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Effects of Histone Deacetylase Inhibitors on p55*CDC*/Cdc20 Expression in HT29 Cell Line

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Abstract In a previous work, taking advantage of the gene-array screening technology, we analysed the effects of histone deacetylase (HDAC) inhibitor sodium butyrate (NaBt), on gene transcription in HT29 human adenocarcinoma cell line. In this study, we focused our attention on p55*CDC*/Cdc20 gene, whose expression was dramatically reduced by NaBt treatment. Mammalian p55*CDC*/Cdc20 interacts with the anaphase promoting complex/cyclosome (APC/C), and is involved in regulating anaphase onset and late mitotic events. Using NaBt and trichostatin A (TSA), a member of the HDAC inhibitor family, we showed that both HDAC inhibitors totally downregulated p55*CDC*/Cdc20 transcription and expression. Cell cycle analysis demonstrated that NaBt arrested HT29 cells in G0/G1 phase, while TSA caused a double block in G0/G1 and G2/M phases. Moreover, p55*CDC*/Cdc20 showed maximal expression in S and G2/M phases of HT29 cell division cycle. Based on this evidence, and by means of specific cell cycle modulators, such as nocodazole and hydroxyurea, we demonstrated that both TSA and NaBt were responsible for loss of p55*CDC*/Cdc20 expression, but with different mechanisms of action. Taken together, these results suggest that targeting molecules involved in spindle mitotic checkpoint, such as p55*CDC*/Cdc20, might account for the high cytotoxicity of HDAC inhibitors versus malignant cells. J. Cell. Biochem. 99: 1122–1131, 2006.

Key words: sodium butyrate; trichostatin A; histone deacetylase inhibitors; p55*CDC*/Cdc20; HT29 cell line

Modulation of gene expression is mediated by various mechanisms, such as transcription factors, DNA methylation, ATP-dependent chromatin remodelling and post-translational modifications of histones, which include the dynamic acetylation and deacetylation of ε-amino groups of lysine residues present in core histones [Legube and Trouche, 2003; Sengupta and Seto, 2004]. The enzymes responsible for reversible acetylation/deacetylation processes are the histone acetyltransferases (HATs) and the histone deacetylases (HDACs), respectively [Legube and Trouche, 2003; Sengupta and Seto, 2004]. HATs act as transcriptional co-activators, and HDACs are part of transcriptional co-repressor complexes. Inhibitors of HDAC induce hyperacetylation of histones, modulating chromatin structure and gene expression [Monneret, 2005]. These inhibitors also cause growth arrest, cell differentiation and apoptosis of tumour cells in vivo [Archer and Hodin, 1999; Mei et al., 2004]. Thus, HDAC inhibitors have been thought as a new class of potential drugs in cancer therapy [Villar-Garea and Esteller, 2004; Drummond et al., 2005].

Sodium butyrate (NaBt), a short chain fatty acid, has been the mostly studied HDAC inhibitor, since the mid-1970s [Riggs et al., 1977; Davie, 2003]. Treatment of cultured cells with NaBt produces reversible hyperacetylation of histones as a consequence of HDAC inhibition [Davie, 2003]. This modification is important in the modulation of chromatin structure and transcriptional activity. In fact, NaBt effects on gene expression, cell growth regulation and differentiation have been described in a large number of cell lines (reviewed in [Russo et al., 1999a]). In vivo, NaBt is generated by colonic fermentation of dietary fibre and starch [Miller, 2004]. The molecule is quickly taken up by the colonic epithelium and used as the main energy source via β-oxidation [Hague et al., 1997]. The intestinal production of NaBt is considered chemopreventive against colon

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cancer, supporting the view of a protective effect of a fibre-rich diet [Howe et al., 1992; Wollowski et al., 2001]. In fact, several studies on animal models confirmed the effects of NaBt on cell cultures, supporting the beneficial properties of the molecule in preventing carcinogenesis [McIntyre et al., 1993; D'Argenio et al., 1996]. Based on these evidence, pre-clinical studies on human subjects have been reported [Bradburn et al., 1993; Vernia et al., 2003], and several clinical trials on the use of NaBt, or its analogs, as anti-cancer drug have been approved [Patnaik et al., 2002; Chen et al., 2003]. However, reports suggested caution in interpreting the epidemiological data on the protective effects of dietary fibre against cancer [Alberts et al., 2000; Schatzkin et al., 2000]. Consequently, the potential chemopreventive and therapeutic role of NaBt is actually questioned [Schatzkin et al., 2000; Lupton, 2004].

