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Expression profiling of sodium butyrate (NaB)-treated cells: identification of regulation of genes related to cytokine signaling and cancer metastasis by NaB

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Histone deacetylase (HDAC) inhibitors induce growth arrest and apoptosis in a variety of human cancer cells. Sodium butyrate (NaB), a short chain fatty acid, is a HDAC inhibitor and is produced in the colonic lumen as a consequence of microbial degradation of dietary fibers. In order to dissect out the mechanism of NaB-induced growth inhibition of cancer cells, we carried out expression profiling of a human lung carcinoma cell line (H460) treated with NaB using a cDNA microarray. Of the total 1728 genes analysed, there were 32 genes with a mean expression value of 2.0-fold and higher and 66 genes with a mean expression value 3.0-fold and lower in NaB-treated cells. For a few selected genes, we demonstrate that their expression pattern by semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis is matching with the results obtained by microarray analysis. Closer view at the expression profile of NaB-treated cells revealed the downregulation of a total of 16 genes associated with cytokine signaling, in particular, interferon γ (IFNγ) pathway. In good correlation, NaB-pretreated cells failed to induce interferon regulatory factor 1, an INFγ target gene, efficiently upon IFNγ addition. These results suggest that NaB inhibits proinflammatory cytokine signaling pathway, thus providing proof of mechanism for its anti-inflammatory activity. We also found that NaB induced three genes, which are known metastatic suppressors, and downregulated 11 genes, which have been shown to promote metastasis. Upregulation of metastatic suppressor Kangai 1 (KAI1) by NaB in a time-dependent manner was confirmed by RT–PCR analysis. The differential regulation of metastasis-associated genes by NaB provides explanation for the anti-invasive properties of NaB. Therefore, our study presents new evidence for pathways regulated by NaB, thus providing evidence for the mechanism behind anti-inflammatory and antimetastatic activities of NaB.
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Introduction

The acetylation and deacetylation of histones of the core proteins of the nucleosomes in chromatin play an important role in the regulation of gene expression. (Gray and Ekstrom, 2001; Khochbin *et al*., 2001; Urnov, 2003). The acetylation status of the histones is mainly controlled by two classes of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDAC). A number of HDAC inhibitors (HDIs) have been identified that induce growth arrest, differentiation and apoptosis in cancer cells (Gore and Carducci, 2000; Marks *et al*., 2000; Weidle and Grossmann, 2000). HDIs belong to a heterogeneous class of compounds that includes derivatives of short chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides.

HDI sodium butyrate (NaB), a short chain fatty acid, occurs naturally in the body from the acetyl-CoA-dependent catabolic oxidation of long chain saturated fatty acids (Lehninger *et al*., 1993; Widmer *et al*., 1996). NaB is produced in the colon of mammals as a result of anaerobic bacterial fermentation of dietary fiber, undigested starch and proteins (Cummings, 1981; Bugaut and Bentejac, 1993; McIntyre *et al*., 1993; Mcintosh *et al*., 1996; Whiteley *et al*., 1996). NaB serves as an energy source of colonic epithelium. In addition, NaB also has growth inhibitory effect on cancer cells, which has been attributed to its ability to induce cell cycle arrest, differentiation and apoptosis (Medina *et al*., 1997; Richon *et al*., 1998; Wang *et al*., 1999; Butler *et al*., 2000). NaB has been shown to induce $p21^{WAF1/CIP1}$ in a p53-independent manner (Xiao *et al*., 1997; Chai *et al*., 2000; Siavoshian *et al*., 2000; Iacomino *et al*., 2001; Pellizzaro *et al*., 2001), modulate cyclin D1 (Lallemand *et al*., 1996; Siavoshian *et al*., 2000), cdc2 (Charollais *et al*., 1990), cdk2 (Siavoshian *et al*., 2000; Clarke *et al*., 2001) and proliferating cell nuclear antigen (Ranganna *et al*., 2000). In addition to the above functions, an anti-

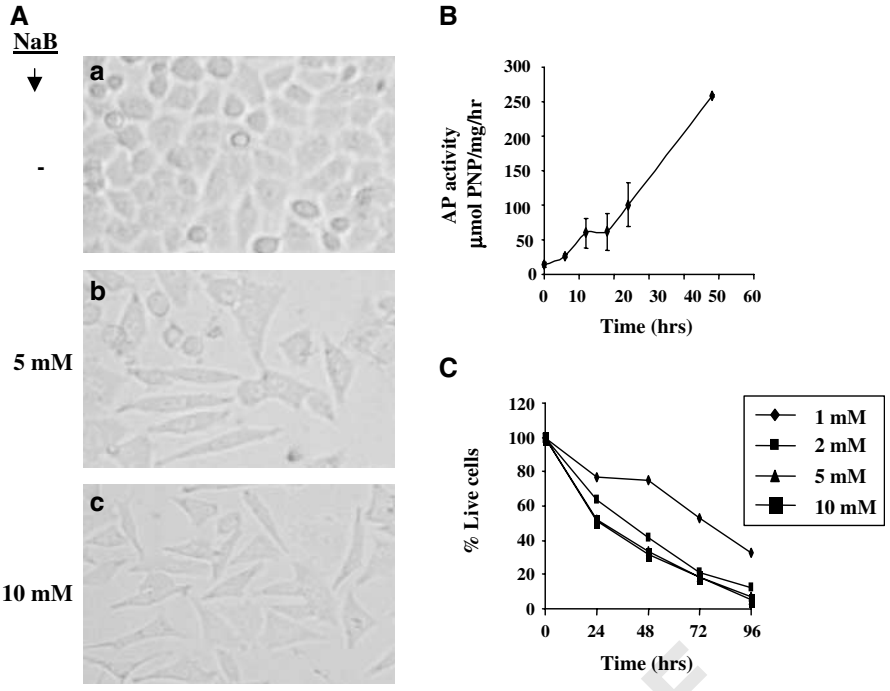

Figure 1 NaB induces differentiation and inhibits the growth of H460 cells. (**A**) H460 cells were either untreated (panel a) or treated with 5 mM (panel b) and 10 mM (panel c) concentrations of NaB for 24 h. The cells were photographed under bright field. (**B**) H460 cells were treated with NaB (2 mM) for different periods of time as indicated in the figure. At the end of the indicated time points, AP activity was quantified as described in Materials and methods. (**C**) H460 cells were treated with increasing concentrations (1, 2, 5 and 10 mM) of NaB for different periods of time as indicated in the figure. At the end of indicated time points, proportion of live cells was quantified by MTT assay as described in Materials and methods. The absorbance of control cells was considered as 100%

inflammatory role for NaB in certain stages of mucosal inflammation has also been proposed. Modulation of certain proinflammatory cytokines by NaB has been found to be the mechanism behind anti-inflammatory functions of this short chain fatty acid (Saemann *et al*., 2000).

