N-methylformamide and 9-hydroxystearic acid: two anti-proliferative and differentiating agents with different modes of action in colon cancer cells

Eleonora Pagnotta[a], Natalia Calonghi[a], Carla Boga[b] and Lanfranco Masotti[a]

***N*-methylformamide (NMF) is an anti-proliferative, differentiating agent studied in several cell lines as well as in preclinical and clinical trials, whose mechanisms of action are still unclear. 9-Hydroxystearic acid (9-HSA) is an endogenous product of lipid peroxidation recently identified as a new histone deacetylase 1 inhibitor. Both agents show the same anti-proliferative effects by arresting colon cancer cell growth in G_0/G_1. We addressed two questions. (i) Do they act by regulating G_0/G_1 checkpoint proteins? (ii) Does 9-HSA have differentiating effects comparable to those of NMF? The effects of NMF and 9-HSA on growth, differentiation and invasiveness of HT29, a colon cancer cell line, have been compared by using immunoprecipitation analysis, confocal microscopy, enzyme assays and invasiveness tests. The results show that the G_1 arrest caused by NMF is a cell cycle exit due to $p27^{KIP1}$ induction, whereas 9-HSA has no effect on the induction of this inhibitor. Evidence is presented that the arrest in early G_0/G_1 induced by 9-HSA is associated with the conversion of HT29 characteristics to those of a more benign phenotype, whereas the arrest in the late G_1 in response to NMF is not followed by a decrease in tumorigenicity. The failure of NMF in cancer therapy indicates that both anti-proliferative and differentiating characteristics are required for an anti-tumoral agent to be effective. *Anti-Cancer Drugs* 17:521–526 © 2006 Lippincott Williams & Wilkins.**

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[a]Department of Biochemistry 'G. Moruzzi', Bologna, Italy and [b]Department of Organic Chemistry 'A. Mangini', Bologna, Italy.

Correspondence to L. Masotti, Department of Biochemistry 'G. Moruzzi', University of Bologna, Via Irnerio 48, 40126 Bologna, Italy.
Tel: +39 051 2091221; fax: +39 051 2091230;
e-mail: eleonora.pagnotta3@unibo.it

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Introduction

Eukaryotic cells have developed control mechanisms that restrain cell cycle transitions in response to stress: they can be arrested transiently at cell cycle checkpoints to allow for the repair of cellular damage. Alternatively, if damage is irreparable, checkpoint signaling activates pathways that lead to apoptosis. Defective checkpoint functions may result in genetic modifications that induce dysregulation of oncogenes and tumor-suppressor genes. Checkpoint control has important implications for the optimization of current therapeutic regimens and the selection of novel cell cycle targets. Thus, identification of genes coding for proteins that control checkpoints, definition of their normal functions and responses to cellular stress have important implications for the development of new anti-cancer drugs [1].

Preclinical studies indicate that cells with defective checkpoint functions are more vulnerable to some anti-cancer agents [1,2]. In particular, because the activity of cyclin-dependent kinases (CDKs) is often deregulated in tumors, compounds that inhibit CDK functions can be effective anti-proliferative agents. CDK–cyclin complexes (CDK4–cyclin D, CDK2–cyclin E) phosphorylate the retinoblastoma protein (Rb), helping to inhibit its transcriptional repression of those genes whose activities are required for S-phase entry. A separate, non-catalytic action of these complexes is the sequestration of CDK inhibitors (CKIs) [3]. CKIs are of two types: INK4 proteins ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4C}$) that interfere with cyclin D binding to CDK4 or CDK6 and so inhibit their kinase activity, and CIP/KIP family members ($p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) that bind to and inhibit the activity of most CDK complexes including CDK2, CDK3, CDK4 and CDK6 with different efficacy [3]. In particular, $p21^{CIP1}$ and $p27^{KIP1}$ promote the assembly of the CDK4–cyclin D complex in the cytoplasm and enhance its nuclear translocation. Once in the nucleus, the complex titrates $p21^{CIP1}$ and, especially, $p27^{KIP1}$ from CDK2, thus promoting cell cycle progression. When cell cycle arrest occurs, the levels of the two CIP/KIP proteins increase and, once D-type proteins are saturated, they bind to CDK2–cyclin E and block its kinase activity, causing viable cells to accumulate in the late G_1 phase.