Recently, taking advantage of the gene-array screening technology, we and others analysed the effects of NaBt on mRNA transcription in human cell lines [Mariadason et al., 2000; Iacomino et al., 2001; Williams et al., 2003]. In our screening, performed on HT29 cell line, genes linked to apoptosis, oxidative metabolism and cell growth appeared the most significantly affected by NaBt [Iacomino et al., 2001]. Among these, we focused our attention on p55*CDC*, a human protein that shows high homology to the cell cycle proteins Cdc20p of budding yeast *Saccharomyces cerevisiae* and to the product of the *Drosophila fizzy* (fzy) gene, both of which contain WD repeats required for the metaphase–anaphase transition [Weinstein, 1997; Kallio et al., 1998; Lin et al., 2003]. In addition, mammalian p55*CDC*/Cdc20 mediates association of the spindle checkpoint protein Mad2 with the anaphase promoting complex/cyclosome (APC/C), and is involved in regulating anaphase onset and late mitotic events [Kallio et al., 1998]. Furthermore, the involvement of p55*CDC*/Cdc20 in cell cycle regulation is supported by its physical interaction with the cyclin A/Cdk complex [Ohtoshi et al., 2000]. Additional roles of mammalian p55*CDC*/Cdc20 have been described in controlling cell proliferation, differentiation and apoptosis [Lin et al., 2003].

Here, we studied in details the ability of NaBt and a different HDAC inhibitor, namely trichostatin A (TSA), to modulate p55*CDC*/Cdc20 gene expression in HT29 cell lines. TSA is a fungistatic antibiotic purified from *Streptomyces platensi*s able to induce accumulation hyperacetylated histones by strongly inhibiting the activity of HDAC [Yoshida et al., 1990; Vanhaecke et al., 2004]. Numerous and well documented are the effects of TSA on cell differentiation, cell cycle regulation and apoptosis [Wu et al., 2001; Komata et al., 2005]. However, the mode of action of TSA is specific and different respect to other HDACs, including NaBt, as clarified by crystal structure studies [Finnin et al., 1999; Davie, 2003]. Therefore, TSA appears to be useful in analysing the role of NaBt in mediating cellular proliferation and differentiation.

MATERIALS AND METHODS

Cell Culture and Reagents

HT29 human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin at 37°C in a 5% CO_2 incubator. All cell culture reagents were from Invitrogen (Milan, Italy). TSA, NaBt, nocodazole, hydroxyurea and propide iodide were from Sigma Chemical Co. (Milan, Italy). Cells were seeded at a density of 5×10^5/ml and allowed to grow for 1 day before exposure to 4 mM NaBt or 0.5 μM TSA. Medium with or without HDAC inhibitors was replaced every 24 h. Cell growth was monitored by crystal violet dye assay [Russo et al., 1999b], and cell viability was assessed by trypan blue dye exclusion test. HT29 cells were synchronized in G0 phase by 24 h serum starvation.

Immunoblotting Analysis

Cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.4; 500 mM NaCl; 1% Nonidet P-40; 10 mM EDTA; 10% glycerol; 0.5 mM dithiothreitol) containing protease inhibitors (100 mg/L phenylmethylsulfonyl fluoride; 100 mg/L tosyl-phenyl-hloromethyl ketone; 1 mg/L leupeptin; 0.83 mg/L chymostatin; 10 mg/L soybean trypsin inhibitor; 1 mg/L pepstatin). Total protein (25–30 μg) were loaded on 12% SDS polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) that were subsequently incubated with the following commercially available antibodies: anti-Cdc20, -cyclin A, B1,

D1, E, Cdk6 (Santa Cruz Biotechnology, Heidelberg, Germany), anti-α-tubulin (Sigma). Immunoreactivity was visualised by chemoluminescence reagents according to manufacturer's instructions (ECL plus; Amersham Biosciences, Cologno Monzese, MI, Italy).

Cell Cycle Analysis

HT29 cells were trypsinized and fixed in 70% cold ethanol for 2 h at −20°C. Cells were washed, resuspended in phosphate buffer saline solution containing 200 μg/ml RNAse A and stained with 25 μg/ml propide iodide for 2 h at room temperature. DNA content was analysed by flow cytometry (Facscalibur; BD Biosciences, Sparks, MD). ModFit LT software (Verity Software House, Inc., Topsham, ME) was used to generate DNA content histograms.

Nocodazole and hydroxyurea synchronization was performed essentially as described [Russo et al., 1992]. Briefly, HT29 cells were incubated in the presence of 1 mM hydroxyurea or 10 μg/ml nocodazole for 18 h. Subsequently, cells were harvested and analysed by flow cytometry and immunoblotting.