In the present study, we carried out expression profiling of the NaB-treated human non-small-cell lung cancer line H460 using cDNA microarrays. We have identified several previously known genes and some novel genes, which are differentially expressed upon NaB treatment. We demonstrate that NaB downregulates the expression of a total of 16 genes belonging to cytokine signaling pathways, particularly that of IFNγ, suggesting that NaB interferes with cytokine signaling, which explains its anti-inflammatory functions. In addition, we also show that a total of 14 genes associated with metastasis are differentially regulated by NaB, thus providing evidence for the mechanism behind antimetastatic properties of NaB.

Results

Effect of NaB on H460 cells

NaB has been reported to inhibit the growth and induce differentiation and/or apoptosis in many cancer cells (Siavoshian *et al*., 2000; Pellizzaro *et al*., 2001). We carried out experiments to explore the effect of NaB in H460 cells. NaB treatment of H460 cells resulted in morphological changes characteristic of differentiating cells. While untreated cells appeared hexagonal, NaB-treated cells looked elongated (Figure 1A compare panels b and c with a). Alkaline phosphatase (AP) is a marker for cell differentiation (Morita *et al*., 1982; Tsao *et al*., 1982; Siavoshian *et al*., 2000; Tabuchi *et al*., 2000). Upon NaB treatment, the AP activity increased in a time-dependent manner (Figure 1B), which is indicative of differentiation. The addition of NaB also inhibited the growth of H460 cells in a dose-dependent and time-dependent manner (Figure 1C). To study the mechanism of growth inhibition by NaB, we monitored the cellular DNA synthesis and level of apoptosis. Cellular DNA synthesis was measured by BrdU incorporation assay and cell cycle analysis. Treatment of H460 cells with NaB for 48 h resulted in a drastic reduction in the percentage of cells incorporating BrdU compared to control cells (Figure 2A compare panels d and f with b; 2b compare bars 2 and 3 with 1). Fluorescence-activated cell sorting (FACS) was carried out to analyse the cell cycle profile of NaB-treated cells. The proportion of cells actively replicating DNA, which represent S phase cells, decreased to 50% by 24 h (50.36–24.04%) in NaB-treated cells (Figure 2C). The reduction in S phase cells resulted in concomitant change in G1 and G2/M phase cells. The proportion of cells containing 2*N* amount of

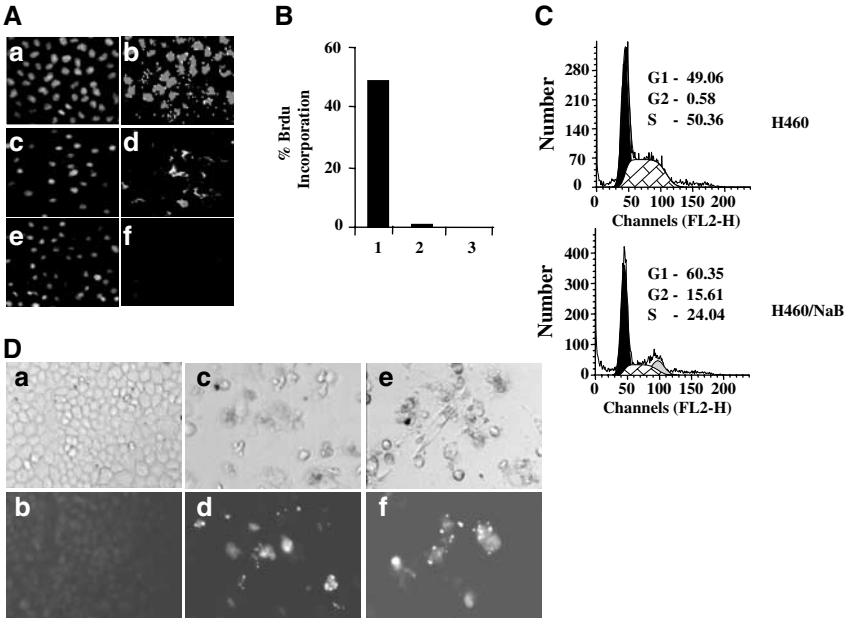

Figure 2 NaB inhibits cellular DNA synthesis and induces apoptosis in H460 cells. (**A**) H460 cells were either untreated (panels a and b) or treated with 1 mM (panels c and d) and 2 mM NaB (panels e and f). The cells were allowed to incorporate BrdU for last 4 h. After 24 h of the addition of drugs, BrdU-positive cells were identified by staining cells using anti-BrdU antibody and Texas red-conjugated anti-mouse secondary antibody (panels b, d and f). Panels a, c and e are of DAPI staining of cells of the same region as panels b, d and f, respectively. (**B**) BrdU-positive cells were calculated as %BrdU incorporation from (**A**) and shown (control – bar 1; 1 mM NaB – bar 2; and 2 mM NaB – bar 3). %BrdU incorporation was calculated by dividing the number of cells showing BrdU incorporation by total number of cells counted. (**C**) H460 cells were either untreated or treated with 2 mM concentrations of NaB for 24 h. The cells were harvested at 24 h after NaB addition and subjected to flow cytometry analysis as mentioned under 'Materials and methods'. The proportion of cells having a 2*N* amount of cells (G1), which corresponds to the G1 phase of cell cycle, 4*N* content of DNA (G2/M), which correspond to G2/M phase of the cell cycle and varying amount (2–4*N*) of DNA (S), which correspond to S phase of cell cycle are quantified, and the values are given on the right side. (**D**) H460 cells were either untreated (panels a and b) or treated with 0.2 μg/ml of adriamycin (panels c and d) and 1 mM of NaB (panels e and f). After 48 h of the addition of drugs, 1 μl of 1 mg/ml DAPI was added to each well and observed under UV microscopy immediately. The picture of the same area was taken under bright field and fluorescent microscopy (panels a and b, c and d, e and f, respectively). Note that the spots with bright blue fluorescence in panels d and f indicate the presence of nuclear fragmentation

DNA, which represent G1 phase cells, increased significantly in NaB-treated cells (49.05–60.35%). Similarly, the fraction of cells with 4*N* amount of DNA, which represent G2/M phase cells, increased several times (0.58–15.61%). These results suggest that treatment of H460 cells with NaB results in the inhibition of cellular DNA synthesis leading to the arrest of cells in G1 and G2/M phase of the cell cycle. NaB-treated cells were also monitored for nuclear fragmentation during apoptosis by staining the cells with DAPI and visualizing them by fluorescence microscopy (Figure 2D). H460 cells treated with adriamycin, a known inducer of apoptosis, showed bright blue fluorescence, which is indicative of nuclear fragmentation in comparison to control cells (Figure 2D compare panel d with b). NaB-treated H460 cells also showed nuclear fragmentation in comparison to control cells (Figure 2D compare panel f with b). These experiments suggest that H460 cells undergo differentiation, cell cycle arrest and apoptosis upon NaB treatment.

