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Genome-wide expression profiling of 8-chloroadenosine- and 8-chloro-cAMP-treated human neuroblastoma cells using radioactive human cDNA microarray

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Abbreviations: 8-Cl-adenosine, 8-chloro-adenosine; 8-Cl-cAMP, 8-chloro-cyclic adenosine 3,5-monophosphate; PKA, protein kinase A; RFXAP, regulatory factor X-associated protein; DCC, deleted in colorectal cancer; CDKs, cyclin-dependent kinases; MHC, major histocompatibility complex; TGF-β, transforming growth factor-β; DYRK2, dual specificity tyrosine phosphorylated and regulated kinase 2

Abstract

Previous reports raised question as to whether 8-chloro-cyclic adenosine 3,5-monophosphate (8-Cl-cAMP) is a prodrug for its metabolite, 8-Cl-adenosine which exerts growth inhibition in a broad spectrum of cancer cells. The present study was carried out to clarify overall cellular affects of 8-Cl-cAMP and 8-Cl-adenosine on SK-N-DZ human neuroblastoma cells by systematically characterizing gene expression using radioactive human cDNA microarray. Microarray was prepared with PCR-amplified cDNA of 2,304 known genes spotted on nylon membranes, employing ^{33}P-labeled cDNAs of SK-N-DZ cells as a probe. The expression levels of approximately 100 cDNAs, representing about 8% of the total DNA elements on the array, were altered in 8-Cl-adenosine- or 8-Cl-cAMP-treated cells, respectively. The genome-wide expression of the two samples exhibited partial overlaps; different sets of up-regulated genes but the same set of down-regulated genes. 8-Cl-adenosine treatment up-regulated genes involved in differentiation and development (LIM protein, connexin 26, neogenin, neurofilament triplet L protein and p21$^{WAF1/CIP1}$) and immune response such as natural killer cells protein 4, and down-regulated ones involved in proliferation and transformation (transforming growth factor-β, DYRK2, urokinase-type plasminogen activator and proteins involved in transcription and translation) which were in close parallel with those by 8-Cl-cAMP. Our results indicated that the two drugs shared common genomic pathways for the down-regulation of certain genes, but used distinct pathways for the up-regulation of different gene clusters. Based on the findings, we suggest that the anti-cancer activity of 8-Cl-cAMP results at least in part through 8-Cl-adenosine. Thus, the systematic use of DNA arrays can provide insight into the dynamic cellular pathways involved in anticancer activities of chemotherapeutics.

Keywords: 8-Cl-adenosine, 8-Cl-cAMP, anticancer activity, radioactive cDNA microarray, genome-wide expression

Introduction

8-Cl-adenosine, dephosphorylated metabolite of antineoplastic agent 8-Cl-cAMP has a growth-inhibitory effect, which is produced from 8-Cl-cAMP by the action of serum phosphodiesterase and 5-nucleotidase activity (Figure. 1) (Taylor *et al*., 1990; Lange-Carter *et al*., 1993; Halgren *et al*., 1998). 8-Cl-cAMP, a site-selective cAMP analogue, exhibits growth inhibition in a broad spectrum of cancer cell lines. However, its mechanism of action remains unelucidated. One possible mode of action is to induce cytotoxicity by acting as competitive inhibitor in biochemical pathway involving cAMP-dependent protein kinase [protein kinase A (PKA)] (Cho-Chung, 1989; Cho-Chung *et al*., 1995; Noguchi *et al*., 1998). PKA is a serine/threonine protein kinase that is activated by cAMP. Two isozymes of PKA, PKA-I and PKA-II, are distinguished by the association of type R subunit (R) or type R subunit (R) with common C subunits, respectively. Four different R subunits-RIa, RIb, RIIa, and RIIb-have been identified (Taylor *et al*., 1990). Expression of the PKA RIa subunit is correlated with active cell growth and transformation, and increased in various human tumors and cell lines. Furthermore, overexpression of the PKA RIa subunit is correlated with malignancy and poor prognosis in cancer patients (Miller *et al*., 1993a; Miller *et al*., 1993b; Simpson *et al*., 1996; Miller *et al*., 1997). In contrast, a high level of Rb is correlated with growth arrest and differentiation (Cho-