RT-PCR Analysis

Total RNA was obtained by guanidinium/phenol:chloroform procedure using RNA-Fast reagent (Molecular Systems, San Diego, CA) following manufacturer's instructions. Reverse transcriptions were performed on 2 μg total RNA in a reaction volume of 20 μl containing 4 U of Omniscript-RT (Qiagen, Milan, Italy), 2 μl of 10× RT buffer (Qiagen), 1 μM Oligo-dT primer (Invitrogen), 2 mM dNTP mix (Amersham Biosciences), 4 U RNasin ribonuclease inhibitor (Promega, Milan, Italy). Reactions were incubated for 60 min at 37°C before volume adjustment to 200 μl with TE buffer. Semiquantitative PCR amplifications were performed with 1, 2 and 4 μl (1:2 dilution) of the cDNA solution supplemented with 2 mM $MgCl_2$; 1 mM dNTP; 20 pmol of each PCR-specific primer (MWG-Biotech AG, Ebersberg, Germany); 1.25 U recombinant Taq DNA polymerase (Invitrogen) in PCR buffer to a final volume of 50 μl. The following primers were used for PCR: p55*CDC*/Cdc20 (sense 5′-GG CAC CAGTGATCGACACATTCGCAT-3′; antisense 5′-GCCATAGCCTCAGGGTCTCATCTG CT-3′); *GAPDH* (sense 5′-GGCTCTCCAGAA-CATCATCCCTGC-3′; anti-sense 5′-GGGTGTC GCTGTTGAAGTCAGAGG-3′). PCR reactions included an initial cycle of denaturation at 94°C for 2 min, followed by 21–23 cycles of denaturation at 92°C for 45 s, annealing at 60°C for 60 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min. PCR reactions were carried out in a PTC-100 thermocycler (M.J. Research, Inc., Waltham, MA). The amplified products were separated by electrophoresis on a 1.2% agarose gel. The DNA bands were visualised by Vistra Green staining (Amersham Biosciences) and the images were digitised on the ImageDoc 2000 Instrument (Bio-Rad Laboratories, Segrate, MI, Italy). Gene expression was normalised to *GAPDH* transcript. Control experiments confirmed that RT-PCR assays were entirely dependent on the RT reaction, and the reaction products accumulated linearly with respect to cDNA amount and PCR cycle numbers.

RESULTS AND DISCUSSION

In previous works, we showed that NaBt was able to inhibit cell growth of HT29 cell line without any significant cytotoxic effect [Russo et al., 1997; Iacomino et al., 2001], confirming the ability of the molecule to cause cell cycle arrest in G0/G1 phase of the cell cycle (reviewed in [Russo et al., 1999a; Davie, 2003]). Figure 1 shows that TSA was able to inhibit HT29 cell proliferation in a dose-dependent manner.

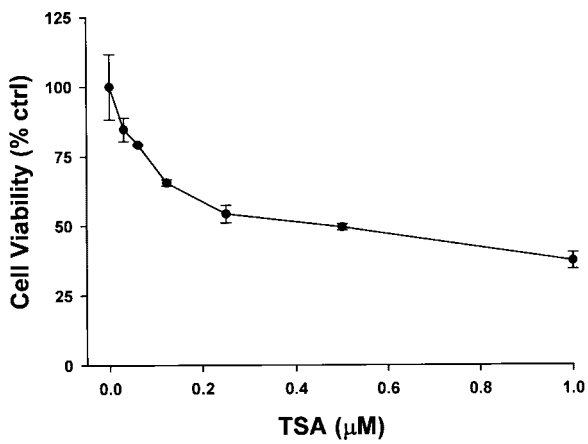


Fig. 1. Effect of TSA on HT29 cells viability. HT29 cells were cultured in the presence of the indicate concentrations of TSA for 24 h. Cell viability was assayed by crystal violet dye. Control (Ctrl in ordinate) was represented by cells added with 0.1% dimethylsulphoxide (DMSO; vehicle), the same concentration of solvent present in the experimental points considered (0–1 μM TSA). DMSO, at 0.1% final concentration, was not cytotoxic. Percentage in ordinate has been calculated respect to DMSO-treated cells. Each value corresponds to the average of three different experiments ± SD.

However, at 0.5 μM concentration, TSA arrested HT29 cell growth without affecting cell viability; in fact, trypan blue exclusion test and microscopic analyses of cell morphology indicated that 0.5 μM TSA was not cytotoxic (data not reported). Therefore, this concentration was employed in the next experiments. We attributed the effect of TSA to its ability to interfere with the cell cycle machinery. In fact, cytofluorimetric analysis showed that HT29 cells treated with TSA accumulated in both G0/G1 and G2/M phases of the cell division cycle (Fig. 2). The interpretation of TSA effects on cell cycle block is partially controversial. There are authors describing a TSA-depend G2/M arrest in different cell lines [Li et al., 2003; Noh and Lee, 2003], while others suggest an effect of TSA on both G0/G1 and G2/M phases of HT29 cell cycle [Siavoshian et al., 2000]. Our data support this latter view. In fact, in an asynchronous population of HT29 cells treated with TSA, the absence of an S phase demonstrates that cells that already passed G1/S restriction point arrest at G2/M. This block prevents an enrichment of cell population in G1. On the opposite, cells that did not reach G1/S transition remained arrested in G0/G1. The logical consequence of this hypothesis is that cell number in G1 phase remains constant, while cells in G2/M increase, as clearly reported in Figure 2 and by others [Siavoshian et al., 2000]. In conclusion, even if NaBt and TSA belong to the class of HDAC inhibitors, they affect cell cycle machinery with different mechanisms: the former arrest cells in G0/G1 phase, the latter causes a double block in G0/G1 and G2/M phases. We ruled out the possibility that an increase of NaBt concentration would have mimic TSA effects on cell division cycle. In fact, at values higher than 4 mM, NaBt resulted cytotoxic on HT29 cell line (data not shown).