Analysis of gene expression changes by NaB treatment

We used microarray slides containing 1728 human genes (1.7K human chip) obtained from University Health Network, Toronto, Canada. Total RNA was isolated from H460 cells untreated or treated with NaB for 24 h, labeled and hybridized to arrays. A total of three independent experiments were carried out. Genes containing values at least in two of the experiments were taken into further consideration. Among the total of 696 genes, which fit this criteria, there were 32 genes with a mean expression value of 2.0-fold or higher and 66 genes with a mean expression value 3.0-fold or higher in NaB-treated cells. The list of 32 genes, upregulated in NaB-treated cells are described in Table 1. Table 2 gives the list of 66 downregulated genes in NaB-treated cells. These genes are classified into several groups that include cell proliferation, metabolism, signal transduction, transport, cell death and other groups. To verify our microarray data, we selected a few genes from both upregulated and downregulated gene lists and verified their expression levels in NaB-treated cells by semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) (Figure 3). The expression levels of retinoic acid induced 3 (RAI3), S100 calcium-binding protein (S100P), serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), metallothionein 2A (MT2A), Ras homolog gene family,

Table 1 List upregulated genes by NaB

Acc. no.[a]	*Symbol*	*Gene name*	*Fold change*[b]	*Biological process*[c]					
				1	*2*	*3*	*4*	*5*	*6*
AL578517	RAI3	Retinoic acid induced 3	7.0±3.4	Y	—	—	—	—	—
AA128249	FABP4	Fatty acid-binding protein 4, adipocyte	4.5±3.0	—	Y	—	—	—	—
N92185	NR4A1	Nuclear receptor subfamily 4	4.5±0.7	Y	—	—	—	—	—
W68191	CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	4.0±1.6	—	—	—	Y	—	—
H48072	COX6A1	Cytochrome *c* oxidase subunit via polypeptide 1	3.6±1.7	—	—	—	Y	—	—
AA001324	TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	3.1±0.8	—	—	Y	Y	—	—
BG565696	PRDX1	Peroxiredoxin 1	2.6±0.8	—	—	Y	—	Y	—
W04882	CETN2	Centrin, EF-hand protein, 2	2.5±0.1						
BM263733	ATP5G3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c	2.5±0.9	Y	Y	—	—	—	—
R14058	PRKCB1	Protein kinase C, beta 1	2.5±0.1	Y	—	—	Y	—	—
AA041319	GDI1	GDP dissociation inhibitor 1	2.4±0.4	Y	Y	—	—	—	—
AA133258	HADHB	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), β subunit	2.4±0.4	—	—	—	Y	—	—
R09570	POLR2L	Polymerase (RNA) II (DNA directed) polypeptide L (7.6 kDa)	2.3±1.0	—	—	—	Y	—	—
H53667	GSTP1	Glutathione *S*-transferase pi	2.3±1.1	—	—	—	—	Y	—
R59926	PACE	Paired basic amino acid cleaving enzyme (furin, membrane-associated receptor protein)	2.3±1.4	—	—	—	Y	—	—
W39107	ARHA	Ras homolog gene family, member A	2.2±0.8	Y	—	—	—	—	Y
BI666621	NME1	Nonmetastatic cells 1, protein (NM23A) expressed in domains proteoglycan (testican) group A, member (subunit 9) isoform 3 pathways	2.1±1.2	—	—	Y	Y	—	—
W67766	PSAP	Prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	2.1±0.3	—	Y	—	Y	—	Y
H74119	SEC61B	Protein translocation complex beta	2.1±0.9	—	Y	—	Y	—	—
H16888	PCMT1	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase	2.0±0.6	—	—	—	Y	—	—
BM263123	GGT1	Gamma-glutamyltransferase 1	2.0±1.1	Y	Y	—	—	—	—
R32131	IGF2	Insulin-like growth factor 2 (somatomedin A)	2.0±1.2	Y	—	Y	Y	Y	Y
AI093233	SPOCK	Sparc/osteonectin, cwcv and kazal like	1.9±2.9	—	—	Y	—	Y	—
Others:									
AA136789	S100P	S100 calcium-binding protein P	4.3±2.0						
H50992	SERPINB2	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	3.6±1.6						
H91613	MT2A	Metallothionein 2A	2.8±3.8						
AI762390	KIAA1228	KIAA1228 protein	2.6±0.8						
W24688	IFI30	Interferon, gamma-inducible protein 30	2.5±0.6						
AA056280	SAT	Spermidine/spermine N1-acetyltransferase	2.4±0.8						
BE874451		EST	2.3±0.4						
N77716	QP-C	Low molecular mass ubiquinone-binding protein (9.5 kDa)	2.0±0.2						
AA047157	KAI1	Kangai 1 (suppression of tumorigenicity 6, prostate; CD82 antigen (R2 leukocyte antigen, antigen detected by monoclonal and antibody IA4))	2.0±1.3						

[a]GenBank Accession number. [b]Average fold change of three experiments. [c]Biological process: 1 –signal transduction; 2 – transport; 3 – cell prolofiration; 4 – metabolism; 5 – organogenisis; 6 – cell organization and biogenesis; Y – involved in that process

member A (RAHA), Sparc/osteonectin (SPOCK) and Kangai 1 (KAI1) are increased in NaB-treated cells, thus confirming the results obtained from microarray experiment (Figure 3a). Similarly, the expression levels of follistatin-like 1 (FSTL1), interleukin 6 (IL-6) (interferon (IFN), beta 2; IL6), nucleolin (NCL) and cyclin-dependent kinase 4 (CDK4) are downregulated in NaB-treated cells.

NaB specifically downregulates cytokine signaling related to inflammation

Upon closer look at the expression profile of NaB-treated cells, we found that a total of 16 genes associated with cytokine signaling, particularly related to interferon γ (IFNγ), were downregulated. The list includes cytokines, cytokine receptors, intracellular signaling molecules as well as target genes (Table 3). For visual