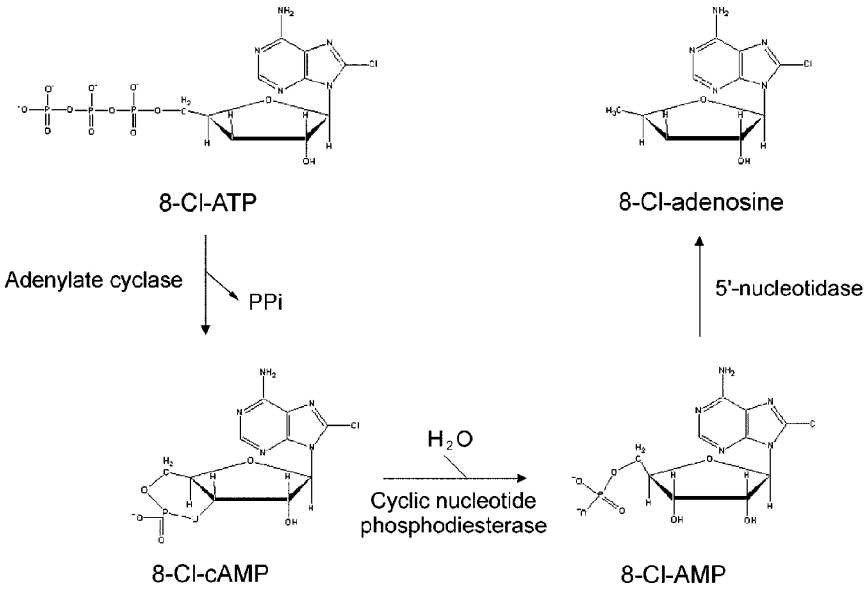

Figure 1. Metabolic conversion of 8-Cl-cAMP to 8-Cl-adenosine

Chung, 1990). Cancer cells treated with 8-Cl-cAMP showed a decrease of Ra and an increase of Rb, leading to inhibition of cancer cell growth (Ally *et al*., 1988; Ciardiello *et al*., 1990; Rohlff *et al*., 1993).

The mechanism of 8-Cl-cAMP action is not yet clarified as to whether it is a prodrug for its metabolite, 8-Cl-adenosine. On the one side, 8-Cl-cAMP exerts its growth-inhibitory effect by binding to PKA in a competitive manner against cAMP (Rohlff *et al*., 1993; Noguchi *et al*., 1998) and on the other side, the effect of 8-Cl-cAMP is at least in part due to its metabolite 8-Cl-adenosine. Furthermore, the anticancer effect of 8-Cl-cAMP has been suggested to be independent of residual activity of PKA or concentration of cAMP (Lange-Carter *et al*., 1993; Cummings *et al*., 1996; Langefeld *et al*., 1997; Halgren *et al*., 1998; Carlson *et al*., 2001; Yin *et al*., 2001).

In order to evaluate the molecular portrait of anti-cancer actions of 8-Cl-adenosine versus 8-Cl-cAMP, we have selected human cDNA microarray comprised of 2,304 nonredundant human clones to examine whole spectrum of genes affected by both drugs. The microarray-based genomic survey is one of the high-throughput well known approaches, and has allowed parallel studies on expression patterns of thousands of genes (Schena *et al*., 1995; Duggan *et al*., 1999; Konu *et al*., 2001; Vawter *et al*., 2001). In the present study, we investigated genome-wide expression profiling under the treatment of 8-Cl-adenosine or 8-Cl-cAMP as compared to untreated control. As a cancer cell model, SK-N-DZ human neuroblastoma cell line was chosen, because it has higher concentration of PKA than other types of cancer cells (Cho *et al*., 2000).

Materials and Methods

Cell culture and RNA preparation

SK-N-DZ cells (ATCC #CRL-2149) grown in RPMI 1640 medium with 10% fetal bovine serum, 50 units/ml penicillin, 500 μg/ml streptomycin, and 1 mM glutamine (GIBCO) were seeded at $5x10^5$ cells per dish at humidified atmosphere of 5% CO_2 at 37°C. Cells were counted on a Coulter Counter, and were treated with 5 μM 8-Cl-adenosine (Biology Life Science Institute, Bremen, Germany) or 8-Cl-cAMP (National Cancer Institute, Drug Synthesis and Chemistry Branch, Bethesda, MD. USA) for 24 h, respectively. Total RNA was prepared by using RNeasy Midi Kit (Qiagen, Chatsworth, CA, USA).

Human cDNA microarray

A human cDNA microarray was primarily derived from a commercially available master set of approximately 15,000 human verified-sequences (Research Genetics, Inc.). The 15,000 human cDNA clone set was sorted for a list of genes (2,304 elements) representing families such as differentiation, development, proliferation, transformation, cell cycle progression, immune response, transcription and translation factors, oncogenes, and molecules involved in cell growth and maintenance. PCR-amplified cDNAs were spotted on nylon membranes. The general methodology of arraying is based on the procedures of DeRisi *et al*. (1996).