Although the role of the members of CKIs, such as $p21^{WAF1}$, in oncogenesis is still unclear, their functional

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role suggests that they may act as tumor suppressors [2]. In normal tissue adjacent to a tumor, $p21^{WAF1}$ is expressed exclusively in terminally differentiated cells. In tumors themselves, $p21^{WAF1}$ is frequently expressed, occasionally with the highest expression in the more malignant lesions; yet other tumors show decreased $p21^{WAF1}$ expression correlated with invasion and metastasis [2]. In lung cancer, cell cycle arrest in early G_1 and $p21^{WAF1}$ increased expression are accompanied by increased expression of cell surface proteins that are associated with the suppression of tumor growth and metastasis [4]. $p27^{KIP1}$ is implicated in mediating several growth-inhibitory signals including transforming growth factor (TGF)-β and contact inhibition. Mice lacking $p27^{KIP1}$ are abnormally big, show multiple organ hyperplasia and are predisposed to developing pituitary tumors. Patients with colon cancer and low or absent $p27^{KIP1}$ protein have median survival less than half that of patients with tumors that show high expression of $p27^{KIP1}$ [3].

9-Hydroxystearic acid (9-HSA) is an intermediate product of endogenous lipid peroxidation [5]. In recent studies we demonstrated that in HT29, a human colon adenocarcinoma cell line, it acts as a growth inhibitor through $p21^{WAF1}$ induction via a p53-independent mechanism involving histone hyperacetylation. $p21^{WAF1}$ appears to be an absolute requirement for cell growth arrest by 9-HSA [6,7].

N-methylformamide (NMF) belongs to a class of polar-planar agents that has been demonstrated to show anti-proliferative activity against colon, mammary and lung tumor xenografts. However, the last clinical trials have demonstrated that NMF therapy results neither in complete nor partial response [8]. In HT29, both NMF and 9-HSA induce a pronounced G_1 cell cycle arrest [6,9]: do the two agents exert their blocking activity through the same mechanisms and is this early arrest important for their different effects? We have addressed the issue whether different control mechanisms of proliferation lead to distinct differentiation programs. $p21^{WAF1}$ and $p27^{KIP1}$ have been studied to distinguish an early from a late arrest in G_1, while cell differentiation has been followed by measuring alkaline phosphatase (AP) activity, actin cytoskeletal architecture, cell invasion through a basement membrane model and α_5 integrin subunit as well as TGF-β1 expression.

Materials and methods

Cell line, antibodies and reagents

HT29 cells were obtained from Istituto Zooprofilattico Sperimentale (Brescia, Italy). RPMI 1640 was from Eurobio, Labtek (Milan, Italy). FBS was from Euroclone, CelBio (Milan, Italy). L-glutamine, trypsin, EDTA, NP-40, RNase, ethanol, propidium iodide (PI), HEPES, *p*-nitrophenylphosphate, glycerol, glycerophosphate, BSA, glycine, PBS, Na_3PO_4, NaCl, Triton X-100, PMSF, β-mercaptoethanol, SDS, deoxycholate, Trizma base and AP assay kit were purchased from Sigma (Milan, Italy). NMF was from Merck (Whitehouse Station, New Jersey, USA). Aprotinin, leupeptin, pepstatin and antipain were from Calbiochem (Darmstadt, Germany). The mouse monoclonal antibodies against $p21^{WAF1}$ and α_5 integrin subunit were from Oncogene (Darmstadt, Germany), the rabbit polyclonal antibodies against $p27^{KIP1}$ and TGF-β1 were from Santa Cruz Biotechnology (Hayward, California, USA). The Protein A–Sepharose and secondary antibodies conjugated with horseradish peroxidase were from Amersham Biosciences (Milan, Italy). Secondary antibodies conjugated with Alexa Fluor 568 or Alexa Fluor 498 and Texas Red-X phalloidin were from Molecular Probes (Poort Gebouw, The Netherlands). Mowiol was from Hoechst (Frankfurt, Germany). 9-HSA was synthesized in our laboratory.

