene whose

accession number was shown at the bottom of the panel. Genes were arranged according to the similarity of their expression pattern among the reference sets by applying the “Gene Tree” hierarchical clustering function of GeneSpring. The dendrogram constructed by GeneSpring is shown at the top of the panel. There were three apparent branches, one constituted from the group of genes whose expression was relatively higher in natural retinoid-treated cells (cluster A), another branch contained genes whose expression pattern was opposite to the first group, *i.e.*, relatively lower in natural retinoid-treated cells (cluster C), and the genes in the third branch (cluster B) were expressed relatively highly only in the all-trans retinoic acid-treated cells. The clear contrast in the expression pattern of the 50 genes among the control, all-trans retinoic acid-treated, and 9-cis retinoic acid-treated samples indicated the usefulness of those 50 genes as predictor genes for the characterization of synthetic retinoids. The identification of predictor genes along with “predictive strength” calculated by Predict Parameter Value function is listed in Table 1. A gene with larger predictive strength has more power to discriminate each class from all the others. Comparison of predictive strength values of genes in each cluster indicated that each cluster contributed equally to the prediction of each class.

**Characterization of Synthetic Retinoids and Retinoid Synergists on the Basis of Their Gene Expression Pattern.** Because we had obtained 50 predictor genes, we tried to characterize the synthetic retinoids and retinoid synergists based on their effects on the expression of these genes. The expression pattern of the predictor genes is shown in the bottom panel of Fig. 3 (test set). There was also a charac-

Table 1 List of predictor genes for retinoid classification

Cluster	Accession no.	Gene description	Predictive strength
A	M92287	Cyclin D3	6.6
	U36500	Nuclear body protein Sp140	13.5
	M24398	Parathymosin	13.5
	M19722	v-fgr oncogene homologue	7.3
	AB020499	RP105-associated MD-1	13.5
	X04828	G protein, $\alpha$ inhibiting activity polypeptide 2	13.5
	X83368	PI3k, catalytic, $\gamma$ -polypeptide <sup>a</sup>	13.5
	AF062075	Leupaxin	13.5
	AI800499	Absent in melanoma 1	13.5
	M33308	Vinculin	13.5
	Z23090	Heat shock 27 kDa protein 1	13.5
	Y00451	$\delta$ -Aminolevulinic synthase 1	7.3
	U90313	Glutathione transferase $\omega$	10.7
	X82456	LIM and SH3 protein 1	13.5
	AA873858	Histone H2B, member D	13.5
	U53831	IFN regulatory factor 7	13.5
	AI743299	EST	13.5
	AL022398	EST	13.5
	AL022398	EST	13.5
U21931	EST	13.5	
B	L19182	IGF-binding protein 7	13.5
	U80982	C/EBP, $\epsilon$	9.7
	AI688812	RAS guanyl releasing protein 2	6.6
	X02994	Adenosine deaminase	13.5
	M13792	Adenosine deaminase	13.5
	D14697	Farnesyl diphosphate synthase	6.6
	X06956	Tubulin, $\alpha$ 1	13.5
	HG2259-HT2348	Tubulin, $\alpha$ 1	13.5
C	X89750	TG-interactin factor	7.3
	U86602	EBNA1-binding protein 2	13.5
	U10324	Interleukin enhancer-binding factor 3	13.5
	X75252	Prostatic-binding protein	13.5
	AF014398	Inositol(myo)-1 (or 4)-monophosphatase 2	13.5
	AB019408	Nuclear distribution gene C ( <i>A nidulans</i> ) homologue	13.5
	D82348	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase	13.5
	L33842	Inosine monophosphate dehydrogenase 2	13.5
	U04953	Isoleucine-tRNA synthetase	13.5
	D89678	hnRNP D-like <sup>l</sup>	13.5
	D21852	R3H domain containing	13.5
	AJ007042	Wolf-Hirschhorn syndrome candidate 1	13.5
	AA151716	Apg12 (autophagy 12, <i>S. cerevisiae</i> ) like	6.6
	U24183	Phosphofructokinase	8.4
	X73424	Propionyl coenzyme A carboxylase, $\beta$ polypeptide	13.5
	X59434	Thiosulfate sulfurtransferase	13.5
	X02317	Superoxide dismutase 1	13.5
	AF039656	Brain acid-soluble protein 1	13.5
	Y00097	Annexin A6	13.5
	W26334	Casein kinase 2, $\alpha$ -1	13.5
	AF055001	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	7.3
	AB002370	EST	8.4