cDNA Radiolabeling

Total RNAs prepared from SK-N-DZ cells treated with 8-Cl-adenosine or 8-Cl-cAMP and untreated control cells

were used to synthesize ^{33}P-labeled cDNAs by reverse transcription. Briefly, 3~10 μg RNA were labeled in a reverse transcription reaction containing 5X first strand PCR buffer, 1 μg of 24-mer poly dT primer, 4 μl of 20 mM each dNTP excluding dCTP, 4 μl of 0.1 M DTT, 40 U of RNase inhibitor, 6 μl of 3000 Ci/mmol α-^{33}P dCTP to a final volume of 40 μl. The mixture was heated at 65°C for 5 min, followed by incubation at 42°C for 3 min. Two μl (specific activity: 200,000 U/ml) of Superscript reverse transcriptase (Life Technologies) was then added and the samples were incubated for 30 min at 42°C, followed by the addition of 2 μl of Superscript reverse transcriptase and another 30 min of incubation. Five μl of 0.5 M EDTA was added to chelate divalent cations. After the addition of 10 μl of 0.1 M NaOH, the samples were incubated at 65°C for 30 min to hydrolyze remaining RNA. Following the addition of 25 μl of 1 M Tris (pH 8.0), the samples were purified using Bio-Rad 6 purification columns (Hercules, CA, USA). This resulted in 5x10^6 to 3x10^7 cpm per reaction (Vawter *et al*., 2001).

Hybridization and Scanning

cDNA microarrays were pre-hybridized in hybridization buffer containing 4.0 ml Microhyb (Research Genetics), 10 μl of 10 μg/ml human Cot 1 DNA (Life Technologies), and 10 μl of 8 mg/ml poly dA (Pharmacia, NJ). Both Cot 1 and poly dA were denatured at 95°C for 5 min prior to use. After 4 h of pre-hybridization at 42°C, approximately 10^7 cpm/ml of heat-denatured (95°C, 5 min) probes were added and incubation was continued for 17 h at 42°C. Hybridized arrays were washed three times in 2X SSC and 0.1% SDS for 15 min at room temperature. The microarrays were exposed to phosphorimager screens for 1-5 days, and the screens were then scanned in a FLA-8000 (Fuji Photo Film Co) at 50 μm resolution (Vawter *et al*., 2001).

Data analysis

Microarray images were trimmed and rotated for further analysis using L-Processor (Fuji Photo Film Co). Gene expression of each microarrays was captured by the intensity of each spot produced by radioactive isotopes. Pixels per spot were counted by Arrayguage (Fuji Photo Film Co) and were exported to Microsoft Excel (Microsoft, Seattle, WA, USA). The data were normalized with Z transformation to obtain Z scores by subtracting each average of gene intensity and dividing with each standard deviation. Z scores provide each of 2,304 genes with the distance from the average intensity and were expressed in units of standard deviation. Thus, each Z score provides flexibility to compare different sets of microarray experiments by adjusting differences in hybridization intensities.

Gene expression difference as compared to untreated control cells was calculated by comparing the Z score differences (Z differences) among the same genes. This facilitates to compare each gene that had been up- or downregulated as compared to the control cells. Z differences were calculated first by subtracting Z scores of the control from each Z score of the samples. These differences were normalized again to distribute their position by subtracting the average Z difference and dividing with the standard deviation of the Z differences. These distributions represent the Z ratio value, and provide the efficiency for comparing each microarray experiment (Vawter *et al*., 2001).

Spotfire produced scatter plots of intensity values. (Spotfire, Inc., Cambridge, MA) (Tanaka *et al*., 2000). Cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisens laboratory (http://rana.lbl.gov). Clustering of changes in gene expression was determined by using public domain Cluster based on pair wise complete-linkage cluster analysis (Eisen *et al*., 1998).

Western analysis

SK-N-DZ cells were seeded at a density of 1×10^6 cells/100-mm plate and treated with 8-Cl-adenosine or 8-Cl-cAMP (5 μM each) for 12 or 24 h. Cells were washed twice with ice-cold PBS, lysed in buffer (20 mM Tris/HCl, 100 μM NaCl, 5 mM $MgCl_2$, 1% NP-40, 0.5% sodium deoxycholate, 100 μM pepstatin, 100 μM antipain, 100 μM chymostatin, 10 μg/ml leupeptin, 0.5 μM phenylmethylsulfonyl fluoride, 5 mg/ml trypsin inhibitor, and 1 mM benzamidine, pH 7.5), and placed on ice for 15 min. Protein concentration was determined by the Bradford assay using the Bio-Rad protein assay kit (Bio-Rad). Cell extracts were subjected to SDS-PAGE, and Western analysis was performed according to the described method (Srivastava *et al*., 1999). Primary antibodies for P21$^{WAF1/CIP1}$ (Cat. No. 33-7000) and human growth hormone-dependent insulin-like growth factor-binding protein (IGFBP), and FITC-labeled secondary antibody (Cat. No. 62-6511) were products of Zymed lab. (San Francisco, CA, USA).