Cell culture and treatments

Human colon adenocarcinoma cells HT29 were grown in RPMI 1640, supplemented with 10% FBS and 2 mmol/l L-glutamine at 37°C/5% CO_2. HT29 were seeded at 2×10^4 cells/cm^2 and exposed to 100 µmol/l 9-HSA or 1% NMF after 24 h of growth. The effects on cell growth were studied after 24 h of treatment. The effects of 9-HSA and NMF on cell differentiation and invasiveness were measured 120 h after administration of the two agents.

Cell lysis and immunoprecipitation

For immunoprecipitation, cells were lysed for 1 h in lysis buffer (40 mmol/l HEPES, pH 7.4, 60 mmol/l glycerophosphate, 20 mmol/l *p*-nitrophenylphosphate, 0.5 mmol/l Na_3PO_4, 250 mmol/l NaCl, 1% Triton X-100, 0.5 mmol/l PMSF, and 10 µg/ml each of aprotinin, leupeptin, pepstatin and antipain) at 0°C. Cell lysates were centrifuged at 12 000 *g* for 20 min. Supernatants were collected and protein concentration determined by using the Bio-Rad protein assay method (Bio-Rad, Hercules, California, USA). Aliquots of 500 µg of proteins were incubated overnight at 4°C with 1 µg of anti-$p21^{WAF1}$ monoclonal antibody or anti-$p27^{KIP1}$ polyclonal antibody. Then, 50 µl of Protein A–Sepharose (50% v/v) was added to each sample, incubated for 1 h, centrifuged at 12 000 *g* for 20 min at 4°C and washed 4 times with 0.1 mol/l potassium phosphate buffer, pH 8.0. Immunoprecipitated $p21^{WAF1}$ and $p27^{KIP1}$ were extracted from the complex with 30 µl of denaturing buffer (65 mmol/l Tris–HCl, 65 mmol/l β-mercaptoethanol, 1% SDS and 10% glycerol, pH 7.5) by heating at 100°C for 5 min. The proteins were resolved by SDS–PAGE and immunoblotted with anti-$p21^{WAF1}$ or anti-$p27^{KIP1}$ antibody, respectively. Detection of immunoreactive bands was performed with a secondary antibody conjugated with

horseradish peroxidase and developed with an enhanced chemiluminescence (ECL) system.

Confocal microscopy

Cells were seeded at 2×10^4 cells/cm^2 on glass coverslips, treated as described above, and then washed twice with PBS, fixed with 3% paraformaldehyde, washed with 0.1 mol/l glycine in PBS and permeabilized in 70% ice-cold ethanol. After fixing, the cells were incubated with primary antibodies (monoclonal antibody against α_5 integrin subunit or rabbit polyclonal antibody against TGF-β1) overnight at 4°C. Subsequently the samples were washed with 1% BSA in PBS and incubated with secondary antibodies conjugated with Alexa Fluor 498 or Alexa Fluor 568 for 1 h at room temperature. Some samples were labeled with Texas Red-X phalloidin for 25 min at room temperature. Preparations were embedded in Mowiol and analyzed using a laser scanning confocal microscope (MRC 1024; Bio-Rad) equipped with a Nikon Eclipse microscope.

AP activity

Cells were lysed in ice by a 1 ml solution of 40 mmol/l HEPES, 110 mmol/l NaCl, 0.25% deoxycholate and 1 mg/ml aprotinin, pH 7.4. The homogenate was used for the AP spectrophotometric assay by using *p*-nitrophenyl-phosphate as a substrate, according to the instructions of the manufacturer. AP activity was normalized for the protein content. Proteins were measured by the Bio-Rad protein assay method. One unit of AP activity is defined as the amount of protein capable of transforming 1 μmol of substrate in 1 min at 25°C.

