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Journal of Nutritional Biochemistry 19 (2008) 587 – 593
Journal of Nutritional Biochemistry

Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon

Markus Waldecker, Tanja Kautenburger, Heike Daumann, Cordula Busch, Dieter Schrenk*

Food Chemistry and Environmental Toxicology, University of Kaiserslautern, D-67663 Kaiserslautern, Germany

Received 7 May 2007; received in revised form 12 July 2007; accepted 1 August 2007

Abstract

Colorectal cancer is the most abundant cause of cancer mortality in the Western world. Nutrition and the microbial flora are considered to have a marked influence on the risk of colorectal cancer, the formation of butyrate and other short-chain fatty acids (SCFAs) possibly playing a major role as chemopreventive products of microbial fermentation in the colon. In this study, we investigated the effects of butyrate, other SCFAs, and of a number of phenolic SCFA and *trans*-cinnamic acid derivatives formed during the intestinal degradation of polyphenolic constituents of fruits and vegetables on global histone deacetylase (HDAC) activity in nuclear extracts from colon carcinoma cell cultures using *tert*-butoxycarbonyl-lysine (acetylated)-4-amino-7-methylcoumarin (Boc-Lys(Ac)-AMC) as substrate. Inhibition of HDAC activity, e.g., by butyrate, is related to a suppression of malignant transformation and a stimulation of apoptosis of precancerous colonic cells. In nuclear extracts from HT-29 human colon carcinoma cells, butyrate was found to be the most potent HDAC inhibitor (IC_{50}=0.09 mM), while other SCFAs such as propionate were less potent. In the same assay, *p*-coumaric acid (IC_{50}=0.19 mM), 3-(4-OH-phenyl)-propionate (IC_{50}=0.62 mM) and caffeic acid (IC_{50}=0.85 mM) were the most potent HDAC inhibitors among the polyphenol metabolites tested. Interestingly, butyrate was also the most potent HDAC inhibitor in a whole-cell HeLa Mad 38-based reporter gene assay, while all polyphenol metabolites and all other SCFAs tested were much less potent; some were completely inactive. The findings suggest that butyrate plays an outstanding role as endogenous HDAC inhibitor in the colon, and that other SCFAs and HDAC-inhibitory polyphenol metabolites present in the colon seem to play a much smaller role, probably because of their limited levels, their marked cytotoxicity and/or their limited intracellular availability.

Keywords: Colorectal cancer; Histone deacetylation; HT-29 cells; Inhibition; Short-chain fatty acids; Polyphenol metabolites

1. Introduction

One of the most important mechanisms of regulation of gene expression is the modulation of histones via acetylation [1]. The acetyl moieties are attached to the N-terminus of histones by histone acetyltransferases while histone deacetylases (HDACs) remove histone-bound acetyl groups. These complex modifications result in a characteristic pattern of acetylation which allows access of transcription factors and other regulatory proteins to certain DNA sequences but prevents access to others [2].

Such mechanisms of selective regulation of gene expression are thought to explain the observation that HDAC inhibitors can induce differentiation and/or lead to apoptosis of precancerous and tumor cells. Therefore, their use in cancer treatment has been investigated [3,4]. Furthermore, HDAC inhibitors were suggested to be involved in chemoprevention since they may keep genetically modified putative preneoplastic cells in a more differentiated state and/or enhance their removal via apoptosis [5–7].

In the colon, the short-chain fatty acid (SCFA) butyrate is a product of fermentation depending on the intestinal microflora. Butyrate plays a major role not only as an important energy source for the mucosa but also as an apoptosis activator [8]. In particular, the production of

Abbreviations: Boc-Lys(Ac)-AMC, *tert*-butoxycarbonyl-lysine (acetylated)-4-amino-7-methylcoumarin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FCA, fetal calf serum; HDAC, histone-deacetylase; IC_{50}, 50%-inhibitory concentration; NEAA, nonessential amino acids; PBS, phosphate-buffered saline; SCFA, short-chain fatty acid.