Results and Discussion

Microarray reproducibility

To assess the reliability of microarray technique used, we calculated microarray reproducibility between duplicates. The duplicate genetic elements for each microarray resulted in two separate Z normalizations and an average Z score for each gene. The scatter plot for Z scores in untreated control and 8-Cl-adenosine- or 8-Cl-cAMP-treated cells showed R^2 across duplicates of 0.81, 0.83, and 0.95, respectively (Figure. 2). A perfect

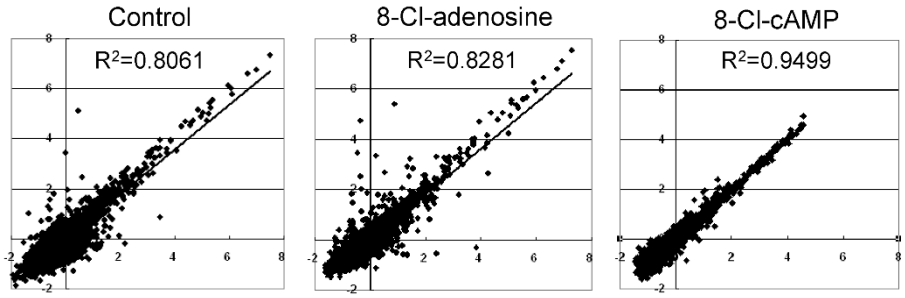

Figure 2. Microarray reproducibility. Each microarray contains two identical grids. Regression analysis of Z scores from two independent samples of untreated control and 8-Cl-adenosine- or 8-Cl-cAMP-treated SK-N-DZ cells were performed. Z scores of individual genes from each member of duplicates were plotted, and the relationship between two samples was calculated to obtain R^2. Based on R^2, microarray hybridization patterns were found to be highly consistent between the samples. A perfect relationship between samples would be a slope of 1.

relationship between duplicates would be a slope of 1. The average coefficient of variation for duplicate Z scores within each microarray was below 20% approximately, indicating a high reliability of our microarray data.

Expression profiles between 8-Cl-adenosine- and 8-Cl-cAMP-treated SK-N-DZ cells

The mechanism of 8-Cl-cAMP action has been controversial among some academics that begun more than a decade ago (Ally *et al*., 1988; Ciardiello *et al*., 1990; Rohlff *et al*., 1993). One of the possible mechanisms has been suggested to be through direct modulation of PKA activity and the other via its metabolite, 8-Cl-adenosine. In order to identify the genomic mechanism

Table 1. Expression profile of genes altered in SK-N-DZ cells treated with 8-Cl-adenosine or 8-Cl-cAMP

Genes	Fold change	
	8-Cl-adenosine	8-Cl-cAMP
Homo sapiens (clone s153) mRNA fragment	7.82	-0.44
H.sapiens mRNA for M130 antigen	6.85	-0.39
Human LIM protein MLP mRNA, complete cds	5.54	0.32
Gap junction protein, beta 2, 26kD (connexin 26)	5.43	-0.22
Hexokinase 1	5.06	-1.26
Prostate specific antigen	4.54	0.98
Neurofilament triplet L protein	4.17	5.53
H.sapiens RFXAP mRNA	3.96	0.17
Histidine ammonia-lyase	3.74	1.24
Hepatoma transmembrane kinase	3.54	0.45
Cyclin-dependent kinase inhibitor 1A (p21Waf1/Cip1)	3.40	0.33
Homo sapiens clone 22 mRNA, alternative splice variant alpha	3.28	-0.64
Human mRNA for golgi antigen gcp372, complete cds	3.03	0.27
Neogenin (chicken) homolog 1	3.02	2.32
Heterogeneous nuclear ribonucleoproteins C1/C2	2.91	-0.70
H.sapiens insulin-like growth factor binding protein 2	0.16	3.55
Vacuolar H+ ATPase proton channel subunit	-2.66	-1.91
Urokinase-type plasminogen activator	-2.66	-1.29
General transcription factor IIF, polypeptide 1 (74kD subunit)	-2.74	-2.03
ERGIC-53 protein precursor	-2.75	-1.46
Neutrophil gelatinase-associated lipocalin precursor	-2.76	-2.69
Probable G protein-coupled receptor LCR1 homolog	-2.82	-2.29
Human clone 23839 mRNA sequence	-2.90	-1.33
Laminin B1 chain	-2.96	-2.30
Transcription factor 12 (HTF4, helix-loop-helix transcription factors 4)	-3.03	-2.47
Cartilage linking protein 1	-3.08	-3.06
Dihydropyrimidine dehydrogenase	-3.27	-3.04
DYRK2	-4.43	-3.55
Carbonic anhydrase lib	-5.68	-4.90
Human mRNA for stac, complete cds	-6.20	-4.60
Human mRNA for TGF-beta superfamily protein, complete cds	-7.32	-6.29