Table 2 List of downregulated genes by NaB

Acc. no.[a]	*Symbol*	*Gene name*	*Fold change*[b]	*Biological process*						
				1	*2*	*3*	*4*	*5*	*6*	*7*
H79188	ERCC2	Excision repair crosscomplementing rodent repair deficiency, complementation group	−9.1±2.7	—	Y	—	—	Y	—	—
AA149475	IFNAR2	Interferon (alpha, beta and omega) receptor 2	−7.6±1.7	—	—	Y	—	Y	—	—
N34004	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	−7.5±5.6	—	Y	Y	—	—	—	—
R94457	GMPS	Guanine monophosphate synthetase	−5.5±4.5	—	Y	—	—	—	—	—
R48041	GAA	Glucosidase, alpha; acid (Pompe disease, glycogen storage disease type II)	−5.4±1.9	—	Y	—	—	—	—	—
R81839	TXK	TXK tyrosine kinase	−4.9±2.7	—	Y	Y	—	—	—	—
W17249	ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)	−4.6±0.3	—	Y	Y	—	—	—	—
W47038	HAK	Heart alpha-kinase	−4.5±3.1	—	Y	—	—	—	—	—
R88469	DPP6	Dipeptidylpeptidase VI	−4.5±3.3	—	Y	—	—	—	—	—
AI820764	CMRF35	CMRF35 leukocyte immunoglobulin-like receptor	−4.5±1.2	—	—	—	—	Y	—	—
AA043457	ZNF137	Zinc-finger protein 137 (clone pHZ-30)	−4.3±0.9	—	Y	—	—	—	—	—
W24394	A2M	Alpha-2-macroglobulin	−4.0±2.7	—	—	—	Y	—	—	—
R73063	CSF1	Colony-stimulating factor 1 (macrophage)	−3.9±1.5	Y	—	—	—	—	—	Y
AW607760	CLCN3	Chloride channel 3	−3.9±1.7	—	—	—	Y	—	—	—
R23132	CUL1	Cullin 1	−3.9±2.3	Y	—	—	—	—	Y	—
W44684	MPP3	Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	−3.7±2.5	—	—	Y	—	—	—	—
R23778	C7	Complement component 7	−3.7±2.8	—	—	—	—	Y	Y	—
BI493041	TIMP3	Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	−3.7±2.5	—	Y	—	—	—	—	—
N98673	GRM3	Glutamate receptor, metabotropic 3	−3.6±3.2	—	—	Y	—	—	—	—
W24348	IL11	Interleukin 11	−3.6±1.7	Y	—	—	—	Y	—	Y
H75353	ALAD	Aminolevulinate, delta-, dehydratase	−3.6±0.8	—	Y	—	—	—	—	—
R90829	VEGFB	Vascular endothelial growth factor B	−3.6±2.2	Y	—	Y	—	—	—	—
H27400	HMGB1	ESTs, highly similar to S02826 nonhistone chromosomal protein HMG-1 (*Homo sapiens*)	−3.6±0.3	Y	Y	—	—	—	—	—
R23436	PRKAR2A	Protein kinase, cAMP-dependent, regulatory, type II, alpha	−3.6±1.5	—	—	Y	—	—	—	—
N41908	FES	Feline sarcoma oncogenes	−3.6±0.1	Y	Y	Y	—	—	—	—
BM049821	EMP3	Epithelial membrane protein 3	−3.5±1.6	Y	—	—	—	—	Y	—
AA040255	COL6A3	Collagen, type VI, alpha 3	−3.3±1.9	—	—	—	—	—	—	Y
AA042836	IRF2	Interferon regulatory factor 2	−3.3±1.5	Y	Y	—	—	Y	—	—
W56121	CAPN7	Calpain 7	−3.3±0.7	—	Y	—	—	—	—	—
R01959	USP7	Ubiquitin-specific protease 7	−3.3±1.9	—	Y	—	—	—	—	—
N77287	F10	Coagulation factor X	−3.2±2.2	—	Y	—	—	—	—	—
BM044542	CDK4	Cyclin-dependent kinase 4	−2.2±0.4	Y	—	—	—	—	—	—
R99156	CKS1B	CDC28 protein kinase regulatory subunit 1B	−3.1±1.3	Y	—	—	—	—	—	—
BG759096	EIF4G2	Eukaryotic translation initiation factor 4 gamma, 2	−3.1±1.0	Y	Y	—	—	—	Y	—
H58668	ELA2	Elastase 2, neutrophil	−3.1±0.1	—	Y	—	—	—	—	—
H28534	AQP1	Aquaporin 1 (28 kDa)	−3.1±0.3	—	—	—	Y	—	—	—
R96617	ABCC2	ATP-binding cassette, subfamily C, member 2	−3.0±1.2	—	—	—	Y	—	—	—
R18562	ATP7B	ATPase, Cu2+ transporting, beta polypeptide	−3.3±0.2	—	—	—	Y	—	—	—
Others										
R23999	HSPG2	Heparan sulfate proteoglycan 2 (perlecan)	−10.0±6.8							
W92014	ADH6	Alcohol dehydrogenase 6 (class V)	−10.0±7.1							
N34169	NF2	Neurofibromin 2 (bilateral acoustic neuroma)	−7.1±0.2							
N95415	LOC54466	Spindlin like	−7.1±5.6							
W38462	STE	Sulfotransferase, estrogen preferring	−4.8±3.0							
W84867	CYP4A11	Cytochrome *P*450, subfamily IVA, polypeptide 11	−4.8±0.2							
AA088647		ESTs	−4.8±2.6							
N31846	ACP2	Acid phosphatase 2, lysosomal	−4.7±1.9							
H25460	CDH11	Cadherin 11, type 2, OB cadherin (osteoblast)	−4.3±0.9							
R47859	NPR1	Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	−4.2±0.7							
AL046955	FSTL1	Follistatin-like 1	−4.1±1.5							
W25614		KIAA0960 protein	−4.1±0.9							
AI668645	MYL4	Myosin, light polypeptide 4, alkali; atrial, embryonic	−4.0±1.9							
W38732	MLLT2	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*); translocated to, 2	−3.8±1.3							
W33064	TUBA1	Tubulin, alpha 1 (testis specific)	−3.6±1.4							
W39129	NUCB1	Nucleobindin 1	−3.4±1.1							
W31016	IL6	Interleukin 6 (interferon, beta 2)	−3.4±1.1							
T81285	PFKL	Phosphofructokinase, liver	−3.4±0.4							
AA042836	IRF2	Interferon regulatory factor 2	−3.3±1.5							
R02471	G6PC	Glucose-6-phosphatase, catalytic (glycogen storage disease	−3.3±0.9							

Table 2 (*continued*)

Acc. no.[a]	*Symbol*	*Gene name*	*Fold change*[b]	*Biological process*						
				1	*2*	*3*	*4*	*5*	*6*	*7*
		type I, von Gierke disease)								
Unresolved	ZFP36L1	Zinc-finger protein 36, C3H type-like 1	−3.3±0.6							
H48784	ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	−3.2±1.2							
BG054547	RNF141	Ring-finger protein 141	−3.2±1.4							
H30138	THBS4	Thrombospondin 4	−3.1±0.6							
R74161	PYGL	Phosphorylase, glycogen; liver	−3.1±0.8							
R67197	Thra1	Thyroid hormone receptor alpha	−3.1±1.1							
BE738657	PBEF	Pre-B-cell colony-enhancing factor	−3.0±0.0							
BG675524	NCL	Nucleolin	−2.3±0.6							

[a]GenBank Accession number. [b]Average fold change of three experiments. [c]Biological process: 1 – cell proliferation; 2 – metabolism; 3 – signal transduction; 4 – transport; 5 – response to biotic stimulus, response to pest/pathogen/parasite; 6 – cell death; 7 – organogenesis; Y – involved in that process