* Corresponding author. Tel.: +49 631 2053217; fax: +49 631 2054398.
E-mail address: schrenk@rhrk.uni-kl.de (D. Schrenk).

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doi:10.1016/j.jnutbio.2007.08.002

butyrate has been speculated to play a major role in preventing colorectal cancer. Therefore, attempts have been made to enhance butyrate formation, e.g., by increasing the amount of nutritional fiber in the diet. In particular, certain carbohydrates resistant towards enzymatic degradation in the small intestine can provide a substrate for colonic butyrate formation and can thus be propagated as chemopreventive food ingredients or additives [9].

In fact, butyrate was identified as the first compound which acts as an HDAC inhibitor [10–13] while other SCFAs such as propionate or valerate, also formed by intestinal microorganisms, were less active [13,14]. Another putative nutritional source of HDAC inhibitors are polyphenols which are transformed during the gastrointestinal passage, e.g., into aromatic SCFA derivatives such as phenylacetate or phenylbutyrate detectable in human fecal water [15]. Phenylbutyrate and, to a lower extent, phenylacetate were found to be active as HDAC inhibitors [16–19]. Other intestinal metabolites of nutritional polyphenols are 3-OH-phenylacteate, 3-phenyl-propionate, 3-(4-OH-phenyl)-propionate and a number of aromatic acids bearing an unsaturated side-chain such as *trans*-cinnamic acid, *p*-coumaric acid and caffeic acid [15]. In this study, we have investigated the HDAC inhibitory potency of a number of SCFAs, and of aromatic SCFA derivatives formed, e.g., during the intestinal degradation of nutritional polyphenols. The results obtained in genetically modified HeLa Mad 38 cells bearing an HDAC-sensitive reporter gene, and in nuclear extracts from the human colon carcinoma cell line HT-29, indicate that a number of the latter compounds have the potential to inhibit intestinal HDACs, while butyrate seems to be not only the most abundant but also the most potent HDAC inhibitor.

2. Materials and methods

2.1. Materials

Sodium acetate, sodium propionate, valeric acid, the sodium salts of phenlyacetate, 3,4-diOH-phenylacetate, 3-OH-phenylacetate, phenylpropionate, 3-(4-OH-phenyl)-propionate, caffeic acid, *trans*-cinnamic acid and *p*-coumaric acid were from Sigma-Aldrich (Steinheim, Germany); bovine serum albumin was from Bio-Rad (Hercules, CA, USA); BOC-Lys(Ac)-AMC was from Bachem (Bubendorf, Switzerland).

DMEM, fetal bovine serum and penicillin/streptomycin (100×) were from PAA Laboratories (Pasching, Germany). Puromycin and neomycin were from AppliChem (Darmstadt, Germany). All chemicals were of the highest purity commercially available.

2.2. Cell culture

HT-29 cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and reporter gene-expressing HeLa Mad 38 cells were a gift from Dr. T. Heinzel, Georg-Speyer-Haus, Institute for Biomedical Research, Frankfurt, Germany. All cells were cultured in DMEM (low glucose) supplemented with 1% Pen/Strep solution in fully humidified air containing 5% carbogen at 37°C. Furthermore, the following supplements were added: HT-29 cells: 10% FCS; HeLa Mad 38 cells: 10% FCS, 1 ml of a neomycin solution containing 400 μg/ml, and 20 μl of a puromycin solution containing 1 μg/ml.