specific for anticancer activity of 8-Cl-adenosine or 8-Cl-cAMP by using DNA microarray technology, three independent experiments were performed and a representative one was selected. The expression levels of ≈100 cDNA, representing ≈8% of the total DNA elements on the array, were found to be altered (≥2-fold up-regulated or down-regulated as compared to untreated control) in 8-Cl-adenosine- and 8-Cl-cAMP-treated cells, respectively. The changes ranged from -7.3 fold to +7.8 fold in 8-Cl-adenosine-treated cells, and -6.29 fold to +8.1 fold in 8-Cl-cAMP-treated cells. There were 42 up-regulated and 59 down-regulated genes in 8-Cl-adenosine-treated cells and 65 up-regulated and 41 down-regulated genes in 8-Cl-cAMP-treated cells.

Genes showing highly altered expression levels in treated cells were aligned in the order of the magnitude of altered expression in 8-Cl-adenosine-treated cells (Table 1). The expression profile of 8-Cl-adenosine- or 8-Cl-cAMP-treated cells exhibited a partial overlap with each other; the same set of down-regulated genes and different sets of up-regulated genes (except a few genes) between the two samples on the basis of Z ratio. A scatter plot revealed a single type of down-regulation but a bifurcated type of up-regulation, confirming the result (Figure. 3). Representative genes differentially or coincidentally regulated are presented in Table 2. These results indicated that two drugs shared some common genomic pathways leading to the down-regulation of certain genes, but used distinct pathways for most up-regulated gene clusters involved in different categories

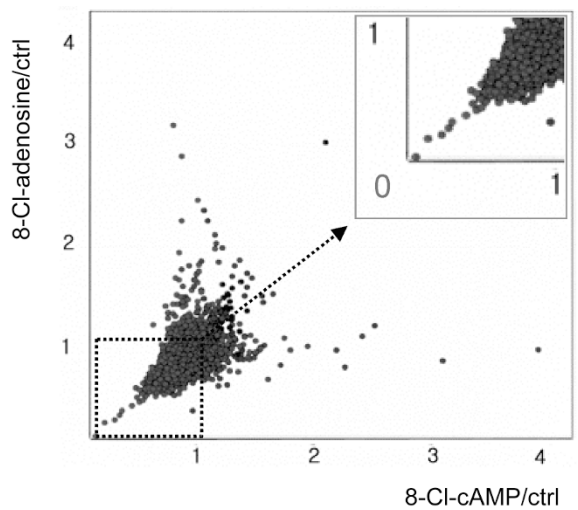

Figure 3. Scatter plot for comparison of expression profile between 8-Cl-adenosine- or 8-Cl-cAMP-treated SK-N-DZ cells. Expression profiles of 8-Cl-adenosine-treated versus 8-Cl-cAMP-treated cells are shown as bivariate scatter plot of 2,304 genes from the microarray. The values are corrected intensities relative to untreated control cells, representing levels of expression for the DNA elements of the microarrays. Scatter plot shows a bifurcated type of up-regulation indicating different sets of up-regulated genes and a single type of down-regulation indicating a same set of down-regulated genes between two samples. The inset is a magnified drawing.

of cellular processes. Based on the findings, it could be hypothesized that the anti-cancer activities of cAMP were due to direct modulation of PKA independent of 8-Cl-adenosine, or via its metabolite, 8-Cl-adenosine, which explains the striking overlaps between down-regulated genes by the two drugs, or a concerted action of the both pathways.

Differential gene up-regulation by 8-Cl-adenosine and 8-Cl-cAMP detected by microarray was verified by Western analysis

To confirm the differential up-regulation of genes by 8-Cl-adenosine and 8-Cl-cAMP identified by microarray, Western blotting of two differentially regulated genes was performed to examine the direction of changes as well as the magnitude of altered expression (Figure. 4). P21WAF1/CIP1 plays a pivotal role in cell cycle arrest. The expression of the gene in SK-N-DZ cells was preferentially increased by 8-Cl-adenosine treatment in agreement with the previous report (Carlson *et al*., 2001), but not by 8-Cl-cAMP. Insulin like growth factor binding protein (IGFBP) expression increased remarkably by 12 h of treatment with 8-Cl-cAMP, which decreased up to ≈one fourth the level at 24 h. By contrast, 8-Cl-adenosine did not change IGFBP expression. Both results closely matched the microarray data (Table 1).