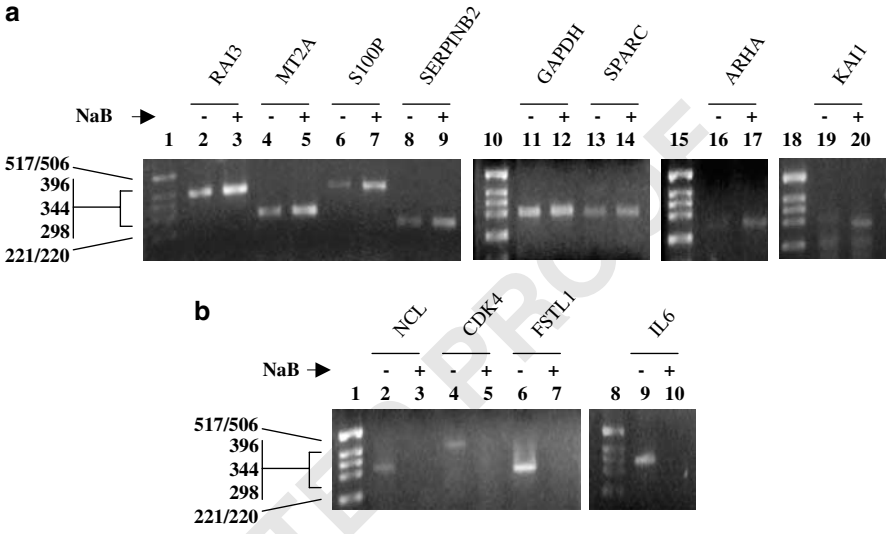

Figure 3 Semiquantitative RT–PCR verification of selected gene. H460 cells either untreated (−) or treated (+) with NaB (2 mM) for 24 h, total RNA was isolated, first-strand cDNA was made as described in the Materials and methods and used for subsequent gene specific PCRs. (**a**) Using gene specific primers, PCR was carried out for the following upregulated genes: RAI3 (lanes 2 and 3), MT2A (lanes 4 and 5), S100P (lanes 6 and 7), SERPINB2 (lanes 8 and 9), SPARC (lane 13 and 14), ARHA (lanes 16 and 17) and KAII (lanes 19 and 20). GAPDH was used as control as it did not show any change in expression levels upon NaB treatment (lanes 11 and 12). (**b**) The following downregulated genes in NaB-treated cells were verified by PCR using gene specific primers: NCL (lanes 2 and 3), CDK4 (lanes 4 and 5), FSTL1 (lanes 6 and 7) and IL6 (lanes 9 and 10)

presentation, the same list of genes was subjected to hierarchical clustering by using Gene Cluster version 3.0 and the dendrogram was generated using TreeView (Figure 4a). An anti-inflammatory role for NaB in certain mucosal inflammatory conditions has been reported. IFNγ is a major proinflammatory cytokine upregulated in inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease (Fais *et al*., 1991; Breese *et al*., 1993). IFNγ exerts its function by activating the latent cytoplasmic transcription factor STAT1 (signal transducer and activator of transcription 1) (Darnell *et al*., 1994). Binding of IFNγ to the type II IFN receptor initiates signaling through transphosphorylation and activation of the Janus kinases JAK1/JAK2, which further activates STAT1 by phosphorylation. Activating phosphorylation of STAT1 results in STAT1 dimerization, nuclear translocation, DNA binding and stimulation of the expression of the IFN-responsive genes (Darnell *et al*., 1994; Decker and Kovarik, 2000; Jove, 2000). Since our expression profiling data suggest that NaB downregulates the expression level of several genes at different stages of IFNγ signaling pathway, we hypothesized that NaB might inhibit IFNγ-mediated signaling, thereby providing explanation for its anti-inflammatory effects. To verify this possibility, we analysed the ability of IFNγ to induce its target gene interferon regulatory factor 1 (IRF1) in the presence of NaB. IRF-1 has been shown to be an essential mediator

Table 3 Downregulation of genes associated with cytokine signaling by NaB

Acc. no.[a]	*Symbol*	*Gene name*	*Fold change*[b]
W31016	IL6	Interleukin 6 (interferon, beta 2)	−3.4±1.1
W24348	IL11	Interleukin 11	−3.7±1.7
W47225	IL1B	Interleukin 1, beta	−2.5±1.0
AA149475	IFNAR2	Interferon (alpha, beta and omega) receptor 2	−7.6±1.7
W23815	IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	−2.2±0.8
W31707	IL1R1	Interleukin 1 receptor, type I	−1.8±0.4
AA125917	IL1RL1	Interleukin 1 receptor-like 1	−1.4±0.3
N34004	STAT6	Signal transducer and activator of transcription 6 (IL-4 induced)	−7.6±5.6
AA042836	IRF2	Interferon regulatory factor 2	−3.4±1.5
AA011445	CASP3	Caspase 3, apoptosis-related cysteine protease	−2.7±1.2
H49853	IFITM1	Interferon induced transmembrane protein 1 (9–27)	−2.6±0.8
N31546	IFI16	Interferon, gamma-inducible protein 16	−2.3±1.5
AA131850	GBP2	Guanylate-binding protein 2, interferon-inducible	−2.1±0.8
R34567	OAS2	2′–5′ oligoadenylate synthetase 2 (69–71 kD)	−2.0±1.2
W52254	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	−2.0±1.3
T97408	BAG1	BCL2-associated athanogene	−1.8±0.1

[a]Genbank Accession number. [b]Average fold change of three experiments

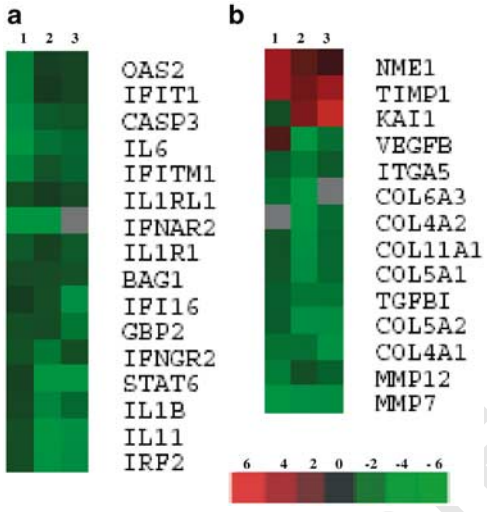

Figure 4 Hierarchical clustering of expression levels of genes associated with cytokine signaling described in Table 3 (**a**) and cancer metastasis described in Table 4 (**b**) in NaB-treated cells. The values from three experiments (as indicated 1, 2 and 3) were used for clustering. A visual dual color code is utilized with red and green indicating relatively either upregulation or downregulation, respectively, in NaB-treated cells compared to untreated cells. Cluster and Treeview programs were used to generate these figures (Eisen *et al*., 1998). Gene symbols are given at the right side. The scale at the bottom shows the relationships between color saturation and the normalized gene expression levels