2.3. Cytotoxicity assays

Cytotoxicity testing with resazurin was performed according to O'Brien et al. [20] with modifications. Cells (8×10^3) were seeded on each well of a 96-well plate. After attachment, cells were treated with vehicle (1% DMSO or PBS) or the test substances, dissolved in vehicle. After 24 and 48 h, the medium was removed, the cell layers were washed with 200 μl/well of PBS, and 200 μl of a freshly prepared resazurin solution (first, 110.5 mg resazurin is dissolved in 1 ml dimethylformamide; then, 100 μl of this solution is diluted 1:1000 with NaCl/phosphate buffer; finally, this is then diluted 1:10 with fresh medium) was added in the dark. After 1 h at 37°C in the incubator, fluorescence was determined in a fluoroscan plate reader (Ascent FL, Thermo Fisher Scientific, Waltham, MA, USA) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

Staining with 4′,6-diamidino-2-phenylindole (DAPI) was used to quantify DNA in cell cultures [21], thus also obtaining an indirect measure of the density of (vital) cells. Before staining, cell cultures were washed with PBS, and 100 μl methanol was added and removed after 5 min. Residues of methanol were allowed to evaporate over 15 min at room temperature. Then 100 μl of a 20-μM DAPI solution (in PBS) was added to each well. The wells were incubated for 30 min at 37°C and the fluorescence was determined in a fluoroscan plate reader at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

2.4. Preparation of nuclear extracts

About 10^7 cells were trypsinized, transferred to a centrifugation tube and centrifuged at 1300×*g* at room temperature. The sediment was suspended in 1 ml ice-cold lysis buffer (aqueous solution of 10 mM Tris-HCl, 10 mM NaCl, 15 mM $MgCl_2$, 250 mM saccharose, 0.1 mM EGTA, 0.5% NP-40, pH 7.5), kept on ice for 15 min and pipetted carefully over a layer of 4 ml saccharose buffer (aqueous solution of 30% saccharose, 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$; pH 7.5). After centrifugation (1300×*g*; 4°C, 10 min), the sediment was resuspended in Tris-HCl buffer (aqueous solution of 10 mM Tris-HCl, 10 mM NaCl; pH 7.5) and centrifuged at 1300×*g*, 4°C for 10 min. The nuclear fraction (sediment) was resuspended in extraction buffer (aqueous solution of 50 mM HEPES, 420 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 10% glycerol) and, after ultrasonification for 30 s, kept on ice for 30 min. Then, the

mixture was centrifuged at 10,000×*g*, 4°C for 10 min. The supernatant (nuclear extract) was separated and kept at −80°C for analysis of nuclear HDAC activity.

2.5. HDAC activity in nuclear extracts

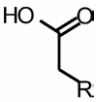
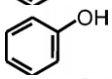
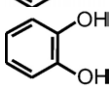
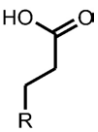
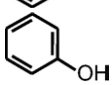
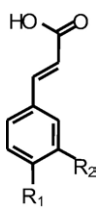
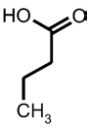
A volume of 150 µl assay buffer (aqueous solution of 25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$; pH 8.0) was pipetted into each well of a black 96-well fluorescence multiwell plate. Then 10 µl inhibitor solution or solvent blank (DMSO or culture medium) and 10 µl nuclear extract were added. The reaction was started by adding 10 µl HDAC substrate solution (4 mM BOC-Lys(Ac)-AMC in DMSO). The plate was protected against light and agitated for 30 min. Then 40 µl developer [aqueous solution of 2.5% 10×trypsin, 5.12 µM trichostatin A (TSA)] was added to each well. After 15 min at room temperature the fluorescence was monitored (excitation at 355 nm, emission at 460 nm).

2.6. HDAC-linked (whole-cell) reporter gene assay

HDAC activity was also measured in a cell-based reporter gene assay as described by Göttlicher et al. [4]. Briefly, the assay uses the cell line HeLa Mad 38 which bears two stably integrated plasmid sequences. One encodes a luciferase reporter with a thymidine kinase (TK) promoter and a 3′-located UA sequence. The UA sequence binds the transcription factor Gal4, which is encoded by the second plasmid. Gal4 forms a dimer with the protein Mad, which recruits class I and II HDACs to the TK promoter. If these HDACs are active, they are thought to repress the constitutively active TK promoter. Upon HDAC inhibition, TK promoter acetylation increases, and TK promoter activity and reporter gene expression are enhanced.