<sup>a</sup> PI3k, phosphatidylinositol 3'-kinase; SH3, Src homology 3; EST, expressed sequence tag; IGF, insulin-like growth factor; C/EBP, CAAT/enhancer binding protein; RAS, rat sarcoma virus oncogene; EBNA, Epstein-Barr nuclear antigen; hnRNP, heterogeneous nuclear ribonucleoprotein.

teristic pattern similar to those of the samples in the reference set. The classification of the synthetic retinoids and retinoid synergists was done by applying Predict Parameter Value function in GeneSpring. The classification procedure was described in "Materials and Methods," and the result is depicted in Fig. 3 as a color. GeneSpring successfully classified 21 of 22 samples into three classifications. One case

that was without a predicted classification by GeneSpring was the sample treated PA024 with Am80 for 72 h in experiment 1. In this case, the sample could not be classified into any class, because the two of five nearest neighboring reference samples were all-*trans* retinoic acid-treated samples, and the other three were 9-*cis* retinoic acid-treated samples (data not shown). As a result, Predict Parameter Value func-

tion could not make the decision of classification of this sample.

When each chemical was used, it was classified as expected from the results of the differentiation assays, *i.e.*, Am80 and Tp80, which were able to differentiate HL60 cells alone, were classified into all-*trans* retinoic acid-like class, and the retinoid synergists, which were not able to differentiate HL60 cells by itself, were classified into control-like class. Retinoid synergist alone changed the expression of a limited number of genes (data not shown). One point that should be mentioned here is that the gene expression pattern induced by Tp80 had the appearance of being intermediate between that by all-*trans* retinoic acid and control, although the Predict Parameter Value function classified it into the all-*trans* retinoic acid-like class. Actually, one of the five nearest neighboring reference samples of Tp80-treated samples was control sample, and the rest was all-*trans* retinoic acid-treated samples (data not shown). In Am80 cases, all of the five nearest neighboring reference samples were all-*trans* retinoic acid-treated ones. In the original study by Ebisawa *et al.* (15), Tp80 alone differentiated HL60 cells poorly, but its differentiation activity was improved strongly by the concomitant use of HX630. These results indicate that Tp80 might be a partial agonist rather than a fully active agonist like Am80. The gene expression pattern in Fig. 3 reflected the character of Tp80 as a partial agonist.

When the retinoid synergists were used in combination with Am80, the classification was unexpected. When Am80 and PA024 were used simultaneously, they acted like 9-*cis* retinoic acid. However, interestingly, the combination of Am80 with either HX630 or TZ335 appeared to belong to the all-*trans* retinoic acid-like class. These results suggested the existence of two subclasses among the retinoid synergists. And also, these results demonstrated that chemicals as well as tumor samples could be classified on the basis of the gene expression pattern.

**Characterization of Synergistic Effects of HX630 or TZ335 and Am80 on HL60 Cells.** According to the results of the transient transfection assay, the retinoid synergists used in this study were able to activate RXR (6).<sup>5</sup> Thus, when synthetic retinoids (an RAR-specific ligand, Am80 in this study) and retinoid synergists are used simultaneously, both RARs and RXRs should be activated like 9-*cis* retinoic acid. As a result, retinoid synergists + Am80 should be classified into the 9-*cis* retinoic acid-like class. Against this expectation, HX630 + Am80 and TZ335 + Am80 were classified into the all-*trans* retinoic acid-like class.