of IFNγ-mediated growth arrest and apoptosis (Kano *et al*., 1999; Yim *et al*., 2003). We checked the ability of IFNγ to induce IRF1 transcript as well as IRF1 protein levels in NaB-pretreated cells. IFNγ induced IRF1 protein level several fold in H460, HCT116 and SW620 cells (Figure 5a). However, if the cells are pretreated with NaB, IFNγ induced the level of IRF1 very inefficiently (Figure 5a). Similarly, IFNγ induced IRF1 transcript levels less efficiently in NaB-pretreated cells compared to control cells (Figure 5b). In addition to the inhibition of IFNγ signaling pathway, NaB also downregulated the levels of another proinflammatory cytokine IL-1 and its receptors IL-1 receptor, type I and interleukin 1 receptor-like 1 (Table 3). These results suggest that NaB interferes with signaling pathway associated with proinflammatory cytokines, in particular, IFNγ.

Differential regulation of metastasis associated genes by NaB

We also identified another set of 14 genes associated with cancer metastasis differentially regulated by NaB (Table 4). Of these genes, NaB induced the levels of three genes, which are known for their metastatic suppressor activity. KAI1, a metastatic suppressor gene, is up regulated by twofold in NaB-treated cells. To further confirm KAI1 upregulation by NaB, we analysed the levels of KAI1 at different time points of NaB treatment. KAI1 levels increased in time-dependent manner after NaB treatment in H460, HCT116 and SW620 cells with varying kinetics (Figure 6). Tissue inhibitor of metalloprotease 1 (TIMP1), which complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them, is also induced by NaB by 3.18-fold. Similarly, the level of nm23, known metastatic suppressor gene, is also increased 2.13-fold in NaB-treated cells. In contrast to these three genes, we found in the list a group of 11 genes, which have been reported to promote metastasis, downregulated in NaB-treated cells. This list includes matrix metalloproteinases, integrin, cadherin, vascular endothelial growth factor B and several genes encoding different types of collagens, which are essential component of extracellular matrix (ECM). These results suggest that NaB differentially regulates the metastasis-associated genes, thus providing proof for the antimetastatic properties of NaB reported in the literature.

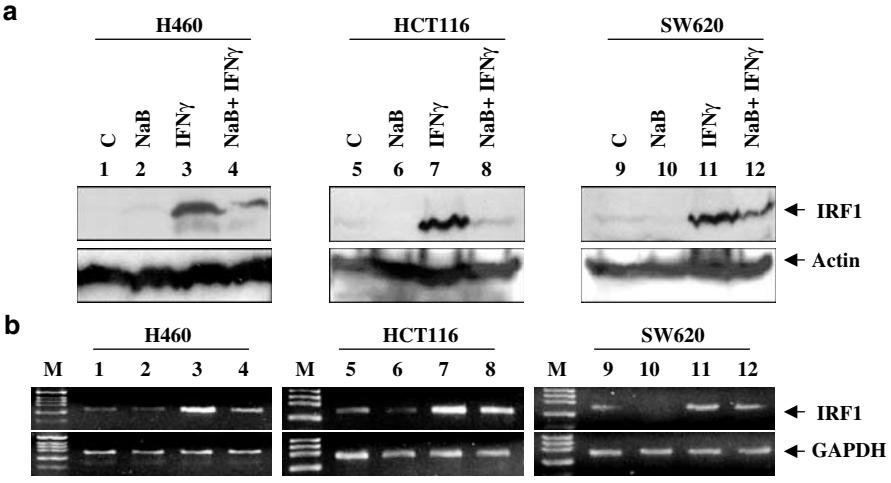

Figure 5 NaB inhibits IFNγ-mediated activation of IRF1. (**a**) Total cell lysate was prepared from H460, HCT 116 and SW620 cells that were either left untreated (lanes 1, 5 and 9) or were treated with 2 mM NaB for 24 h (lanes 2, 6 and 10), 2000 U of IFNγ for 24 h alone (lanes 3, 7 and 11), or after preincubation with NaB for 24 h (lanes 4, 8 and 12). The levels if IRF1 and actin were detected by Western blot analysis. (**b**) Total RNA was prepared from H460, HCT116 and SW620 cells that were either left untreated (lanes 1, 5 and 9) or were treated with 2 mM NaB for 24 h (lanes 2, 6 and 10), 2000 U of IFNγ for 24 h along (lanes 3, 7 and 11), or after preincubation with NaB 24 h (lanes 4, 8 and 122). The levels of IRF1 and GAPD transcripts were detected by semiquantitative RT–PCR

Table 4 List of metastasis associated genes regulated by NaB

Acc. no.[a]	*Symbol*	*Gene name*	*Fold change*[b]
AA001324	TIMP1	Tissue inhibitor of metalloproteinase 1	3.9±0.7
BI666621	NME1	Nonmetastatic cells 1, (NM23A)	2.2±1.2
AA047157	KAI1	Kangai 1 (suppression of tumorigenicity 6)	2.0±2.9
AA151197	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	−1.8±0.4
AA031513	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	−3.5±1.0
N30844	MMP12	Matrix metalloproteinase 12 (macrophage elastase)	−1.8±0.5
AW965882	COL4A1	Collagen, type IV, alpha 1	−2.5±0.6
W23823	COL4A2	Collagen, type IV, alpha 2	−2.7±0.9
W52829	COL5A1	Collagen, type V, alpha 1	−2.0±0.6
N43019	COL5A2	Collagen, type V, alpha 2	−2.4±0.7
AA040255	COL6A3	Collagen, type VI, alpha 3	−3.4±1.9
W31189	COL11A1	Collagen, type XI, alpha 1	−2.1±0.9
R90829	VEGFB	Vascular endothelial growth factor B	−1.9±3.3
BM005966	TGFBI	Transforming growth factor, beta-induced, 68 kDa	−2.0±0.3

[a]GenBank Accession number. [b]Average fold change of three experiments

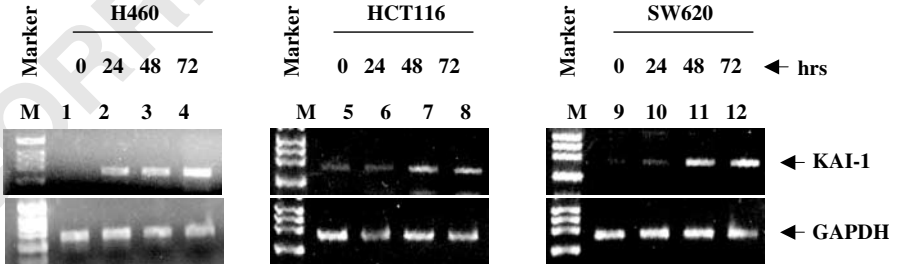

Figure 6 NaB activates KAI1. Total RNA was isolated from H460, HCT116 and SW480 cells either untreated or treated with 2 mM amounts of NaB for 24, 48 and 72 h as indicated. First-strand cDNA synthesis was made as described in the Materials and methods and gene specific PCR was carried out for KAI1 and GAPDH