HeLa Mad 38 cells were seeded into six-well plates (80,000 cells/well) and grown over 24 h under standard conditions. Then medium was replaced by fresh medium and test compounds were added. After 24 h, the medium was removed and cell layers were washed with ice-cold PBS. After the addition of 400 µl/well of PLB, cells were lysed on ice and scraped-off after 5 min. The lysate was centrifuged at 15,000×*g* for 30 s, and the supernatant was removed and kept on ice. Luminescence was measured after mixing 65 µl of LAR (Luciferase Assay Reagent; Promega, Mannheim, Germany) with 20 µl cell lysate supernatant. Afterwards the protein concentration was determined with a NanoDrop analyzer (NanoDrop Technologies, Wilmington,

Table 1
Cytotoxicity of SCFAS, *trans*-cinnamic acid and some phenolic derivatives in HT-29 and HeLa Mad 38 cells after an incubation period of 48 h using the resazurin assay and DNA staining with DAPI

Compound	Substituents	Name	EC_{50} of cytotoxicity (mM)			
			Resazurin HT-29	HeLa Mad 38	DAPI HT-29	HeLa Mad 38
	H	Acetate	>90	>90	>90	>90
		Phenylacetate	>10	>10	>10	>10
		3-OH-Phenylacetate	>20	>20	>20	>20
		3,4-diOH-Phenylacetate	0.52±0.12	0.04±0.03	0.41±0.10	0.06±0.04
	H	Propionate	>90	>90	68.3±13.5	12.8±3.2
		3-Phenyl-propionate	21.2±3.5	16.1±3.2	15.1±4.3	16.1±3.2
		3-(4-OH-phenyl)-Propionate	21.4±0.5	19.3±3.0	17.6±1.9	16.4±2.8
	R_1=H; R_2=H	*trans*-Cinnamic acid	11.7±1.7	10.2±2.3	6.1±3.0	7.2±2.6
	R_1=OH; R_2=H	*p*-Coumaric acid (hydroxy-cinnamic acid)	13.6±0.2	5.6±1.8	5.4±1.3	6.3±1.7
	R_1=OH; R_2=OH	Caffeic acid (3,4-diOH-cinnamic acid)	0.24±0.16	0.03±0.02	0.20±0.14	0.04±0.03
		Butyrate	15.2±2.1	13.6±2.7	5.1±3.0	5.2±2.5

Data are shown as lower limits or as means±S.D. from *n*=3 independent experiments.

DE, USA) using the absorbance of the sample at 280 nm. Activities were expressed as luciferase activity in percent of solvent control.

2.7. Statistical analysis

Data are expressed as means±S.D. from n=3 independent experiments if not otherwise stated. Tests for significant differences between vehicle-treated controls and treated cells or nuclear extracts were carried out using one-way ANOVA with Dunnett's post test. Curve fits and IC_{50} estimates were obtained by linear or logarithmic regression analysis, respectively.

3. Results

3.1. Cytotoxicity

Cytotoxicity of the test compounds was measured using the resazurin reduction assay and DNA staining with DAPI after an incubation period of 24 or 48 h. The values at 48 h were used for the calculation of EC_{50} values (Table 1), while after 24 h similar but less pronounced effects were seen (data not shown). With the simple SCFAs acetate and propionate, no EC_{50} values could be determined in the resazurin assay because of their low cytotoxicity, while propionate was cytotoxic in the DAPI assay. Also for the aromatic derivatives phenylacetate and 3-OH-phenylacetate, EC_{50} values above 10 or 20 mM were found (Table 1). The concentration–response curves were too flat (not shown) to identify an EC_{50} value. Similar results were obtained for the SCFAs *iso*-propionate, *iso*-butyrate, valerate and *iso*-valerate (data not shown). In contrast, butyrate, 3-phenyl-propionate, 3-(4-OH-phenyl)-propionate and the unsaturated side chain acids *trans*-cinnamic acid and *p*-coumaric acid exhibited clear concentration–response curves allowing the determination of EC_{50} values in a range between 5 and 22 mM for the two cell lines tested. In particular with butyrate, the EC_{50} value in the DAPI assay was lower than in the resazurin assay, indicating growth-suppressive and/or pro-apoptotic effects of butyrate. With the two acids bearing catecholic OH groups at the aromatic ring, 3,4-diOH-phenylacetate and caffeic acid, much lower EC_{50} values, i.e., <1 mM in HT-29 cells and <0.1 mM in HeLa Mad 38 cells, were determined in both assays.