To better characterize the effects of NaBt and TSA on HT29 cell division cycle, we measured the expression of several cell cycle related proteins, such as cyclins A, B1, D1, E and Cdk6. Immunoblotting in Figure 3 reports a clear downregulation of cyclins A, B1 and D1, but not cyclin E, after 48 h treatment of HT29 cells with both HDAC inhibitors. We expected to observe an increased expression of cyclin B after TSA treatment, because of the G2/M arrest shown in Figure 2, but this was not the case. Probably, other mechanisms, different than cyclin B accumulation, should be evoked to explain the G2/M block caused by TSA. On the opposite, both HDACs increased cyclin E expression (Fig. 3); in fact, according to other works [Boutillier et al., 2003; Florenes et al., 2004], TSA treatment led to cyclin E accumulation, probably through inhibition of HDAC activity that blocks Rb-mediated repression of E2F-regulated promoter [Brehm et al., 1998]. Moreover, a slight downregulation of Cdk6 was detected in Figure 3, while Cdk2 and Cdk4 did

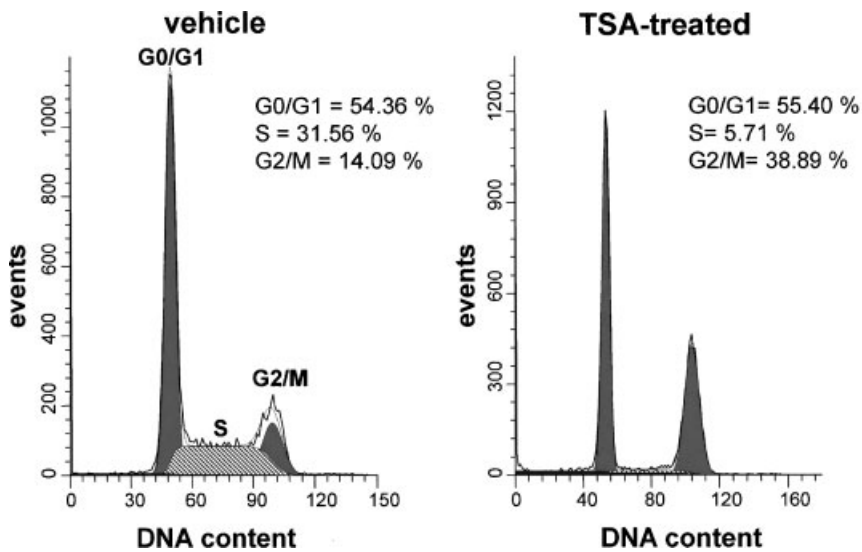


Fig. 2. Effect of TSA on DNA distribution evaluated by cytofluorymetric analysis on HT29 cells. HT29 cells were added with 0.5 μM TSA in 0.1% DMSO (vehicle) for 48 h, fixed and stained with propide iodide. The distribution of the different cell cycle phases (%) for each treatment is indicated within each panel.

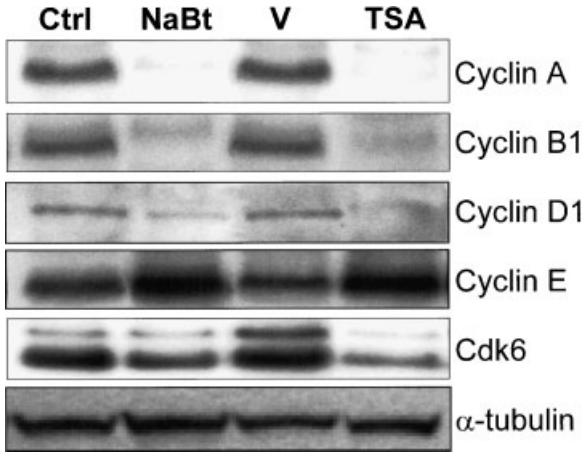


Fig. 3. Expression of the indicated cell cycle regulated proteins after treatment of HT29 cells for 48 h with 4 mM NaBt or 0.5 μM TSA. Immunoblots were performed using commercially available antibodies (Santa Cruz Biotechnology). α-Tubulin expression was employed to assure equal loadings of the samples. In the experiment with NaBt, Ctrl (control) indicates untreated cells, while in the experiment with TSA, V (vehicle) indicates cells treated with 0.1% DMSO. In all panels, the images presented are representative of a single experiment out of three performed.

