Genes modulated by histone acetylation as new effectors of butyrate activity

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Abstract A wealth of evidence correlates the chemopreventive activity of a fiber-rich diet with the production of butyrate. In order to identify the genes transcriptionally modulated by the molecule, we analyzed the expression profile of butyrate-treated colon cancer cells by means of cDNA expression arrays. Moreover, the effect of trichostatin A, a specific histone deacetylase inhibitor, was studied. A superimposable group of 23 genes out of 588 investigated is modulated by both butyrate and trichostatin A. Among them, a major target was *tob-1*, a gene involved in the control of cell cycle. *tob-1* is also up-regulated by butyrate in a neuroblastoma-derived cell line, and its overexpression in the colon cells caused growth arrest. Our findings represent an extensive analysis of genes modulated by butyrate and identify completely new effectors of its biological activities. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Butyrate; Trichostatin A; Histone hyperacetylation; Histone deacetylase; GATA-2; *tob-1*

1. Introduction

Colon cancer is the third most common tumor in the developed countries and the second leading cause of death from cancer, affecting men and women equally. Epidemiological studies indicate that environmental factors are of overwhelming importance in determining the incidence of this disease: in particular, attention has been focused on diet [1,2].

Among the dietary habits which have been linked to a decreased risk of colon cancers, a number of animal investigations and epidemiological studies suggests that the intake of dietary fiber has a positive, but still debated, role [3–5]. Although different mechanisms have been proposed so far, the protective effect is probably mediated by short-chain fatty acids, and particularly by butyrate (BuA), produced abundantly during colonic microbial fiber fermentation [5].

BuA has been indicated as a homeostatic factor of the normal colonic mucosa [2]. Moreover, the molecule might hamper the development of neoplasias by impairing the growth of transformed cells [2]. As a matter of fact, millimolar concentrations of BuA, comparable with those occurring in large bowel, affect cell phenotype in vitro, causing cell division cycle blockade, differentiation or apoptosis in a number of transformed cell lines [6–8]. In addition, an inverse association between tumor size and luminal concentration of BuA has been observed in chemically induced rat colon cancers [9].

The variety of BuA pharmacological activities prompted the use of the molecule and its analogs in the treatment of human diseases, including cancers [10,11], bowel inflammatory pathologies [12], β-thalassemia [13], sickle cell anemia [14], ornithine transcarbamylase deficiency [15], cystic fibrosis [16] and X-linked adrenoleukodystrophy [17].

Despite the growing interest on BuA clinical use, the molecular bases of its effect are so far poorly defined. The modulation of genome expression, as the consequence of chromatin structure changes, is considered a possible mechanism. Indeed, BuA induces histone hyperacetylation in numerous cell systems [18] and inhibits histone deacetylase (HDAC) in vitro [19]. Whether this modification represents the main mechanism of action of BuA on gene transcription is still an open question, since other BuA effects (i.e. modulation of histone phosphorylation and DNA methylation) have been reported [20,21]. However, it must be underlined that the BuA-induced transcriptional activation of some gene promoters was counteracted by HDAC1 overexpression [22].

The importance of HDAC enzymes in controlling genome expression has been widely demonstrated. Eight distinct deacetylases, belonging to two families, have been characterized so far; they remove the acetyl moieties from the ε-acetamido groups of lysine residues of H2A, H2B, H3 and H4 histones at specific chromosomal regions, causing chromatin remodelling and modulation of genome transcription [23]. Moreover, HDACs have been shown to participate in the transcriptional machinery as part of the corepressor complexes which directly hamper transcription of multiple cellular genes [23].

Although the genome effects are relevant in the mechanism of BuA action, no systematic investigation on the genes directly regulated by BuA has been so far reported. Moreover, it has not been clearly established whether the effect of BuA on gene transcription can be ascribed only to histone hyperacetylation or/and to other mechanisms. Very recently, a paper describing the effect of BuA on gene transcription has been published [24]. However, the authors did not distinguish between primary (i.e. due to direct gene modulation) and indi-

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Abbreviations: BuA, butyrate; TSA, trichostatin A; HDAC, histone deacetylase

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rect (i.e. resulting from the synthesis of new proteins) BuA effects.