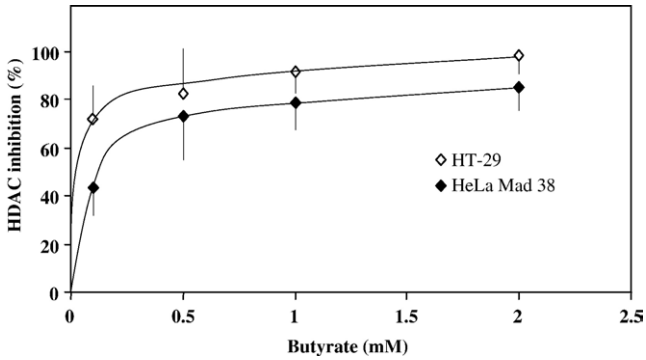


Fig. 2. Inhibition of HDAC activity by butyrate in nuclear extracts from the tested cell lines HT-29 and HeLa Mad 38. Symbols represent means±S.D. from n=3 independent experiments.

3.2. HDAC inhibition with SCFAs

In HeLa Mad 38 cells equipped with an HDAC-dependent reporter gene construct, increased reporter gene expression can be used as a direct measure of HDAC inhibition. With most SCFAs tested, except acetate, HDAC inhibition was obtained at concentrations of ≥10 mM while butyrate showed an outstanding potency being fully effective at 2 mM and almost 40% effective at 1 mM (Fig. 1). Acetate was completely inactive at all concentrations tested.

In nuclear extracts, butyrate was even more effective showing an IC_{50} of HDAC inhibition at about 0.01 mM in HT-29 cells and 0.1 mM in HeLa Mad 38 cells (Fig. 2). In the

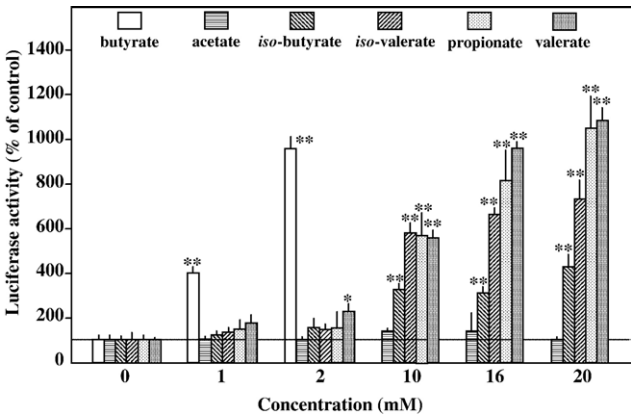


Fig. 1. HDAC inhibition-related reporter gene activity in transfected HeLa Mad 38 cells — effects of SCFAs. Bars represent means±S.D. from n=3 independent experiments. Significantly different from vehicle-treated controls at *P≤.05 or **P≤.01.