Invasion assay

Controls and cells treated for 120 h were detached with trypsin/EDTA, washed and resuspended in RPMI. Cells were seeded in an invasion chamber (Chemicon, Temecula, California, USA) on a basement membrane model formed by proteins derived from the Engelbreth Holm-Swarm (EHS) mouse tumor. Cell invasion was evaluated after 72 h by means of the ratio between colony-forming units (c.f.u.) which migrated through the membrane and the number of seeded cells.

Statistical analysis

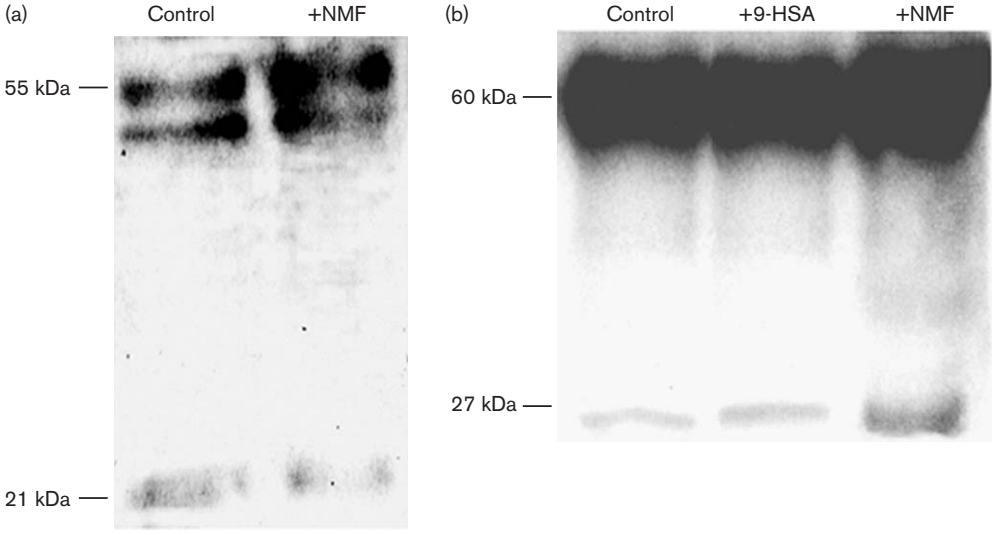
Student's *t*-test was used for repeated measurement values. $P < 0.05$ was considered significant.

Results

Effects of 9-HSA on p27^{KIP1} expression and of NMF on p21^{WAF1} and p27^{KIP1} expression

Since 9-HSA and NMF effectively arrest the cell cycle in G_1, we hypothesized that both agents could act by regulating proteins that control the two checkpoints in G_1. The hypothesis was first tested by examining the effect of the two agents, after 24 h of treatment, on p21^{WAF1} and p27^{KIP1} expression. After exposure of HT29 to 9-HSA, the level of p27^{KIP1} remained comparable with that of the control. However, NMF induced an increase of p27^{KIP1} protein expression, but did not induce that of p21^{WAF1} (Fig. 1).

Fig. 1

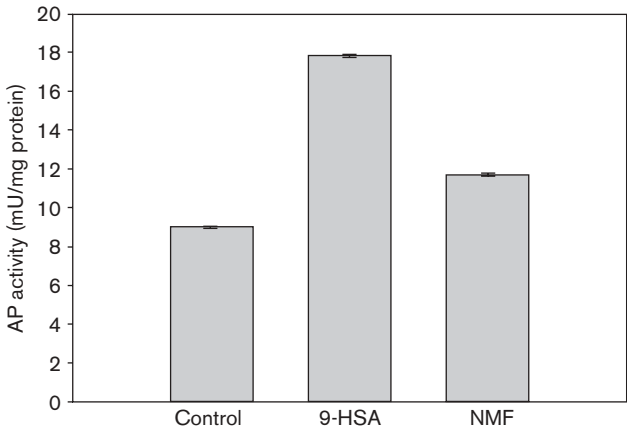


Immunoblot analysis of p21^{WAF1} (a) and p27 ^{KIP1} (b) in control and HT29 cells treated for 24 h. Cell lysates were immunoprecipitated with p21^{WAF1} or p27 ^{KIP1} antibodies and then immunoblotted. Detection of the bands was performed with secondary antibodies conjugated with horseradish peroxidase and developed with ECL; 55 and 60 kDa are the positions of the heavy chains of the immunoglobulins used in the immunoprecipitation. Data reported are representative of three independent experiments.