Thereby, we characterized the expression profile of a colon carcinoma-derived cell line, HT-29, treated, in the presence of cycloheximide, with BuA at a concentration similar to that occurring in the large bowel lumen. The methodology employed is based on cDNA arrays which allow the estimation of mRNA levels of 588 genes simultaneously. The gene repertoire modulated by BuA was compared with that of HT-29 cells treated with trichostatin A (TSA), a selective HDAC inhibitor [23].

2. Materials and methods

2.1. Materials

Anti-GATA-2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The ATLAS cDNA expression array kit, catalog number 7740-1 was furnished by Clontech (Palo Alto, CA, USA). The array filters contain 588 spotted cDNAs specific for genes involved into cell division cycle, signal transduction, stress response, apoptosis, DNA synthesis and repair, and transcriptional regulation. StrataScript RT-PCR kit was purchased from Stratagene (La Jolla, CA, USA) [α-^{32}P]dATP (3000 Ci/mmol) was obtained from Amersham, Bucks, UK.

2.2. Cell lines and treatments

HT-29 cell line was obtained from American Type Culture Collection and grown as reported in [25]. Treatments were performed on cells at 50% confluence, and the culture media were changed 24 h before each experiment. HT-29 cells were incubated with various concentrations of sodium BuA or TSA, as indicated in the text. Differentiation of HT-29 cells was evaluated by alkaline phosphatase assays, performed as in [26]. For the cDNA array analysis, the cells were treated concomitantly with the protein synthesis inhibitor cycloheximide (36 μM). The neuroblastoma cell line, ACN, was kindly given by Dr. Mirko Ponzoni, Giannina Gaslini Children's Hospital, Advanced Biotechnology Center, Genova, Italy. The cells were grown in RPMI 1640 containing 10% fetal calf serum, 10% non-essential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin. ACN were treated with BuA as reported in Figs. 2C and 3C.

2.3. cDNA expression array

Total RNA was prepared using the Atlas pure total RNA Isolation kit (Clontech) following manufacturer's instructions. For analyzing gene expression in HT-29 cells, the ATLAS cDNA expression array (cat. # 7740-1) was employed as described in the user's manual. Briefly, 5 μg of total RNA were converted into ^{32}P-labelled first strand cDNA by means of MMLV reverse transcriptase. Unincorporated ^{32}P-labelled nucleotides were removed by chromatography on CHROMA SPIN-200 (Clontech). cDNA fractions of highest activity were pooled for hybridization to each ATLAS membrane. Equal amounts of cpm (about $4–5\times10^6$ cpm) were employed in each pair of experiments (control and treated cells). After prehybridization, hybridization with probes and washing, the membranes were sealed in sample bags and exposed to X-ray film from 1 to 21 days. Several expositions were taken to select conditions of the highest signals/background ratio. Primary array data were acquired by a laser scanner and the relative intensity of signals was then determined by the NIH Image 1.61 software. Only signals which differed from the control at least 2-fold in all the three independent experiments were taken into consideration. The data in Table 1 were expressed as the mean of three experiments.

2.4. Northern blotting and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA for Northern blot and RT-PCR analyses was prepared as described above. Northern blot experiments were performed as described in [27] by loading 20 μg RNA in each lane. ^{32}P-radiolabelled probes (for *tob-1* and *GAPDH* genes) were prepared from the respective cDNA sequences purified from PCR reactions.

Semiquantitative RT-PCR analyses were carried out as described in [27]. Primer sequences used for *tob-1* and *GATA-2* amplification were obtained from Clontech, while the primers used for glyceraldehyde 3-phosphate dehydrogenase were as in 27. Each experiment was performed at least in triplicate and, in several cases, in quadruplicate.