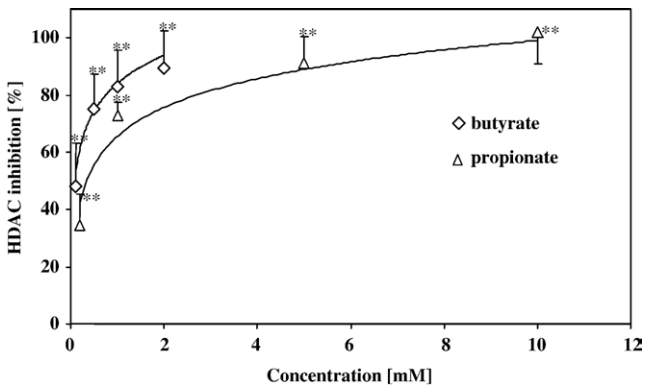


Fig. 3. Inhibition of HDAC activity by butyrate and propionate in nuclear extracts from HT-29 cells. Symbols represent means±S.D. from n=3 independent experiments. Significantly different from vehicle-treated controls at **P≤.01.

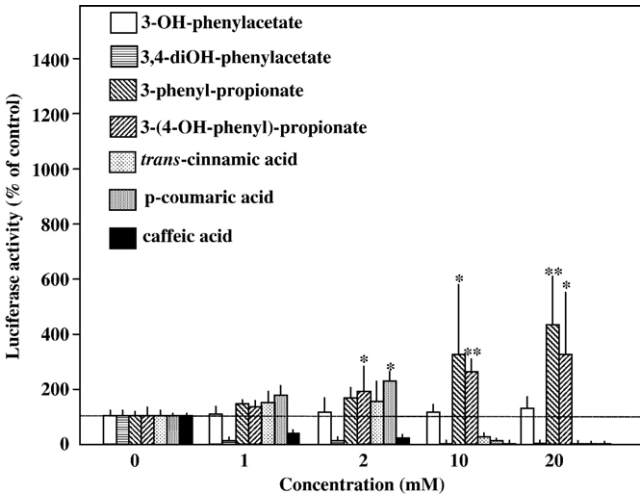


Fig. 4. HDAC inhibition-related reporter gene activity in transfected HeLa Mad 38 cells — effects of various polyphenol metabolites as indicated. Bars represent means±S.D. from n=3 independent experiments. Significantly different from vehicle-treated controls at *P≤.05 or **P≤.01.

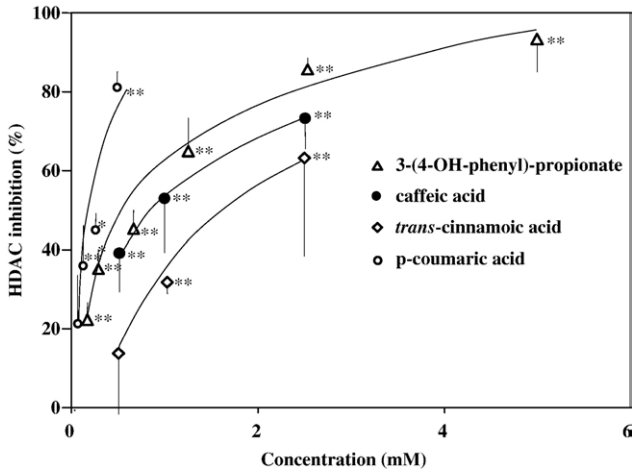


Fig. 6. Inhibition of HDAC activity by 3-(4-OH-phenyl)-propionate, caffeic acid, *trans*-cinnamoic acid and *p*-coumaric acid in nuclear extracts from HT-29 cells. Symbols represent means±S.D. from n=3 independent experiments. Significantly different from vehicle-treated controls at **P≤.01.

range of 2 mM an apparent level of saturation of the inhibitory effect was achieved. Interestingly, propionate was only about fourfold less potent than butyrate in the cell-free HDAC assay (Fig. 3), whereas it showed an at least 10-fold lower potency in the whole-cell assay in HeLa Mad 38 cells. Similar differences between butyrate and propionate as in HT-29 cells were also found in nuclear extracts from HeLa Mad 38 cells (data not shown).

3.3. HDAC inhibition with poylphenol metabolites

The reporter gene assay was also used for testing the HDAC inhibitory capacity of a number of phenolic SCFA derivatives formed from plant polyphenols in the intestine.