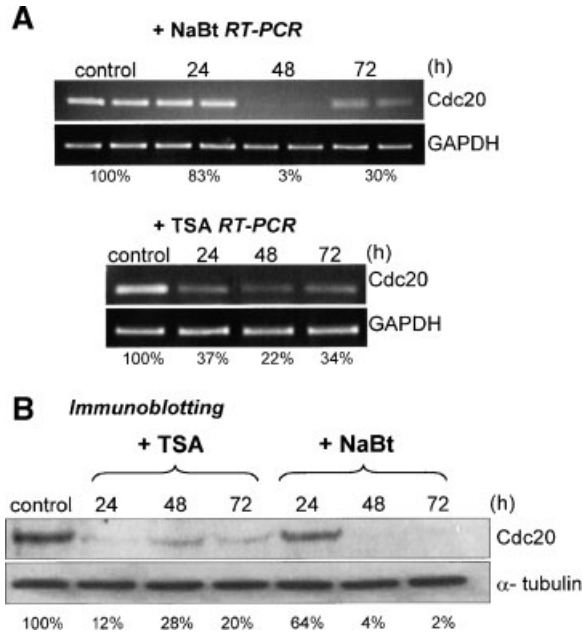


Fig. 4. p55*CDC*/Cdc20 transcription and expression levels by NaBt and TSA. HT29 cells were exposed to medium alone (untreated), 4 mM NaBt or 0.5 μM TSA, as described in Materials and Methods. 'Control' in **panels A** and **B** indicates control cells added with vehicle (0.1% DMSO). **A**: Kinetic analysis of the effect of NaBt and TSA on p55*CDC*/Cdc20 mRNA transcripts. Total RNAs were extracted at the indicated times and RT-PCR products analysed on 1.2% agarose gel. GAPDH levels were used to normalize samples (densitometric analyses are reported as % on the bottom of panels in A). **B**: Effect of NaBt and TSA on p55*CDC*/Cdc20 protein expression. Immunoblots were performed using an anti-p55*CDC*/Cdc20 antibody commercially available (Santa Cruz Biotechnology) at the indicated times using 30 μg of total cell extracts. α-Tubulin expression was employed to assure equal loadings of the samples. Densitometric analyses were reported as % on the bottom of panel B. In both panels, the images presented are representative of a single experiment out of three performed.

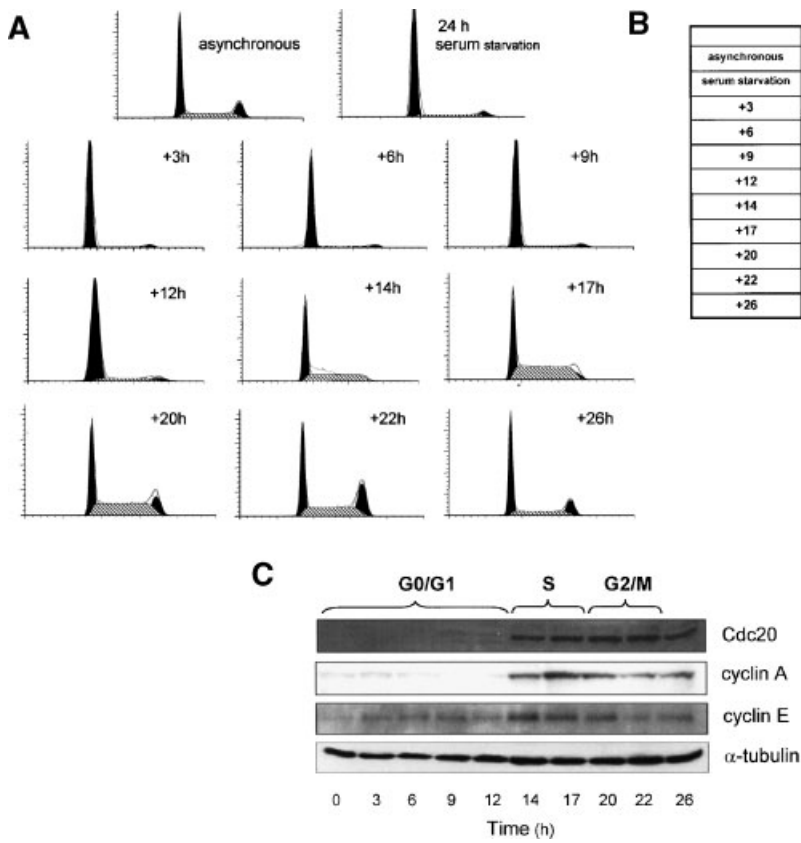
not change (data not shown), according to a previous report [Siavoshian et al., 2000]. Finally, we confirmed on HT29 cells that both TSA and NaBt stimulate p21WAF1 expression (data not shown), as described by others employing the same cell line [Siavoshian et al., 2000].