2.5. Immunoblotting and transfection experiments

Immunoblotting analyses were performed as reported in [25]. Transient transfections were accomplished by using FuGENE 6, following manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, USA). HT-29 cells seeded in a 6-well plate were transfected with 1 μg of pMe-hTob [28] or with the empty vector. After 36 h (about 50% confluence), cells were labelled for 12 h with 2 μCi/well of

Table 1
Human genes induced by BuA and TSA

GenBank accession number	Description	Fold induction		Function
		BuA	TSA	
M68891	GATA-2	13 ± 2	16 ± 2	transcription factor
D38305	TOB-1	10 ± 1	12 ± 2	cell cycle regulator
D10924	HM89	10 ± 2	10 ± 1	chemokine receptor
D10495	PKCδ	10 ± 2	9 ± 1	transduction modulator
X75208	EPH3	8 ± 1	7 ± 1	transduction modulator
U09579	p21^{Cip1}	4 ± 1	5 ± 1	cell cycle regulator
U05340	p55 Cdc	4 ± 1	4 ± 1	cell cycle regulator
X74979	TRKE	3 ± 0.5	4 ± 1	transduction modulator
M11717	HSP70	3 ± 0.8	4 ± 1	stress response
S90469	Cytochrome P450 reductase	3 ± 0.3	3 ± 0.5	detoxication
X67951	PAG	3 ± 0.6	3 ± 0.4	stress response
L25080	RHOA	3 ± 0.7	3 ± 0.2	transduction modulator
L13740	NAK1	3 ± 0.5	3 ± 0.6	transduction modulator
L33264	PISSLRE	3 ± 0.6	3 ± 0.5	cell cycle regulator
U02081	NET1	3 ± 0.5	3 ± 0.4	transduction modulator
M60974	GADD45	3 ± 0.4	3 ± 0.3	cell cycle regulator
J03132	ICAM-1	2 ± 0.2	2 ± 0.2	adhesion molecule
M36717	Placental ribonuclease inhibitor	2 ± 0.2	2 ± 0.2	
X79389	GST-theta 1	2 ± 0.1	2 ± 0.2	detoxication
X54079	HSP27	2 ± 0.2	2 ± 0.1	stress response
U24166	EB1	2 ± 0.1	2 ± 0.2	transduction response

HT-29 cells were treated for 5 h with or without 2 mM sodium BuA or 0.3 μM TSA, in the presence of 36 μM cycloheximide. Total RNA was isolated, retrotranscribed, ^{32}P-labelled and hybridized to the cDNA array representing 588 transcripts for known genes. The signals were then analyzed by NIH Image 1.61 software and expressed as fold of increase with respect to the untreated cells. Data are means of three different experiments.

[^{3}H]thymidine for evaluating the growth rate. Moreover, cell extracts were prepared for analyzing Tob-1 content by immunoblotting technique.

3. Results and discussion

BuA has been reported to inhibit the growth and induce differentiation and/or apoptosis of the colon carcinoma-derived cell line HT-29 [29]. These findings were also confirmed in our preliminary experiments: cell growth was impaired at 0.5 mM concentration, with 50% inhibition at 2 mM BuA after 4 days. Moreover, after 3 days of 2 mM treatment, a 20-fold increase of alkaline phosphatase activity was observable (data not shown). In brief, BuA influences two pivotal phenotypical features, growth and differentiation, of HT-29 cells.

In order to identify genes regulated by BuA, we screened its activity on gene expression by means of cDNA expression arrays. Furthermore, we compared the findings obtained with the effects of a highly specific HDAC inhibitor, TSA, which is structurally unrelated to BuA.

HT-29 cells were treated with 2 mM BuA or with 0.3 μM TSA, the most efficacious and non-toxic concentrations of the molecules [22]. An incubation period of 5 h was selected, since TSA loses its effects on histone acetylation at longer exposition times [29]. In addition, all the experiments were performed in the presence of cycloheximide to identify only direct transcriptional effects and rule out the involvement of de novo synthesized proteins. Examples of the primary array data, regarding some genes of interest (see below), are reported in Fig. 1.

Table 1 shows a summary of the expression profiles upon addition of BuA or TSA to growing HT-29 cells; the fold-induction values are a mean of three independent experiments. Among the genes identified by the arrays, only two, namely *p21^{Cip1}* [22] and *hsp70* [30,31], have been previously found as regulated by BuA. A first conclusion which might be drawn from the profiles obtained is that both BuA and TSA modulate an identical and superimposable set of genes, thus underscoring the importance of HDAC inhibition in the BuA effect on genome transcription.

Only two genes were down-regulated by BuA and TSA, *lactoferrin δ* gene (2-fold) and *MAPKAP kinase* gene (3-fold), while 21 genes were up-regulated. It is noteworthy the large incidence of gene activation compared to down-regulation, which confirms that HDACs function mainly as general transcriptional corepressors.