When HL60 cells are differentiated by retinoids, they subsequently die by apoptosis. According to Nagy *et al.* (11), the roles of RXRs during the terminal differentiation of HL60 cells are the potentiation of differentiation induced by RAR-binding ligands and the induction of apoptosis. The differentiation was accelerated by the combination of retinoid synergists and Am80 in all cases (Fig. 2). However, there is no report on the effects of retinoid synergists with Am80 on apoptosis,

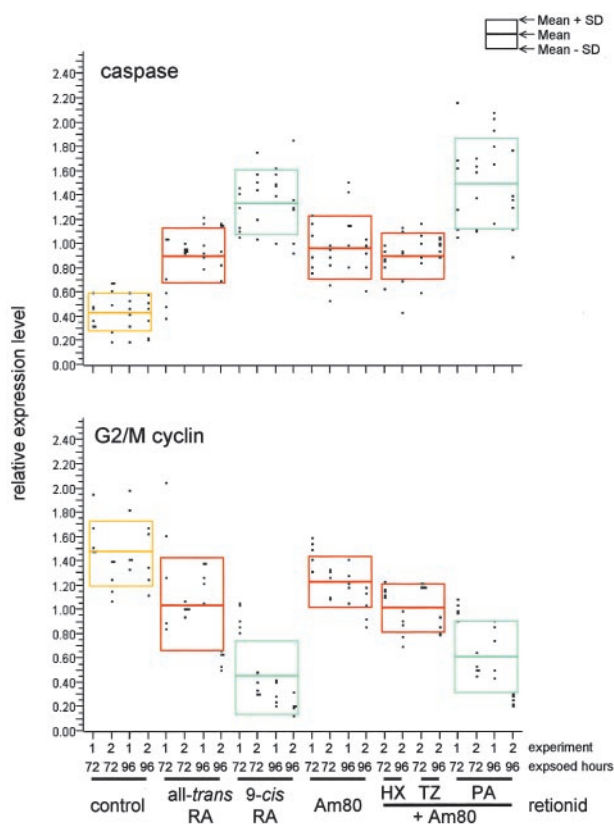
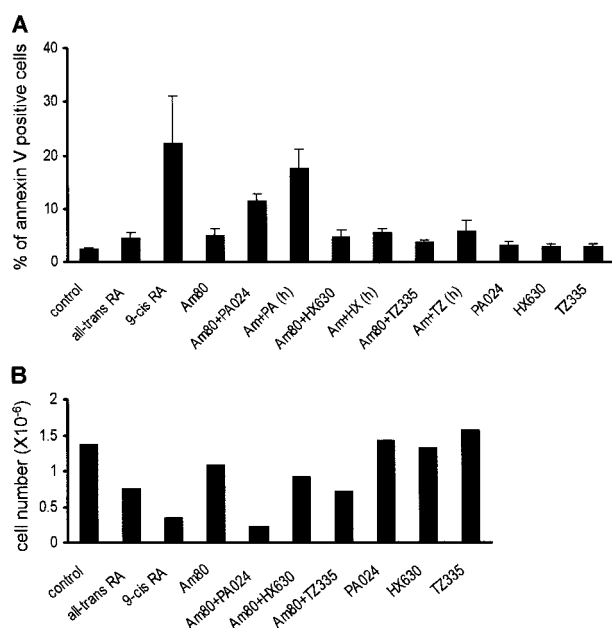


Fig. 4. Comparison of caspase and G<sub>2</sub>-M cyclin gene expression. Five caspase genes (caspase 1, 4, 7, 8, and 9) and three G<sub>2</sub>-M cyclin genes (cyclin A2, B1, and B2) that gave reproducible measurements were picked up, and their relative expression levels were plotted. Because of the duplications of the probes for caspase 1, cyclin A2, and cyclin B1, there are six dots in the top panel and five dots in the bottom panel. Mean  $\pm$  SD was calculated among the retinoid and/or retinoid synergist treatments classified according to the classification given in Fig. 3.