A recent study showed that the cell cycle checkpoint activity of p55*CDC*/Cdc20 depends upon its interaction with HDAC inhibitors [Yoon et al., 2004]. Since the expression of p55*CDC*/Cdc20 is dramatically modulated by NaBt in HT29 cells [Iacomino et al., 2001], we reasoned that the different mode of action of NaBt and TSA might help to understand the effects of HDACs on p55*CDC*/Cdc20-mediated cell cycle regulation. As reported in Figure 4, a semi-quantitative RT-PCR analysis (Fig. 4A) and the relative immunoblotting (Fig. 4B) indicated a dramatic decrease in p55*CDC*/Cdc20 mRNA level at 48 h, following treatment of HT29 cells with NaBt. At 72 h, p55*CDC*/Cdc20 was transcribed at less extent, although immunoblot continued to show absence of protein expression (Fig. 4B, 2% compared to control experiment). TSA behaved similarly; in fact, p55*CDC*/Cdc20 mRNA level decreased respect to control, and was anticipated of 24 h compared to NaBt (Fig. 4A). The resulting immunoblot showed that the inhibition of p55*CDC*/Cdc20 expression by TSA started at 24 h and was maintained for the time of the treatment (Fig. 4B; 12–28% respect to vehicle-treated cells).

Comparing data in Figure 4, the percentage of p55*CDC*/Cdc20 downregulation detected, at different time points, by RT-PCR (Fig. 4A) and immunoblot (Fig. 4B) is consistent with the hypothesis that p55*CDC*/Cdc20 protein level decreases in parallel with the relative mRNA. The only exception is represented by the 72 h time point in the experiment with NaBt, where the appearance of de novo synthesized p55*CDC*/Cdc20 mRNA does not parallel with a corresponding increase in protein expression (compare the last two lanes in the top panel of Figure 4A, with the last two in panel 4B), indicating the presence of other

post-translational regulatory mechanism(s). Based on these data, we can conclude that both NaBt and TSA are able to lower p55*CDC*/Cdc20 expression. The early effect observed for TSA compared to NaBt might be due to a different kinetic of molecule uptake, faster for TSA, or to a different processing.

Previous works demonstrated that p55*CDC*/Cdc20 is expressed in cycling HeLa and Rat1 cells and that the protein is required for normal cell division [Weinstein, 1997]. To identify the stage of HT29 cell cycle where p55*CDC*/Cdc20 was maximally expressed, cells were synchronized in G0/G1 phase by 24 h serum starvation, and subsequently induced to enter the cell cycle by the addition of 10% serum. At each time point reported in Figure 5, cells were harvested and p55*CDC*/Cdc20 expression and DNA content were determined. Immunoblot revealed that p55*CDC*/Cdc20 was barely detectable at 9–12 h after starvation, when cells were in late G1 phase (Fig. 5). Maximal expression of p55*CDC*/Cdc20 was detectable in S (14 h following serum stimulation) and G2/M (18–20 h following serum stimulation) phases of HT29 cell cycle. At metaphase exit (28 h), the amount of p55*CDC*/Cdc20 decreased, according to entry in early G1 phase, and remained low until the next S phase (data not shown). The synchrony of HT29 cells after starvation was also assessed by measuring the level of expression of two S-phase cyclins, namely A and E (Fig. 5C), which were maximal detected by immunoblotting between 14 h and 17 h from serum addition, when cells were in S phase. In conclusion, consistently with previous reports [Weinstein, 1997; Williams et al., 2003], we confirmed that expression of the p55*CDC*/Cdc20 in mammalian cells is



	G0/G1 (%)	S (%)	G2/M (%)
asynchronous	58.41	25.45	16.14
serum starvation	89.63	6.57	3.80
+3	91.07	6.43	2.59
+6	87.29	8.98	3.73
+9	81.28	13.18	5.54
+12	82.13	13.85	4.01
+14	49.44	50.03	0.53
+17	33.86	60.01	6.13
+20	34.37	51.45	14.17
+22	35.33	39.57	25.10
+26	57.35	25.99	16.66

Fig. 5. Expression of p55*CDC*/Cdc20 protein during HT29 cell division cycle. In **panel A**, the flow cytometry analyses of asynchronous HT29 cell line are reported. Cells were synchronized at G0 phase by 24 h serum starvation. At the indicated times (3–26 h), following 10% serum addition, cells were analysed for DNA content and percentage of cells in G0/G1, S and G2/M of the cell cycle was calculated (**panel B**). An aliquot of cells, for each experimental point, was lysed and employed to measure p55*CDC*/Cdc20 expression by immunoblot (**panel C**) as reported in legend of Figure 4. As control experiments, cyclins A and E levels were also assayed by immunoblot as markers of the cell cycle progression, using commercially available antibodies (Santa Cruz Biotechnology). α-Tubulin expression was employed to assure equal loadings of the samples. The image presented is representative of a single experiment out of two performed.

regulated during the cell cycle, peaking at S-G2/M phases.