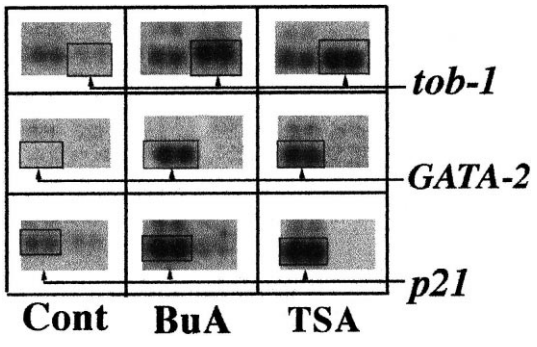

Fig. 1. Effect of HDACs inhibitors on *GATA-2*, *tob-1* and *p21* expression by cDNA array. HT-29 cells were cultured with 2 mM sodium BuA or 0.3 μM TSA for 5 h. Untreated cells are indicated as Cont. The arrows indicate the signal doublet corresponding to the relative gene.

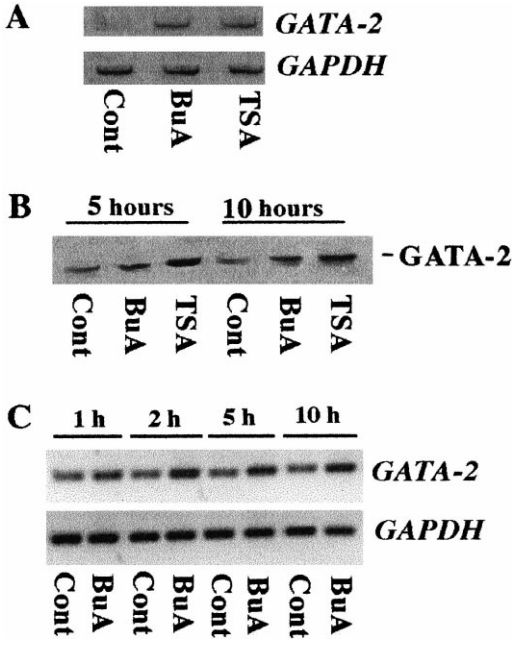

Fig. 2. Effect of HDACs inhibitors on GATA-2 mRNA and protein levels. A, B: HT-29 cells were cultured as in Fig. 1. Untreated cells are indicated as Cont. In the experiments showed in A, cycloheximide (36 μM) was also added to the culture medium. Then, total RNA was prepared and analyzed by RT-PCR. The detection of the amplified product was carried out by silver staining (A). In the experiments showed in B protein cell extracts were analyzed by immunoblotting. C: ACN cells were grown with or without 1 mM BuA. At the indicated time intervals, cells were harvested and total RNA was prepared. The amplified products were separated on 1% agarose gel and the image was acquired by a Fluor-S MultiImager (Bio-Rad, Richmond, CA, USA).

As reported in Table 1, several of the genes overexpressed (*tob-1*, *GATA-2*, *GADD45*, *PKCδ*) control important physiological processes, i.e. cell cycle regulation, signal transduction, DNA repair and genome transcription. Since these genes have not been previously related to BuA or to HDACs inhibition, our findings furnish a novel evidence of their regulation via histone acetylation and provide potential insights into new cellular control mechanisms.

Although our profiling experiments point to a large number of genes as effectors of BuA and TSA activities, we selected the two genes mostly induced, namely *GATA-2* and *tob-1*, for further investigation. In successive studies we will address the role of the other genes up-regulated by histone hyperacetylation.

GATA-2 is a nuclear transcription factor belonging to a family of at least seven members. Although they first emerged as candidate regulators of gene expression in hematopoietic cells [32], GATA proteins were then demonstrated to be involved in a large number of tissutal differentiation processes. In particular, GATA-2 is required for the expansion of erythroid and megakaryocyte precursors [33], the development of placental throphoblasts [34] and urogenital system [35], and in pituitary cell type determination [36].