Molecular profiling of anticancer effects of 8-Cl-adenosine and 8-Cl-cAMP on SK-N-DZ cells

The anticancer activities of 8-Cl-adenosine and 8-Cl-cAMP have been well known in a variety of cancer cells, and phase I clinical studies have been performed against solid tumors with some evidence of clinical response (Halgren *et al*., 1998). Nevertheless, their precise molecular mechanisms of actions are not yet fully understood. To obtain a molecular portrait of these anticancer activities, we used a hierarchical clustering algorithm to group genes on the basis of similarity in their expression patterns (Eisen *et al*., 1998), and the data are presented in a matrix format (Figure. 5). Each row represents all of the hybridization results for a single DNA element of the array, and each column represents the expression levels for all genes in a single hybridization sample. The expression level of each gene is visualized in color, relative to its median expression level across all samples. Red represents an expression greater than the mean and green represents an expression less than the mean, and the intensity of the color denotes the degree of deviation from the mean. Black represents median expression level. Distinct samples representing similar gene patterns from control cells are aligned in adjacent rows. Included in this map are samples from untreated control and 8-Cl-adenosine- or 8-Cl-cAMP-treated SK-N-DZ cells. Coordinately expressed genes were grouped into clusters, which we named on

Table 2. Gene regulation profile in 8-Cl-adenosine- or 8-Cl-cAMP-treated SK-N-DZ cells

Regulation profile and Z ratio		Gene product	Function
8-Cl-adenosine/control	8-Cl-cAMP/control		
Up-regulated	Up-regulated		
4.17	5.53	Neurofilament triplet L protein	Differentiation of nervous system, DCC-related protein
3.02	2.32	Neogenin (chicken) homolog 1	NK cells and T cells activation
2.29	2.80	Natural killer cells protein 4 precursor	Cell cycle control, antigen presentation
2.10	3.40	Human mRNA for proteasome subunit z	
Up-regulated	Down-regulated		
7.82	-0.44	Homo sapiens (clone s153) mRNA fragment	Regulation of the colloidal osmotic pressure of blood
6.85	-0.39	H.sapiens mRNA for M130 antigen	Macrophage scavenger receptor
5.43	-0.22	Gap junction protein, beta 2 (connexin 26)	Human mammary differentiation, murine development
5.06	-1.26	Hexokinase 1	Glycolysis
Down-regulated	Up-regulated		
-0.79	6.14	Vascular endothelial growth factor related protein (VRP)	Vascular development
-0.43	5.59	Alpha-2-HS-glycoprotein alpha and beta chain	Brain development, endocytosis
-0.05	4.37	E2F transcription factor 4, p107/p130 binding	Transcription factor
-0.46	4.33	Cyclin D2	Promotion of cell cycle progression
Down-regulated	Down-regulated		
-7.32	-6.29	Human mRNA for TGF-beta superfamily protein	Growth factor
-6.20	-4.60	Human mRNA for stac, complete cds	Neuron-specific signal transduction
-5.68	-4.90	Carbonic anhydrase II	Reversible hydratation of carbon dioxid
-4.43	-3.55	H.sapiens mRNA for protein kinase, DYRK2	Regulation of cellular growth and/or development

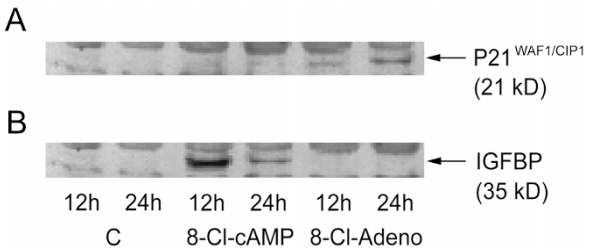

Figure 4. Expression of p21$^{WAF1/CIP1}$ and IGFBP in 8-Cl-adenosine- or 8-Cl-cAMP-treated SK-N-DZ cells identified by Western blot vanalysis. Cells were treated with 8-Cl-adenosine or 8-Cl-cAMP for 12 or 24 h. Untreated and treated cell lysates were subjected to Western blotting as described in Materials and Methods. C, untreated control; 8-Cl-cAMP, 8-Cl-cAMP-treated cells; 8-Cl-Adeno, 8-Cl-adenosine-treated cells.

the basis of the cellular process in which the component genes participate.

The clustergram revealed that 8-Cl-adenosine treatment up-regulated clusters of genes involved in differentiation and development, and down-regulated ones involved in proliferation and transformation, as compared to untreated control cells (Table 1, Figure. 5). The differentiation cluster was characterized by the prevalence of genes encoding developmental proteins (human LIM protein MLP, connexin 26 and neogenin), genes encoding cytoskeleton (neurofilament triplet L protein) and cell cycle proteins (p21$^{WAF1/CIP1}$ and proteasome subunit z). Some immune molecules were also up-regulated, including H.sapiens RFXAP, natural killer cells protein 4 and M130. LIM protein may be involved in specification and maintenance of differentiated phenotypical properties of some neurons (Thor *et al*., 1991) and an essential regulator of myogenic differentiation (Arber *et al*., 1994). Connexin 26 is expressed in normal human mammary epithelial cells but not in mammary tumor cell lines (Lee *et al*., 1992), and has an essential role during an early stage of murine development (Gabriel *et al*., 1998). Neogenin is a tumor suppressor gene DCC-related protein, and believed to be involved in early differentiation of nervous system (Vielmetter *et al*., 1997). Neurofilament triplet L protein is the component of neuronal cytoskeleton found in neurons of both the central and peripheral nervous systems (Carpenter and Wallace, 1996), and the up-regulation of its expression can be regarded as evidence of differentiation or reverse transformation of neuronal cells. P21$^{WAF1/CIP1}$ is a cell cycle inhibitor acting by inhibiting cyclin/CDKs (Carlson *et al*., 2001; Draus *et al*., 2001; Lee *et al*.,