To summarize data reported above, we made the following observations: (1) NaBt and TSA block HT29 cells in specific phases of the cell division cycle (Fig. 2); (2) p55*CDC*/Cdc20 is maximally expressed between S and G2/M phases of HT29 cell division cycle (Fig. 5); (3) NaBt and TSA are both able to downregulate p55*CDC*/Cdc20 expression (Fig. 4). The obvious explanation for the absence of p55*CDC*/Cdc20 after NaBt treatment is probably related to the effect of NaBt on cell cycle arrest: the molecule arrests cells in G0/G1, where p55*CDC*/Cdc20 is not expressed (Fig. 5). Therefore, the effect of NaBt on p55*CDC*/Cdc20 downregulation might be interpreted as an epiphenomenon following G0/G1 cell cycle arrest. More controversial appears the downregulation of p55*CDC*/Cdc20 by TSA; in fact, we expected to observe an increase in p55*CDC*/Cdc20 expression after TSA treatment (Fig. 4), since TSA arrests HT29 cells also in G2/M phase (Fig. 2), where p55*CDC*/Cdc20 is maximally expressed (Fig. 5B). To solve this apparent contradiction, we formulated the hypothesis that the pleiotropic activity of TSA resulted in both cell cycle arrest (G2/M) and changes in p55*CDC*/Cdc20 gene expression. To test this possibility, we performed a control experiment using two cell cycle blockers: hydroxyurea and nocodazole, able to transiently arrest cell cycle at G1/S and G2/M phases, respectively. Figure 6 shows that both molecules arrested the HT29 cell cycle, as expected (panels A and B), and p55*CDC*/Cdc20 was highly expressed after both treatments (Fig. 6C), according to data presented in Figure 5. These results demonstrate that: (1) downregulation of p55*CDC*/Cdc20 by TSA (Fig. 4) was not due to an epiphenomenon related to the cell cycle arrest caused by TSA; in fact, if this would have been the case, we would have observed an increased p55*CDC*/Cdc20 expression after TSA treatment (Fig. 4), due to its ability to cause a G2/M arrest (Fig. 2). (2) TSA probably acts at different levels by inhibiting p55*CDC*/Cdc20 expression and/or accelerating its degradation. The next question to solve is: who comes first? Are the G2/M block and p55*CDC*/Cdc20 downregulation two

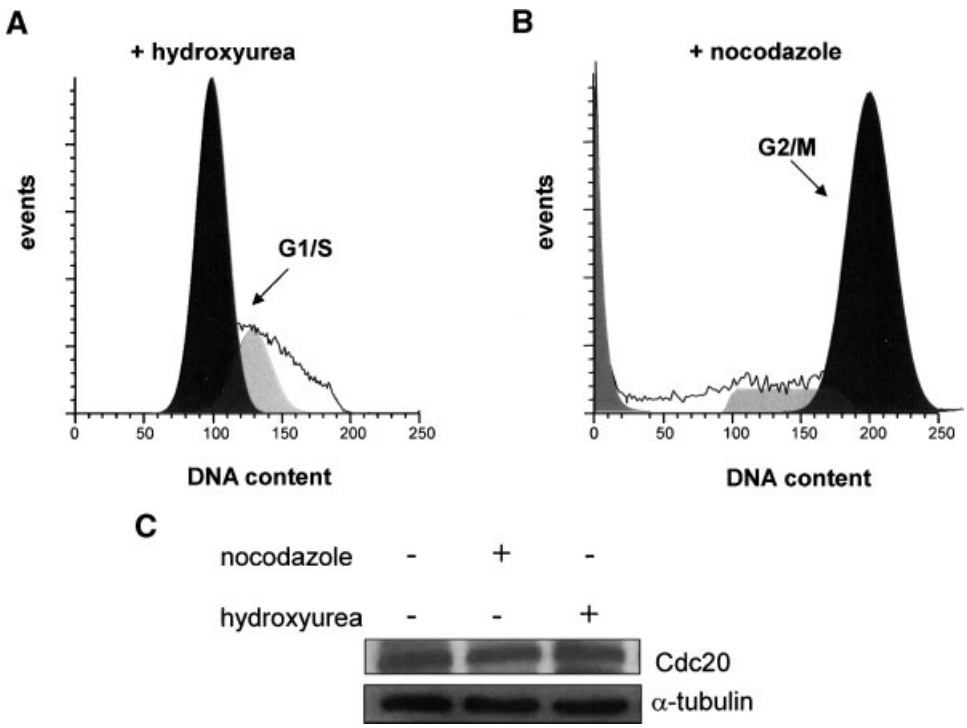


Fig. 6. Effects of cell cycle inhibitors on p55*CDC*/Cdc20 expression. HT29 cells were blocked either in G1/S or G2/M phases by exposing them to 1 mM hydroxyurea (**panel A**), or 10 μg/ml nocodazole (**panel B**), respectively. DNA content was determined by flow cytometry analysis. In **panel C**, immunoblotting did not show significant changes in p55*CDC*/Cdc20 expression in hydroxyurea- and nocodazole-synchronized cells compared to control. α-Tubulin expression was employed to assure equal loadings of the samples. The image presented is representative of a single experiment out of three performed.