The effect of BuA and TSA on the expression of *GATA-2* gene in HT-29 cells was investigated by means of semiquantitative RT-PCR and Western blotting (Fig. 2A,B). The results

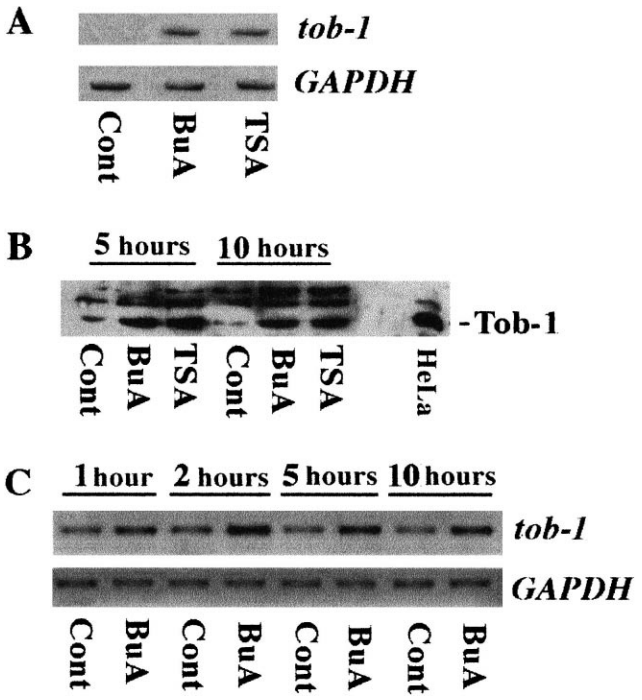

Fig. 3. Effect of HDACs inhibitors on Tob-1 mRNA and protein levels. A, B: The experiments were as in Fig. 2A,B. In B, HeLa cells were employed as positive control for Tob-1 protein. C: ACN cells were grown as in Fig. 2C. RT-PCR was performed as described under Section 2 and in Fig. 2C.

confirmed the data obtained by the cDNA array, demonstrating that *GATA-2* gene is not only up-transcribed, but also efficaciously translated to the respective protein. Moreover, we evaluated the activity of BuA on *GATA-2* expression in a different cell line, namely the neuroblastoma-derived ACN cells. As shown in Fig. 2C, the fatty acid induces the expression of the transcription factor also in these cells. This finding further strengthens the data obtained on BuA-dependent *GATA-2* up-regulation.

Given the role of *GATA-2*, its identification as BuA downstream target gene might help in explaining the differentiative effects of the fatty acid. This could be even more important in view of the chromosome relaxation which follows BuA treatment. Indeed, BuA could (i) increase the levels of GATA-2 protein and (ii) facilitate the transcriptional activity of GATA-2-containing complexes.

The other gene analyzed is *tob-1*, a member of the recently identified *BTG* family whose founder gene, *BTG1* (B-cell translocation gene 1), was firstly cloned near the breakpoint of a chromosomal translocation associated to a chronic lymphocytic leukemia [37]. A wealth of evidence suggests that Btg proteins may be mediators of multiple anti-proliferative activities [28,37–40]. Particularly, Tob-1 is involved in the arrest of cell cycle at G1/S boundary [28,39] and its expression varies during vertebrate development [40]. It was initially identified as a protein specifically interacting with ErbB-2 growth factor receptor, and thus it might negatively regulate the proliferative activity of transmembrane protein kinases [28]. Successive reports showed that Tob-1 also interacts with Caf1 protein, a component of CCR4 (CCR, carbon catabolite repressor) transcriptional complex [39]. Since Caf1 mutations, like those of CCR4, affect cell division cycle, DNA repair and UV-sensitivity [38,39], it is highly probable that Tob-1 protein, via interaction with this transcriptional complex, can regulate key cell functions. Finally, very recently the direct involvement of *tob-1* gene in the control of osteoblastic proliferation and differentiation has been demonstrated by two different approaches. Indeed, mice carrying a targeted deletion of the tob gene have a greater bone mass resulting from increased numbers of osteoblasts [41]. Moreover, a gene array analysis allows the observation of tob-1 up-regulation during osteoblast differentiation [42].