and these retinoid synergists were classified into two subclasses. This result suggests that they might show different effects on HL60 cell apoptosis, *i.e.*, only 9-*cis* retinoic acid-like classes should induce apoptosis effectively. The initial analysis was done by looking closely at the DNA microarray data we had already obtained, because most of the genes on the human genome U95A chip were eliminated during the selection procedure of predictor genes caused by low reproducibility, poor predictive strength, or chance (if there were >50 candidate genes). For this purpose, the mean relative expression level of five caspase genes (caspases 1, 4, 7, 8, and 9) and three G<sub>2</sub>-M cyclin genes (cyclins A2, B1, and B2) that are involved in apoptosis and cell growth was plotted (Fig. 4). There are other caspase genes and G<sub>2</sub>-M cyclin genes on the human genome U95A GeneChip, but the expressions of these genes differed >2-fold in the duplicate DNA chip measurements. Thus, these genes were eliminated from the plot. When all-*trans* retinoic acid, 9-*cis* retinoic acid, and Am80 were compared, caspase genes were induced more in the 9-*cis* retinoic acid-treated samples than in the other samples, and G<sub>2</sub>-M cyclin genes were more suppressed in the 9-*cis* retinoic acid-treated samples than in the

<sup>5</sup> M. Ebisawa, H. Fukasawa, E. Kawachi, and H. Kagechika, unpublished results.





**Fig. 5.** Difference of two predicted classes of retinoid synergists in the effect of HL60 cell differentiation. **A**, induction of apoptosis after 48-h exposure to retinoids and/or retinoid synergists. HL60 cells were cultured with retinoids and/or retinoid synergists for 48 h, and annexin V-positive cells were counted. Experiments were done in triplicate, and mean values are shown with SDs as error bars. The concentrations of retinoids and retinoid synergists were the same as in Fig. 2 except for Am + PA (h), Am + HX (h), and Am + TZ (h). The concentration of retinoid synergists for these three cases was 100 nM. **B**, inhibition of HL60 cell growth after 96-h exposure to retinoids and/or retinoid synergists. HL60 cells were cultured with retinoids and/or retinoid synergists for 96 h, and the cell numbers were counted. The concentration of retinoids and retinoid synergists was the same as in Fig. 2. A representative result is shown.

other samples. The effect of PA024 in combination with Am80 on the expression of caspase genes and G<sub>2</sub>-M cyclin genes was the same as that of 9-*cis* retinoic acid. The combination of HX630 or TZ335 with Am80 showed effects similar to those of all-*trans* retinoic acid or Am80 alone. These results indicated that Am80 + PA024 might be able to induce the apoptosis of HL60 cells like 9-*cis* retinoic acid, but Am80 + HX630 or Am80 + TZ335 might not.

To confirm the initial analysis, the induction of HL60 cell apoptosis was assayed by counting annexin V-positive cells after 48-h exposure to the retinoids (Fig. 5A). 9-*cis* retinoic acid induced apoptosis in 22% of the HL60 cells, contrasting with the <5% induction by all-*trans* retinoic acid. Retinoid synergists alone also induced apoptosis in <5% of the cells. As was expected from the gene expression results, Am80 + PA024 induced apoptosis in 12% of cells and the other synergists, HX630 and TX335, in combination with Am80, induced apoptosis in <5% of the cells. The percentage of apoptotic cells did not increase when 10-fold higher concentrations of HX630 and TX335 were used. In contrast, when the concentration of PA024 was increased 10-fold, a higher percentage of apoptosis was induced (17%). The results of the gene expression analysis of caspase genes and G<sub>2</sub>-M cyclin genes were also reflected in the growth of HL60 cells (Fig. 5B). 9-*cis* retinoic acid suppressed HL60 cell growth

more than all-*trans* retinoic acid did. The suppression of HL60 cell growth by PA024 with Am80 was the same as or even stronger than those by 9-*cis* retinoic acid and Am80. The suppression of HL60 cell growth by HX630 with Am80 or TZ335 with Am80 was weaker than that by all-*trans* retinoic acid. These results confirmed that the combination of PA024 with Am80 acted like 9-*cis* retinoic acid and that the combination of HX630 or TZ335 with Am80 acted like all-*trans* retinoic acid.