Effects of 9-HSA and NMF on differentiation and invasiveness

All the experiments described in the following paragraph were performed after 120 h of treatment with either agent: 9-HSA was at 100 μmol/l and NMF was at 1% (170 mmol/l). AP activity was measured to determine the effects of 9-HSA and NMF on differentiation. As shown in Fig. 2, the enzyme activity increased by 100% in 9-HSA-treated cells and by 30% in NMF-treated cells. Both 9-HSA and NMF induced changes in HT29 cytoskeletal architecture (Fig. 3). Only 9-HSA-treated HT29 regained actin organization and stress fiber formation. Immunofluorescence staining of control, 9-HSA- or NMF-treated HT29 revealed the expression of integrin α_5 subunit in 9-HSA-treated cells, but not in control and NMF-treated cells (Fig. 4, upper). Since previous studies showed that the expression of TGF-β1 has a role in modulating the steady-state expression of $\alpha_5\beta_1$ integrin and, as a result, $\alpha_5\beta_1$ integrin mediated adhesion [10], we tested the effect of the two agents on TGF-β1 expression. As shown in Fig. 4(lower), TGF-β1 expression increased in 9-HSA-treated cells, whereas it remained at control levels in NMF-treated cells. Finally, Fig. 5 shows that cell invasion, measured as c.f.u., diminished by 70% in 9-HSA-treated cells, whereas it increased by about 20% in NMF-treated cells with respect to control.

Fig. 2

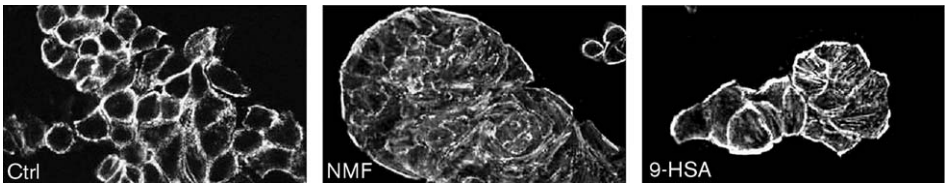


Effects of 9-HSA and NMF on AP activity after 120 h of treatment with 100 μmol/l 9-HSA or 1% NMF. Data reported indicate means ± SD of three independent experiments: AP activity was 9.03 ± 0.05, 17.88 ± 0.08 and 11.76 ± 0.06 mU/mg for lysates from control cells, 100 μmol/l 9-HSA- and 1% NMF-treated samples, respectively.