Discussion

DNA microarray technique is a powerful method to analyse simultaneously the expression levels of multiple genes (Schena *et al*., 1995; DeRisi *et al*., 1996; Cheung *et al*., 1999). In this study, we have carried out gene expression profiling of NaB-treated cells using cDNA microarrays. We identified several genes belonging to

different classes of functions differentially regulated by NaB. We also confirmed the microarray results by semiquantitative PCR for a few selected genes from both up- and downregulated genes by NaB. In addition, we have identified a set of 16 genes related to cytokine signaling, in particular, IFNγ is downregulated by NaB. Moreover, we have also found out a set of 14 genes associated with cancer metastasis are differentially regulated by NaB. Thus we present evidence, from microarray-based expression profiling, for specific regulation of two groups of genes by NaB, thereby providing a possible mechanism behind anti-inflammatory and antimetastatic properties of NaB.

In addition to its well-known function as an essential energy source for colonocytes and growth inhibitory effects on cancer cells, NaB has anti-inflammatory and immunosuppressive effects *in vitro* and *in vivo* (Velazquez *et al*., 1997; Andoh *et al*., 1999; Inan *et al*., 2000; Saemann *et al*., 2000). Several proinflammatory cytokines, including TNF and IL-1, have been shown to activate NF-κB (Pahl, 1999). The deregulation of NF-κB activity has been reported frequently in IBDs, such as UC and Crohn's disease (Rogler *et al*., 1998; Schreiber *et al*., 1998; Segain *et al*., 2000). The anti-inflammatory activity of NaB has been attributed to its ability to interfere with the activity of NF-κB (Inan *et al*., 2000, Segain *et al*., 2000; Yin *et al*., 2001; Luhrs *et al*., 2002a, b). In our study, we found that NaB downregulated the expression of IL-1b and its receptors, thus providing mechanism behind its previously described function of the downregulation of NF-κB activity.

IFNγ is another major proinflammatory and immunoregulatory cytokine, which is found to be increased in IBD (Fais *et al*., 1991; Fuss *et al*., 1996). IFNγ exerts its action through the activation of the latent cytoplasmic transcription factor STAT1, which in turn activates several IFN-responsive genes (Darnell *et al*., 1994; Decker and Kovarik, 2000; Jove, 2000). Our study shows that NaB downregulates the expression of IFNγ receptor 2 (IFNGR2), signal transducer and activator of transcription 6 (STAT6) as well as many downstream target genes of IFNγ (Table 3). These results suggest that downregulation of IFNγ signaling pathway by NaB could be one of the mechanisms by which NaB exerts its anti-inflammatory activity. This hypothesis is rightly supported by our results that IFNγ failed to activate its downstream target genes IRF-1 efficiently (Figure 5a). While this manuscript was under preparation, Klampfer *et al*., 2003 reported that NaB inhibits IFNγ signaling, thus providing more support to our results. Together, these results provide evidence for the fact that NaB downregulates signaling pathways related to proinflammatory cytokines, thus providing a potential mechanism behind its anti-inflammatory activities.

The most damaging change during cancer progression is the switch from a locally growing tumor to a metastatic tumor. This change is believed to involve several alterations that allow tumor cells to dislodge from the origin, migrate to different locations to grow as metastatic tumors (Chambers *et al*., 2002). Deacetylase inhibitors have been shown to be inhibitors of metastasis. The antimetastatic property of deacetylase inhibitors has been attributed to their ability to downregulate matrix metalloprotease 2 (MMP-2; Kim *et al*., 2000; Ailenberg and Silverman, 2002; Liu *et al*., 2003) and upregulate E-cadherin (Masuda *et al*., 2000). Considering the fact that metastasis is a complex process, it is likely that NaB may regulate several more genes related to metastasis. Our results show that NaB activates the expression of three metastatic suppressor genes, KAI1, NME1 and TIMP1. KAI, a surface glycoprotein, has been shown to posses metastatic suppressor activity (Rinker-Schaeffer *et al*., 1994; Dong *et al*., 1995; Phillips *et al*., 1998). NME1 (NM23) has also been shown to act as a metastatic suppressor (Leary *et al*., 1995; Miele *et al*., 1997). Overexpression of TIMP1 has been correlated with decreased metastasis (Kawamata *et al*., 1995). In addition, NaB also repressed the expression of 11 genes, which have been shown to be associated with metastasis (Table 4). This set includes metalloprotease 7 and 12, which belong to the MMP family. MMP family consists of over 20 members and is characterized by their ability to degrade ECM (Chang and Werb, 2001). Increased metastasis has been correlated with high levels of metalloprotease activity in cancer cells and the ability of MMPs to degrade ECM proteins has been found to be the underlying mechanism (Chang and Werb, 2001). Apart from MMPs, several genes that include ECM proteins as well as molecules that regulate their assembly have been shown to promote metastasis (Maniotis *et al*., 1999; Ruoslahti, 1999; Clark *et al*., 2000). Our results show that NaB downregulates the expression of integrin, alpha 5 (fibronectin receptor) and several genes, which encode various subunits of collagens (Table 4). Therefore, this study presents evidence for a differential regulation of metastasis-associated gene by NaB, thus providing a mechanism for its antimetastatic activity.

Thus, we have identified the several up- and downregulated genes in NaB-treated cells from our study. Very specifically, we have identified that NaB regulates two groups of genes related to cytokine signaling and cancer metastasis, thereby providing mechanistic evidence for its anti-inflammatory and antimetastatic properties. We believe that the expression profile of NaB-treated cells developed in the study would help us to further understand the mechanisms behind the biological changes occurring the NaB-treated cells.

Materials and methods

Cell line and culture conditions

H460, a human non-small lung carcinoma cell line is grown in RPMI 1640 medium with 10% fetal calf serum (FCS) (Das *et al*., 2003a). HCT116 and SW620 cells, human colon carcinoma cell lines, are grown in DMEM with 10% FCS (Das *et al*., 2003b). H460 cells were incubated with various concentrations of NaB (Sigma) for indicated times.

MTT assay

MTT assay was carried out as described previously (Das *et al*., 2003b). A total of 1.5×10^3 cells/well were plated in a 96-well plate. After 24 h of plating, the cells were treated with indicated amounts of NaB. A measure of 20 μl (5 mg/ml) of MTT was added to each well 48 h after the addition of the drug. MTT is a tetrazolium salt that is converted by living cells into purple formazan crystals. The medium was removed from the wells 3 h after MTT addition and 200 μl of DMSO was added to dissolve the formazan crystals, and then the absorbance was measured at 550 nm in an ELISA reader.