## Discussion

**Advantages of the Use of DNA Microarray for the Classification of Chemicals.** Recent progress in DNA microarray analysis has enabled us to analyze tens of thousands of gene expressions at once. Several applications of this technology have already been reported. Among them, there are increasing numbers of reports that demonstrate the usefulness of DNA microarray analysis on the phenotyping of tumors (12, 13). This type of analysis is based on the assumption that there should be a gene expression pattern specific to each type of tumor. The strength of this technique, together with bioinformatics analysis, makes it possible to obtain a specific gene expression pattern, the “gene expression profile,” that can predict the clinical status of the tumor equally or sometimes more powerfully compared with traditional histopathological diagnosis.

In this study, we applied the same approach to classify synthetic retinoids and retinoid synergists. These compounds were screened by an HL60 differentiation assay. As with the traditional histopathological diagnosis of tumor samples, it depends on the morphological and functional changes of HL60 cells by these chemicals. Considering the potential of retinoids for chemopreventive and therapeutic applications in dermatology and oncology as well as their side effects, a more precise assay is required for the development of retinoids that are more effective and/or have fewer side effects. We first compared the gene expression patterns obtained by the Affymetrix human genome U95A chip in all-*trans* retinoic acid-treated HL60, 9-*cis* retinoic acid-treated HL60, and control HL60 cells. Next, we selected 50 predictor genes by applying the Predict Parameter Value function in the GeneSpring expression analysis software (SiliconGenetics). These predictor genes were selected because their expression pattern was reproducibly and clearly different in all-*trans* retinoic acid-treated, 9-*cis* retinoic acid-treated, and control HL60 cells. Then, synthetic retinoids and retinoid synergists were classified into an all-*trans* retinoic acid-like, 9-*cis* retinoic acid-like, and control-like classes based on the similarity of the expression patterns of the predictor genes. The expression patterns of the predictor genes were not completely reproducible among the test samples because the predictor genes were selected based on the measurements of reference set, although 21 of 22 samples were still successfully classified. This depicted the robustness of the algorithm and the confirmation experiment with more retinoid and retinoid synergists is planning now. Synthetic retinoids and retinoid synergists were classified as expected when they were used alone. In contrast, the Predict Parameter Value function classified the retinoid synergists

into two subclasses, 9-*cis* retinoic acid-like and all-*trans* retinoic acid-like classes, when they were used together with Am80. All retinoid synergists were thought to represent a 9-*cis* retinoic acid like-class when they are used with Am80, because retinoid synergists activate RXRs, and Am80 activates RARs. But the DNA microarray and bioinformatics analyses gave an unexpected classification in our analyses. These results indicated that the DNA microarray analyses together with bioinformatics analyses are powerful enough to reveal the biological activities of compounds, which could not be predicted by conventional analyses based on cell morphology and function.

**Description of the 50 Predictor Genes and the Relationship to Retinoids.** The usefulness of the predictor genes was confirmed by the fact that the expression patterns of these genes were clearly distinguishable even by sight (Fig. 3). The descriptions of these genes are listed in Table 1. Although these genes were useful to predict the classification of retinoids, they were not necessarily shown to be modulated by RARs or RXRs. This was expected because the incubation period of all-*trans* retinoic acid or 9-*cis* retinoic acid was 72 or 96 h. Thus, the changes of the gene expressions were far downstream of the initial response of the HL60 cells to either retinoic acid. However, several genes were found to have relationships with the retinoidal action or to the development of acute promyelocytic leukemia.

*Insulin-like growth factor binding protein 7* in cluster B was reported to be an all-*trans* retinoic acid inducible gene (16). *TG-interacting factor* (cluster C), which belongs to homeobox proteins, might interfere with the transcription activity of RXR because both proteins can bind the RXR-responsive element from the *cellular retinol-binding protein II* promoter (17). *Epstein-Barr nuclear antigen 1-binding protein 2* (cluster C) was down-regulated when HL60 cells were differentiated by DMSO or TPA (18). Because DMSO and retinoic acids differentiate HL60 cells into the same direction, granulocytes, it is likely that this gene is also regulated during the HL60 differentiation induced by retinoids. Two genes in cluster A, nuclear body protein *Sp140* and *parachymosin*, are interesting because they are related to *PML* oncogene product. *PML* is a major component of an organelle called the nuclear body or POD (19). It was originally found as the t(15;17) chromosomal translocation partner of *RAR $\alpha$*  in acute promyelocytic leukemia, and this translocation was thought to be one of the causes of acute promyelocytic leukemia. Nuclear body protein *Sp140* is a component of POD (20), and *parachymosin* associates with POD (21). Therefore, these two genes might play roles during the development of acute promyelocytic leukemia or its remission by retinoid treatment. *PML* gene does not translocate to the *RAR $\alpha$*  gene in HL60 cells, although the cells were established from an acute promyelocytic leukemia patient (22). Expression of *Sp140* is up-regulated in HL60 cells treated with TPA or DMSO and also in NB4 cells, other human leukemia cells, treated with retinoic acid (20). *c-Fgr* in cluster A is a protein-tyrosine kinase. Katagiri *et al.* (23) reported that *c-fgr* was induced during the granulocytic differentiation of HL60 cells that was induced by retinoic acid. According to