'independent' events occurring at the same time, or the downregulation of p55*CDC*/Cdc20 is the primary cause for the TSA-dependent cell cycle arrest? We incline for the latter hypothesis for two circumstantial observations: (1) decrease of p55*CDC*/Cdc20 expression started after 24 h from TSA treatment (Fig. 4), a time long enough to exclude a previous TSA effect on p55*CDC*/Cdc20 promoter; (2) after TSA addition, asynchronous HT29 cells that already passed G1/S restriction point, continue to cycle until each single cell reaches mitosis; here, we believe that the absence p55*CDC*/Cdc20 caused by TSA is responsible for the G2/M arrest; in fact, untreated cells require high expression of p55*CDC*/Cdc20 to complete cell division (Fig. 5). Similarly, lack of expression of p55*CDC*/Cdc20 in S phase might prevent G1 cells to pass the G1/S checkpoint, explaining the DNA distribution profile reported in Figure 2. To further confirm and extend data obtained on HT29 cells, a different cell line, namely Caco2, deriving from a human adenocarcinoma, has been subjected to NaBt and TSA treatments. Our data suggest that, at least in this cell type, downregulation of p55*CDC*/Cdc20 following TSA or NaBt treatment was conserved with minor differences probably due to changes in HDAC bioavailability (data not shown).

Recent findings suggest that HDAC inhibitors, including TSA and NaBt, might represent one of the few examples of potentially useful chemotherapeutic drugs that specifically kill malignant cells, respect to their normal counterparts, by targeting cell cycle control checkpoints [Warrener et al., 2003]. These authors suggest that HDAC inhibitors block the normal function of the mitotic spindle checkpoint and the resulting cytotoxicity is the result of cell failure to respond to the aberrant mitosis with a mitotic checkpoint arrest [Warrener et al., 2003]. However, they left open the question on the molecules triggered by HDAC inhibitors, indicating HDAC inhibitor-sensitive G2-phase checkpoints and mitotic spindle checkpoints as potential targets. The observations that: (1) p55*CDC*/Cdc20 is involved in the mitotic spindle checkpoint being associated with hsMad2 [Kallio et al., 1998; Wassmann and Benezra, 1998] in different cancer cell lines; (2) p55*CDC*/Cdc20 functional mutants bypass the mitotic arrest inducing apoptosis [Sihn et al., 2003]; (3) p55*CDC*/Cdc20 is downregulated by HDAC inhibitors (this study), represent, all together, circumstantial evidence that p55*CDC*/Cdc20 might mediate the functional link between the cytotoxicity of HDAC inhibitors and the regulation of mitotic spindle checkpoints. Although the specific effects of TSA and NaBt on p55*CDC*/Cdc20 function will be investigated in the next future, the clear observation that p55*CDC*/Cdc20 is deregulated by NaBt/TSA, led us to hypothesize a consequent loss-of-function of its downstream effectors, such as APC/C complex. In fact, it has been reported that p55*CDC*/Cdc20 is essential for the activity of APC/C in mitosis [Eytan et al., 2006].

Our work also suggests caution in interpreting data deriving from initial gene-array screenings. In fact, in a precedent publication, we reported p55*CDC*/Cdc20 as one of the main candidate target of NaBt in HT29 cell line [Iacomino et al., 2001]. Here, we clearly demonstrate that the NaBt-dependent downregulation of p55*CDC*/Cdc20 was an epiphenomenon due to the G0/G1 cell cycle arrest caused by the molecule. This explanation has been possible after a detailed analysis of p55*CDC*/Cdc20 expression during the HT29 cell cycle (this study). Therefore, as suggested by others [van Bakel and Holstege, 2004], caution should be posed when interpreting data generated by microarray analyses: array technology should represent a first line screening method, and results obtained from differential expression studies might be further confirmed by traditional methods, such as Northern blot, qPCR analysis and, overall, by kinetic studies in order to discriminate between primary and consequential events.

The present work supports the promising therapeutic potential of HDACs. In fact, it appears clear the ability of this class of compounds to inhibit cell growth by disruption of cell cycle checkpoints [Warrener et al., 2003]. It results also evident that, at least for TSA, the pleiotropic effect(s) of the molecule involves both trascription-dependent and -independent mechanisms. The redundant activity of HDACs, abundantly reported in the scientific literature, should not be interpreted as an example of drugs with low specificity. On the opposite, a general agreement is growing on the potential use of HDAC inhibitors in combination with other anti-cancer drugs, such as classical chemotherapy drugs and DNA-demethylating agents [Villar-Garea and Esteller, 2004].

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