AP assay

AP assay was carried out as per the manufacturer's instruction (Bangalore Genei, India). Briefly, 200 μg whole-cell lysate of control and NaB- (2 mM) treated (for different time points) samples (in triplicate) were mixed with 100 μl of cocktail containing pNPP (4-nitophenyl phosphate) in 96-well plates and incubated at 37°C for 30 min. The reaction was stopped by the addition of 80 μl of 1 N NaOH and the absorbance was measured at 405 nm in an ELISA reader. Absorbance values obtained were used to calculate the millimoles of the product formed using millimolar extinction coefficient of 17.8.

DNA synthesis inhibition

DNA synthesis inhibition was monitored by assessing BrdU incorporation as described earlier (Wajapeyee and Somasundaram, 2003). BrdU (20 μM) was added 20 h after NaB or adriamycin addition. The experiment was terminated 4 h after the addition of BrdU and the DNA synthesis was measured by using anti-BrdU antibody (Ab-3; Oncogene).

Western blot analysis

Western analysis was performed as described earlier (Das *et al*., 2003a) with mouse anti-human IRF1 and goat anti-human actin polyclonal (I-19; Santa Cruz) antibodies. Cells were harvested after 24 h or appropriate time point as described after drug addition and subjected to analysis.

Flow cytometry

FACS was carried out as described earlier (Wajapeyee and Somasundaram, 2003). H460 cells were treated with NaB (1 mM). After 24 h of NaB addition, the cells were washed with phosphate-buffered saline (PBS) twice and harvested by trypsinization. The cells were washed again with PBS and fixed with cold 70% ethanol for 1 h. The cells were washed with PBS once and then incubated with 4 μg of ribonuclease A (Roche Applied Science) for 30 min at room temperature. Propidium iodide was added to the cell suspension at a final concentration of 20 μg/ml and incubated for 30 min. The cells were then analysed by flow cytometry using FACScan (Becton Dickinson). The results were quantified by using the software Cell Quest (Becton Dickinson).

Total RNA preparation, cDNA labeling and array hybridization

Total RNA was extracted from tissue culture cells by TRIzol method (Life Technologies, Inc.) according to the manufacturer's instructions. The RNA samples were quantified using a spectrophotometer and visualized on a TBE gel for quality assurance. cDNA synthesis and labeling from total RNA were carried out using Micromax direct labeling kit (Perkin-Elmer Life Sciences, Inc.). RNA derived from untreated cells was labeled with cyanine 3-dUTP (Cy3), while that of NaB-treated cells was labeled with cyanine 5 dUTP (Cy5). Total RNA (20 μg) was used per reaction. The quality of cDNA labeling was monitored by separating small amounts of Cy3- and Cy5-labeled cDNA in an agarose gel made on a microscopic slide and scanning the gel using a laser scanner LSIV (Genomic Solutions, USA). Cy3- and Cy5-labeled cDNAs were added to 100 μl of 1 × hybridization buffer (Ultrahyb, Sigma), incubated at 75°C for 5 min before adding to 1.7K human cDNA array (University Health Network, Toronto, Canada). A complete list of gene names and their localization can be found in the following web page: www.microarray.ca/support/glists.html. Hybridization was carried out overnight in GeneTAC Hyb Station (Genomic Solutions) at 65°C for 4 h, 60°C for 4 h and 55°C for 8 h. The slides were washed using medium stringency, high stringency and postwash buffers for 5 min each, dried and scanned using GeneTAC LS IV scanner (Genomic Solutions). The entire experiment from NaB treatment, RNA isolation, cDNA labeling and array hybridization were carried out three times.

Microarray image and data analysis

Image analysis carried out with GeneTAC analyzer version 3.3. (Genomic Solutions). We followed several stringent criteria to select the optimal spots from the image. Spots showing a signal-to-noise ratio greater than 1.1 are used for further analysis. Spots, which show a total intensity less than the mean of the total intensity of blank spots on the array, are excluded from analysis. If the coefficient of variation of ratios of duplicate spots of a given gene is equal or less than 20%, then the gene is taken for further analysis. Normalization was carried out using trimmed median log. The normalized data are subjected to hierarchical Cluster analysis using Gene Cluster-version 3.0 (developed by Michael Eisen, Stanford University) to obtain a more visual representation and substructure of the data (Eisen *et al*., 1998). Functional classifications of differentially regulated genes were carried out by 'Database for Annotation, Visualization and Integrated Discovery (DAVID)' (http://apps1.niaid.nih.gov/david/).

Semiquantitative RT–PCR

RT–PCR was carried out using two-step strategy: cDNA was generated using Reverse Transcription kit (Promega) in the first step; using gene specific primer sets, PCR was carried out with cDNA as templates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used for internal normalization. The sequences of the sense (S) and antisense (AS) primers used for RT–PCR are given below: RAI3 (S: 5′-TAGAGCTGTGTGAGCCAGCG-3′; AS: 5′-CCAGCCTCAAC CACAATTC-3′); MT2A (S: 5′-ATAGCAAACGGTCACGGTCAG-3′; AS: 5′-ATGGATCCCAACTGCTCCTG-3′); S100P (S: 5′-ATTTTCTCGGCCCTGCC-3′; AS: 5′-ACACTTTTGGGAAAGCCTTG-3′); SERPINB2 (S: 5′-TCAAGCATCTGGCAAAAGC-3′; AS: 5′ TCGCATCCAGGATAACTACC 3′); GAPDH (S: 5′-TTTGTCAAGCTCATTTCCTGG-3′; AS: 5′-TGATGGTACATGACAAGGTGC-3′); SPARC (S: 5′-GAGGGGAAATGACATCTGGA-3′; AS: 5′-GAGCCATGCATGATTAGTGG-3′); ARHA (S: 5′-TTAACGATGTCCAACCCGTC-3′; AS: 5′-TGTTACGGAGTAAAGCCCTG-3′); KAI1 (S: 5′-TTTCTGTGAGGAAGGGCTTC-3′; AS: 5′-GGCAGGGAGATGGGGATAG-3′); NCL (S: 5′-CAAACCTAAGGGTGAAGGTG-3′; AS: 5′-TGTCCTTGGAATTGTCCTC-3′); CDK4 (S: 5′-TTTGAGCATCCCAATGTTG-3′; AS: 5′-CTCCACATGTCCACAGGTG-3′); FSTL1 (S: 5′-TTCCATGAATGGTCCTCAG-3′; AS: 5′-TGCCTTGAA-

GAACTCACAG-3′); and IL6 (S: 5′-TACATCCTCGACGG-CATCTC-3′; AS: 5′-GCATTGTGGTTGGGGTCAAG-3′).

Abbreviations
NaB, sodium butyrate|IFNγ, interferon gamma|IRF1, interferon regulatory factor 1.

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