this study, the role of *Fgr* protein-tyrosine kinase is to promote granulocytic differentiation of HL60 cells by preventing them from apoptosis. Considering that 9-*cis* retinoic acid induced more apoptotic cells than all-*trans* retinoic acid (Fig. 5A), *c-fgr* should be expected to be in cluster B, not in cluster A. However, in the preliminary result of time course experiment in which HL60 cells were treated with either all-*trans* retinoic acid or 9-*cis* retinoic acid, the kinetics of *c-fgr* expression showed clear differences between these two treatments (data not shown). The expression level of *c-fgr* increased continuously during the all-*trans* retinoic acid treatment up to 96 h, whereas it increased sharply then decreased during the 9-*cis* retinoic acid treatment. The peak of the expression was 48 h after the addition of 9-*cis* retinoic acid. Considering that *c-fgr* prevents the apoptosis of HL60 cells during their terminal differentiation process, the different kinetics of *c-fgr* expression may explain the different effects of all-*trans* retinoic acid and 9-*cis* retinoic acid on the apoptosis of HL60 cells, *i.e.*, the continuous increase of *c-fgr* expression in all-*trans* retinoic acid-treated HL60 cells prevents them from the apoptosis. This speculation implies that the kinetics of expressions of genes in Table 1 is important for the elucidation of the mechanistic difference between all-*trans* retinoic acid and 9-*cis* retinoic acid. Such a kind of study is now ongoing in our laboratory.

**Molecular Biological Aspects of the Two Classes of Retinoid Synergists.** The transient transfection assay indicated that synthetic retinoids activate RARs, and retinoid synergists activate RXRs (6).<sup>5</sup> Therefore, simultaneous usage of synthetic retinoids and retinoid synergists should result in the same effect as 9-*cis* retinoic acid. Three retinoid synergists, HX630, TZ335, and PA024, behaved similarly in the differentiation assay (Fig. 2). However, the classification based on the DNA microarray analysis (Figs. 3 and 4) and the results of HL60 cell growth and apoptosis (Fig. 5) revealed two subclasses among the retinoid synergists. Our results indicated that the mode of RXR activation by HX630 and TZ335 was enough for the potentiation of differentiation but not for the apoptosis. In contrast, PA024 was potent enough for both differentiation and apoptosis. The activation of the RXR moiety in the heterodimer comprises successive events of releasing corepressors and recruiting coactivators induced by structural changes of RAR-RXR by binding their cognate ligands (9). Our results suggested that two subclasses of retinoid synergists might differently modulate the activation procedure of the RAR-RXR heterodimer. The precise mechanism remains to be clarified, but RAR antagonists might give a clue for understanding the mechanism (24). In this study, there appeared to be two types of RAR antagonists that differentially modulated the coregulator interaction. The same kind of differential modulation might be one of the explanations for the mechanistic difference of the two subclasses of retinoid synergists.