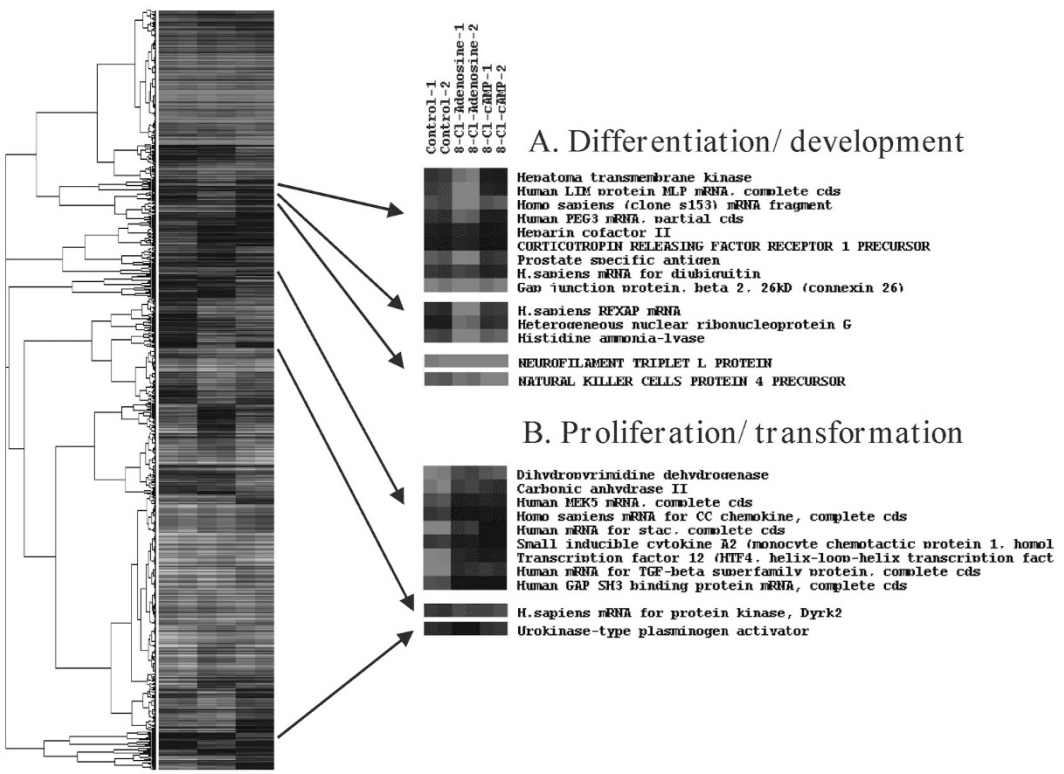

Figure 5. Molecular portrait of anticancer activities of 8-Cl-adenosine or 8-Cl-cAMP on SK-N-DZ cells. Cells were treated with 8-Cl-adenosine or 8-Cl-cAMP for 24 h. Microarray data from untreated control and 8-Cl-adenosine or 8-Cl-cAMP-treated cells were combined and clustered. Cluster analysis was performed on Z-transformed microarray data by using two separate programs available as shareware from Michael Eisens lab. Each gene is represented by a single row of colored boxes; each experimental sample is represented by a single column. The entire clustered image is shown on the left. Full gene names are shown for coordinately expressed clusters containing genes involved in differentiation and development (A), and proliferation and transformation (B). These clusters also contain uncharacterized genes and genes not involved in these processes.

2002). Proteasomes degrade protein-ubiquitin conjugates in an ATP-dependent reaction, and are involved in cell cycle control and in early steps of the immune response (Petit *et al*., 1997). RFXAP is critical to the expression of MHC class II genes and plays an ancillary role in the transcriptional control of MHC class I genes (Nagarajan *et al*., 2000). Natural killer cells protein 4 encodes a product common to the activation pathways of both natural killer (NK) cells and T cells (Dahl *et al*., 1992). M130 is a macrophage scavenger receptor, and not expressed in any cancer cells except mesothelioma where asbestos bound to the scavenger receptors (Frank *et al*., 1998). The coordinate up-regulation of the above genes most likely promotes differentiation and reverse transformation.