In the light of the described biological activities of Tob-1, its up-regulation by BuA treatment appears particularly intriguing, since it may account for the anti-proliferative BuA effects. So far, the BuA-dependent growth impairment has been mainly ascribed to the increased expression of $p21^{Cip1}$, a cyclin-dependent kinase inhibitor [22]. However, this mechanism is strongly challenged by the observation that the growth of primary fibroblasts from $p21^{-/-}$ mice is still inhibited by BuA, through a mechanism which also requires the maintenance of pRb in a hypophosphorylated status [43]. In this scenario, Tob-1 protein could represent a critical effector of BuA, acting synergistic with $p21^{Cip1}$. This is strengthened by data demonstrating that Tob-1, via interaction with Caf1, interferes with cyclin-dependent kinases activity causing pRb hypophosphorylation [39].

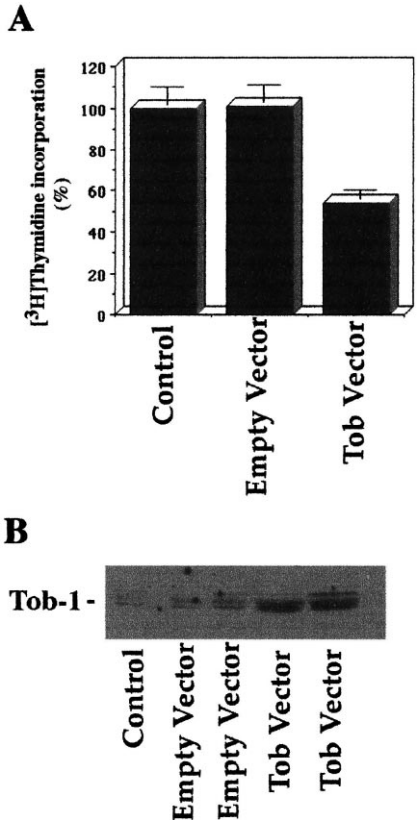

Fig. 4. Effect of transfection of Tob-1 plasmid on cell proliferation of HT-29 cells. A: HT-29 cells were transfected with 1 μg of empty vector or Tob-1 vector. The proliferation was expressed as percentage of [^{3}H]thymidine incorporated with respect of control as described in Section 2. The data shown represent the mean ± S.E.M. of three independent experiments, each performed in duplicate. B: The Tob-1 content of HT-29 cells transfected with Tob-1 vector was determined by immunoblotting.

In order to evaluate this hypothesis, we first verified the cDNA array findings on *tob-1* gene by means of Northern blot (not reported), RT-PCR and Western blot. As shown in Fig. 3A,B, BuA and TSA strongly induce the transcription and translation of *tob-1* in HT-29 cells. Moreover, the fatty acid up-regulates *tob-1* expression in ACN cells as soon as 1 h after the addition of the molecule (Fig. 3C). Afterwards, we investigated whether Tob-1 overexpression caused growth impairment in HT-29 cells. We transiently transfected *tob-1*-encoding cDNA in the colon cell line and evaluated the effect on the proliferation rate by estimating the incorporation of [^{3}H]thymidine into the DNA. A remarkable reduction of the proliferation rate was evident after transfection (Fig. 4A), which correlated with an increase of Tob-1 protein (Fig. 4B). Therefore, Tob-1 up-regulation might contribute to explain the BuA anti-proliferative effect.

In conclusion, this paper reports a study aimed at evaluating the effect of BuA on gene expression by a cDNA array. Since BuA induces histone hyperacetylation, our findings also allow us to identify genes regulated by an increased degree of histone acetylation and nucleosome relaxation. The use of TSA further strengthens our conclusions.

Several of the genes whose expression appears regulated by HDACs activity control important cellular pathways (like *GADD45* and *PKCδ*). These initial observations need to be confirmed and extended by future investigations. Furthermore, we demonstrated by the array technique and by additional approaches that two genes, i.e. *tob-1* and *GATA-2*, are strongly up-regulated by the fatty acid. Neither of them had been previously taken into consideration to explain the action of BuA. However, since they both induce growth impairment and/or differentiation, they can account for the complex phenotypical effects of the fatty acid.

Several reports suggest that BuA and its structural analogs might exert important therapeutical effects, acting both as non-toxic cytostatic drugs and as gene regulators in the treatment of cancers [9,10], hemoglobinopathies [12,13], cystic fibrosis [16] and other diseases. In this context, our findings not only contribute to the elucidation of the molecular effects of BuA (and its analogs), but also might help in the identification of important cellular targets for the development of new pharmacological strategies.

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