**Clinical Aspects of the Two Classes of Retinoid Synergists.** All-*trans* retinoic acid has been used for the treatment of acute promyelocytic leukemia or skin diseases, including acne and psoriasis, *e.g.*, all-*trans* retinoic acid is



the first-choice drug in acute promyelocytic leukemia treatment and is known to induce a high rate of complete remission (2). However, the use of all-*trans* retinoic acid in therapy is limited because of undesirable side effects, including retinoid syndrome (25). And, in the case of acute promyelocytic leukemia treatment, there is another problem. Despite the high rate of complete remission, the duration of remission is generally short, and, once relapse occurs, all-*trans* retinoic acid alone fails to induce a second remission in most cases. Therefore, developing retinoids with more specific effects and fewer side effects is clinically important. One such compound is Am80, which is one of the most potent retinobenzoic acids (14). Am80 was ~10 times more potent than all-*trans* retinoic acid in an *in vitro* differentiation assay, and it selectively binds to RAR $\alpha$  and RAR $\beta$  (8). Am80 has already been used for several clinical treatments (26). When it was applied for the treatment of acute promyelocytic leukemia after the relapse from complete remission induced by all-*trans* retinoic acid, it induced a second complete remission in 58% of the patients (3). Moreover, the patients experienced fewer adverse effects compared with the all-*trans* retinoic acid treatment in their study. Their study depicted that Am80 was more potent and had fewer side effects than all-*trans* retinoic acid. However, 42% of the patients showed resistance to Am80 treatment, and the side effects did not disappear completely. There might be several ways to solve these problems: (a) the addition of retinoidal activities that Am80 does not have might help obtaining a higher complete remission rate; and (b) reducing the amount of Am80 might help to lighten the side effects.

In this study, we showed that there are two classes of retinoid synergists. The effects of these two classes of retinoid synergists were very interesting because one class was able to enhance the retinoidal activities of Am80, and the other class was able to increase the apoptotic effects. Using HX630 or TZ335 with Am80, we could reduce the amount of Am80 while maintaining almost the same differentiation activities. When the apoptotic effects are required, we could use PA024 with Am80. It is interesting to study in the future if we can actually modulate the retinoidal activities of Am80 in leukemia cells that are derived from acute promyelocyte leukemia patients, especially from relapsed ones, by selecting either class of retinoid synergists. Our finding will also provide important information for developing useful retinoid synergists.