Discussion

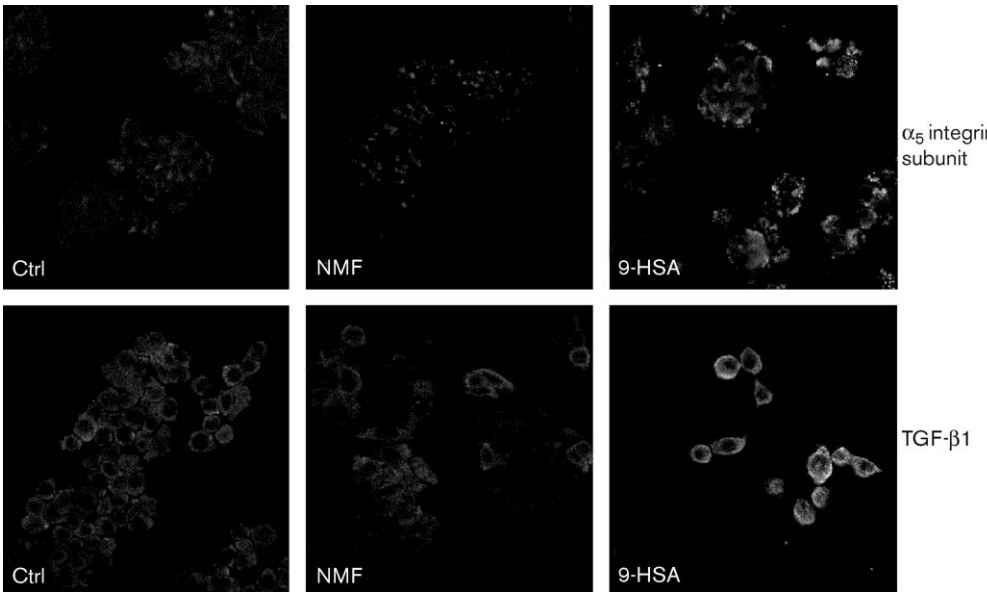
As previously stated, 9-HSA is an endogenous lipid peroxidation product identified in human cancer and normal cell lines [11–13]. In HT29 it acts as a growth inhibitor through $p21^{WAF1}$ induction in an immediate–early fashion, bypassing p53 [6,7]. NMF is a polar-planar molecule which has been used as a solvent for parenteral administration of organic compounds. Data from early studies suggest that it could interfere with tumor growth by inhibiting nucleic acid biosynthesis, but its mechanisms of action are still unknown [8]. In this paper we show that the two anti-proliferative agents, 9-HSA and NMF, which are known to exhibit comparable cytostatic effects characterized by a significant arrest of the cell cycle in G_0/G_1, induce distinct programs of differentiation in HT29 colon cancer cells. Their effects are related to the selective induction of a different CKI: 9-HSA induces $p21^{WAF1}$ [6], but not $p27^{KIP1}$, whereas NMF induces $p27^{KIP1}$, but not $p21^{WAF1}$. As previously demonstrated, 9-HSA acts as a HDAC1 inhibitor. The inappropriate transcriptional repression mediated by histone deacetylases (HDACs) is a molecular mechanism that leads to tumor formation [14]. Blocking the enzymatic activity caused by HDAC inhibitors results in an increase in the acetylation of the core histones, which in turn affects chromatin structure and the regulation of gene expression, both in tumor cells and in normal tissues. Furthermore, they are able to activate differentiation, to arrest the cell cycle in G_1 and/or G_2, and to induce apoptosis in cancer cells. Induction of $p21^{WAF1}$ and suppression of angiogenic-stimulating factors have been observed in tumor cells following exposure to HDAC inhibitors [15]. The increase of $p27^{KIP1}$ protein levels

Fig. 3



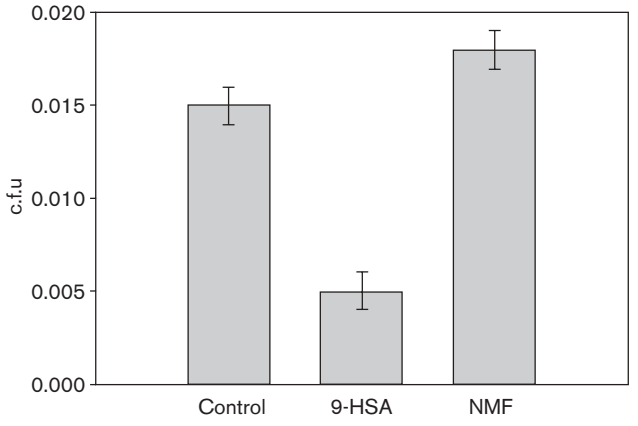
Representative confocal micrographs showing the effect of 100 μmol/l 9-HSA and 1% NMF on actin stress fibers in HT29. Control and HT29 cells treated for 120 h were labeled with Texas Red-X phalloidin. The images were collected using a Nikon Plan Apo ×60, 1.4 NA oil immersion lens. Data reported are representative of three independent experiments.