In the proliferation and transformation clusters, genes encoding proteins involved in proliferation or transformation-related signal transduction (transforming growth factor-b superfamily protein, stac, DYRK2 and LCR1 homolog), malignancy (urokinase-type plasminogen activator, neutrophil gelatinase-associated lipocalin), transcription (transcription factor 12, general transcription factor IIF/polypeptide 1), translation (DYRK2) and nucleic acid metabolism (dihydropyridine dehydrogenase) were preferentially down-regulated by both 8-Cl-adenosine and 8-Cl-cAMP with a similar magnitude of altered expression (Table 1, Figure. 5). Interestingly, the most profoundly down-regulated gene in SK-N-DZ cells by the two drugs was transforming growth factor-β (TGF-β). TGF-β has a multifunctional role in tumorigenesis, acting as a tumor suppressor as well as a tumor promoter by stimulating immunosuppression, angiogenesis and synthesis of extracellular matrix (Heldin *et al*., 1997). Further study is in need on the involvement of TGF-β down-regulation in the up-regulation of some immune molecules [natural killer cells protein 4 precursor (2.29 fold by 8-Cl-adenosine, 2.80 fold by 8-Cl-cAMP), RFXAP (3.96, 0.17)] and down-regulation of some extracellular matrix proteins [cartilage linking protein 1 (-3.08, -3.06), laminin B1 (-2.96, -2.30), collagen type V alpha 1 (-2.55, -2.52), collagen type XV alpha 1 (-2.50, -1.39)] observed in the present experiment. Among the highly down-regulated molecules, stac is likely involved in neuron-specific signal transduction (Suzuki *et al*., 1996). DYRK2 is a member of the dual specificity tyrosine phosphorylated and regulated kinase group. A concerted action of DYRK2 and glycogen synthase kinase-3 regulates translation initiation factor eIF2B, which is involved in switching on overall protein synthesis (Campbell and Proud, 2002). LCR1 is a G-protein-coupled receptor, which likely influences neuronal function at the time after maturation of brain (Wong *et al*., 1996). Urokinase-type plasminogen activator forms part of an important enzymatic system that degraded the extracellular matrix in process of invasion and metastasis, thereby having a strong clinical value in predicting prognosis and survival of cancer patients (Schmitt *et al*., 2000; Nieto-Rodriquez *et al*., 2001). Neutrophil gelatinase-associated lipocalin appears to be induced in epithelial cells under special conditions such as malignancy and inflammation (Nielsen *et al*., 1996; Cowland and Borregaard, 1997). Dihydropyrimidine dehydrogenase (EC 1.3.1.2) is involved in uracil and thymine degradation, and the inhibition of the enzyme can greatly potentiate the therapeutic effectiveness of 5-fluorouracil (Rosenbaum *et al*., 1997). A concomitant down-regulation of the above genes seemed to be of particular relevance to the anticancer activities of the two drugs. The suppressive effect of 8-Cl-adenosine on these gene clusters remarkably resembled that of 8-Cl-cAMP, indicating their use of distal common genomic pathways for the down-regulation of these genes. Furthermore, the altered expression profile generated by 8-Cl-adenosine or 8-Cl-cAMP treatment would reflect true intrinsic differences between the untreated and treated cells rather than variations arising from experimental artifacts.

In summary, we performed radioactive human cDNA microarray to elucidate genomic mechanisms of anticancer activities of 8-Cl-adenosine versus 8-Cl-cAMP, which has been controversial for a long time. In the present study, the down-regulated genes by 8-Cl-adenosine treatment were closely mirrored in those by 8-Cl-cAMP. In contrast, the distinct clusters of genes were up-regulated by the two drugs. Therefore, our data suggest that 8-Cl-adenosine and 8-Cl-cAMP use differential gene regulation mechanisms for their anticancer activities, however, sharing some common distal events with each other. Furthermore, we also suggest that the anti-cancer activities of 8-Cl-cAMP are derived from both direct modulation of PKA independent of its metabolite, 8-Cl-adenosine, and also via 8-Cl-adenosine which explains the striking overlaps among the down-regulated genes by the two drugs. Additionally, 8-Cl-adenosine specifically up-regulated expression of the differentiation and development clusters, and preferentially down-regulated the proliferation and transformation clusters, thereby providing a new level of understanding of the genome-wide mechanism underlying its anticancer activity.

Finally, genome-wide expression profiling using human cDNA microarray has been proven to be an efficient technology to evaluate the genomic mechanism of closely related cancer therapeutics, such as 8-Cl-adenosine and 8-Cl-cAMP, and facilitate the development of new chemotherapeutic combinations based on the dynamic molecular actions of drugs.

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