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### References

- Sporn, M. B., Roberts, A. B., and Goodman, D. S. (eds.). *The Retinoids*, Ed. 2. New York: Raven Press, 1994.
- Zhang, J-W., Gu, J., Wang, Z-Y., Chen, S-J., and Chen, Z. Mechanisms of all-*trans* retinoic acid-induced differentiation of acute promyelocytic leukemia cells. *J. Biosci.*, 25: 275–284, 2000.
- Tobita, T., Takeshita, A., Kitamura, K., Ohnishi, K., Yanagi, M., Hiraoka, A., Karasuno, T., Takeuchi, M., Miyawaki, S., Ueda, R., Naoe, T., and Ohno, R. Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-*trans* retinoic acid. *Blood*, 90: 967–973, 1997.
- Collins, S. J. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood*, 70: 1233–1244, 1987.
- Breitman, T. R., Selonic, S. E., and Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. USA*, 77: 2936–2940, 1980.
- Ohta, K., Kawachi, E., Inoue, N., Fukasawa, H., Hashimoto, Y., Itai, A., and Kagechika, H. Retinoidal pyrimidinecarboxylic acids. Unexpected diaza-substituent effects in retinobenzoic acids. *Chem. Pharm. Bull. (Tokyo)*, 48: 1504–1513, 2000.
- Ebisawa, M., Kawachi, E., Fukasawa, H., Hashimoto, Y., Itai, A., Shudo, K., and Kagechika, K. Novel thiazolidinedione derivatives with retinoid synergistic activity. *Biol. Pharm. Bull.*, 21: 547–549, 1998.
- Umeyama, H., Fukasawa, H., Ebisawa, M., Eyrolles, L., Kawachi, E., Eisenmann, G., Gronemeyer, H., Hashimoto, Y., Shudo, K., and Kagechika, H. Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. *J. Med. Chem.*, 40: 4222–4234, 1997.
- Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G., and Glass, C. K. Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature (London)*, 395: 199–202, 1998.
- Altucci, L., and Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat. Rev. Cancer*, 1: 181–193, 2001.
- Nagy, L., Thomazy, V. A., Shipley, G. L., Fesus, L., Lamph, W., Heyman, R. A., Chandraratna, R. A. S., and Davis, P. J. A. Activation of retinoid X receptors induces apoptosis in HL-60 cell lines. *Mol. Cell. Biol.*, 15: 3540–3551, 1995.
- West, M., Blanchette, C., Dressman, H., Huang, E., Ishida, S., Spang, R., Zuzan, H., Olson, J. A., Jr., Marks, J. R., and Nevins, J. R. Predicting the clinical status of human breast cancer by using gene expression profiles. *Proc. Natl. Acad. Sci. USA*, 98: 11462–11467, 2001.
- Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, T., Yu, X., et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature (Lond.)*, 403: 503–511, 2000.
- Kagechika, H., Kawachi, E., Hashimoto, Y., and Shudo, K. Retinobenzoic acid. 2. Structure-activity relationships of chalcone-4-carboxylic acids and flavone-4'-carboxylic acids. *J. Med. Chem.*, 32: 834–840, 1989.
- Ebisawa, M., Ohta, K., Kawachi, E., Fukasawa, H., Hashimoto, Y., and Kagechika, H. Novel retinoidal tropolone derivatives. Bioisosteric relationship of tropolone ring with benzoic acid moiety in retinoid structure. *Chem. Pharm. Bull. (Tokyo)*, 49: 501–503, 2001.
- Swishelm, K., Ryan, K., Tuchiya, K., and Sager, R. Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. *Proc. Natl. Acad. Sci. USA*, 92: 4472–4476, 1995.
- Bertolino, E., Reimund, B., Wildt-Perinic, D., and Clerc, R. G. A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. *J. Biol. Chem.*, 270: 31178–31188, 1995.
- Reimers, K., Antoine, M., Zapatka, M., Blecken, V., Dickson, C., and Kiefer, P. NoBP, a nuclear fibroblast growth factor 3 binding protein, is cell cycle regulated and promotes cell growth. *Mol. Cell. Biol.*, 21: 4995–5007, 2001.
- Zhong, S., Salomoni, P., and Pandolfi, P. P. The transcriptional role of PML and the nuclear body. *Nat. Cell Biol.*, 2: E85–E90, 2000.
- Bloch, D. B., de la Monte, S. M., Guigaouri, P., Filippov, A., and Bloch, K. D. Identification and characterization of a leukocyte-specific component of the nuclear body. *J. Biol. Chem.*, 46: 29198–29204, 1996.
- Vareli, K., Frangou-Lazaridis, M., van der Kraan, I., and van Driel, R. Nuclear distribution of prothymosin alpha and parathymosin: evi-

dence that prothymosin alpha is associated with RNA synthesis processing and parathymosin with early DNA replication. *Exp. Cell Res.*, 257: 152–161, 2000.

22. Drexler, H. G., Quentmeier, H., MacLeod, R. A., Uphoff, C. C., and Hu, Z. B. Leukemia cell lines: in vitro models for the study of acute promyelocytic leukemia. *Leuk. Res.*, 19: 681–691, 1995.

23. Katagiri, K., Yokoyama, K. K., Yamamoto, T., Omura, S., Irie, S., and Katagiri, T. Lyn and Fgr protein-tyrosine kinase prevent apoptosis during retinoic acid-induced granulocytic differentiation of HL-60 cells. *J. Biol. Chem.*, 271: 11557–11562, 1996.

24. Germain, P., Iyer, J., Zechel, C., and Gronemeyer, H. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. *Nature (Lond.)*, 415: 187–192, 2002.

25. Takeuchi, M., Yano, T., Omoto, E., Takahashi, K., Kibata, M., Shudo, K., Harada, M., Ueda, R., and Ohno, R. Relapsed acute promyelocytic leukemia previously treated with all-*trans* retinoic acid: clinical experience with a new synthetic retinoid, Am-80. *Leuk. Lymphoma*, 31: 444–451, 1998.

26. Kagechika, K. Therapeutic applications of synthetic retinoids. *Drugs*, 3: 73–83, 2000.