Fig. 4



Representative confocal micrographs showing the effect of 100 μmol/l 9-HSA and 1% NMF on α_5 integrin subunit (upper) and TGF-β1 (lower) protein expression in HT29. The confocal sections were collected after 120 h of treatment using a Nikon Plan Apo ×60, 1.4 NA oil immersion lens. Data reported are representative of three independent experiments.

Fig. 5



Effects of 100 μmol/l 9-HSA and 1% NMF on cell invasion. Control and HT29 cells treated for 120 h were allowed to invade a basement membrane model formed by proteins derived from the EHS mouse tumor for 72 h. The invasion index was evaluated as the ratio between c.f.u. counts that migrated through the membrane (719 ± 36, 838 ± 41 and 217 ± 30 for control, NMF- or 9-HSA-treated cells respectively) and the number of seeded cells (50 000 for each test). Data reported indicate means ± SD of three independent experiments.

is mainly regulated at a post-translational level via a ubiquitin–proteasome-mediated proteolysis [16,17], causes inhibition of the CDK2–cyclin E complex and arrests the cells in late G_1. Effects on differentiation in colon cancer cells are observed only when both CDK regulators, $p21^{WAF1}$ and $p27^{KIP1}$, are upregulated [18,19]. No effects on differentiation are observed after the selective increased expression of $p27^{KIP1}$ protein levels [20]. Although the exact mechanisms are poorly understood, the orderly progression from a rapidly dividing pluripotent intestinal stem cell to a terminally differentiated enterocyte is thought to occur through the transcriptional regulation of a small subset of specific genes, which together comprise an overall differentiation program. Among these genes is the intestinal AP, whose expression is often reduced in colon cancer cells. In this work we show that the proliferative arrest induced by the two agents is associated with 100 and 30% AP activity increase in 9-HSA- and NMF-treated HT29, respectively. Furthermore, studies on actin organization in epithelial cells of human colon explants have shown that cells from patients with familial polyposis are found to exhibit reduced microfilament organization as compared with cells from normal individuals [21]. HT29 cells show no actin polymerization and treatment with 9-HSA or NMF induces distinct morphological changes. After 9-HSA administration, cells become more flattened and form monolayers with evident boundaries between cells with concomitant increased actin filament organization in stress fibers. NMF treatment does not seem to greatly modify the general appearance of actin organization in the inner cells; in contrast, at the cluster periphery, the actin microfilaments become aggregated in thick fluorescent bundles. It has been previously shown that $\alpha_5\beta_1$ integrin signaling in colon carcinoma cells restores cytoskeletal

organization. In particular, in HT29, the gene coding for the α_5 subunit is not deleted, but strongly repressed, and its transfection induces markedly greater adhesion to fibronectin, formation of actin stress fibers, growth and experimental lung metastasis inhibition [21,22]. Our results demonstrate that prolonged treatment with 9-HSA induces α_5 subunit expression at the plasma membrane level in this cellular model, with a concomitant increased expression of TGF-β1; in contrast, NMF has no significant effect.

Finally, the invasion assay shows that the c.f.u. count diminishes by 70% in 9-HSA-treated HT29 and surprisingly increases about 20% in NMF-treated cells. As for concentration, 9-HSA exhibits its cytostatic and differentiating effects at concentrations at least 10-fold lower than the other fatty acids (butyrate and derivatives) that act as HDAC inhibitors, and comparable to those of hydroxamic acids. However, NMF in HT29 acts as a cytostatic agent at a concentration as high as 170 mmol/l. These data confirm that the arrest in the early G_0/G_1 phase mediated by 9-HSA is associated with the conversion of HT29 characteristics to those consistent with a more benign phenotype, whereas the arrest in the late G_0/G_1 phase mediated by NMF is not associated with a decrease in tumorigenicity. Finally, we report the first experimental observation that explains NMF's failure as a therapeutic agent and that at the same time strengthens interest in 9-HSA as an anti-cancer drug. Our results underline that an anti-neoplastic drug is required to have both cytostatic and differentiating effects.

Acknowledgments

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