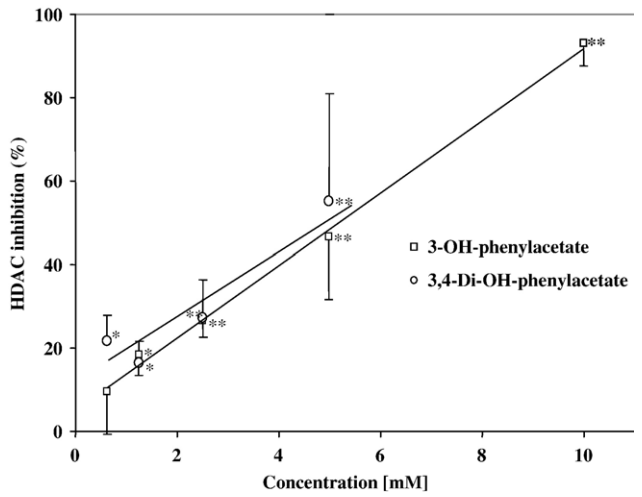


Fig. 5. Inhibition of HDAC activity by 3-OH-phenylacetate and 3,4-diOH-phenylacetate in nuclear extracts from HT-29 cells. Symbols represent means±S.D. from n=3 independent experiments. Significantly different from vehicle-treated controls at *P≤.05 or **P≤.01.

Only 3-OH-phenylpropionate and 3-(4-OH)-phenylpropionate exhibited a relevant increase in reporter gene activity, at a concentration range of 10–20 mM, however (Fig. 4). In the nuclear extract from HT-29 cells, 3-OH-phenylacetate and 3,4-di-OH-phenylacetate, which were both inactive or which suppressed reporter gene activity in the reporter gene assay, were active as HDAC inhibitors (Fig. 5). The most potent HDAC inhibitors (Fig. 6) were *p*-coumaric acid (IC_{50}=0.19

Table 2
Lower limits of IC_{50} values of HDAC-inhibitory concentrations in nuclear extracts from HT-29 cells or in a HeLa Mad 38-based reporter gene assay

Compound	IC_{50} of HDAC inhibition (mM)		Levels in FW[a] or FS[b] (mM)
	Reporter gene (HeLa Mad 38)	Nuclear extract (HT-29)	
Acetate	ND	ND	~70[b]
Phenylacetate	> 10	0.66	0.11–1.10[a]
3-OH-Phenylacetate	ND	5.47	0.00–0.11[a]
3,4-diOH-Phenylacetate	ND	4.31	0.00–0.02[a]
Propionate	~10	0.35	~11[b]
3-Phenyl-propionate	~8	1.11	0.01–0.44[a]
3-(4-OH-phenyl)-Propionate	~5	0.62	0.00–0.2[a]
trans-Cinnamic acid	ND	1.69	<0.01[a]
p-Coumaric acid (hydroxy-cinnamic acid)	ND	0.19	<0.01[a]
Caffeic acid (3,4-diOH-cinnamic acid)	ND	0.85	0.01–0.13[a]
Butyrate	1.13	0.09	~25[b]

Comparison with levels described in human FW or in supernatants from fermentations of human fecal slurry (FS).
ND=not detectable.
[a] Levels in human fecal water (FW) according to Jenner at al. [15].
[b] Mean levels in supernatants (FS) obtained from fermentations with human fecal slurry (unpublished data).

mM), 3-(4-OH-phenyl)-propionate (IC_{50}=0.62 mM) and caffeic acid (IC_{50}=0.85 mM).

The results obtained with nuclear extracts were in clear contrast to the results of the reporter gene assay where *p*-coumaric acid and caffeic acid were inactive. In the case of caffeic acid and 3,4-diOH-phenylpropionic acid, the marked cytotoxicity can easily explain the lack of effectivity in the reporter gene assay. In fact, both compounds decreased reporter gene activity at the respective concentration levels.

A comparison between IC_{50} values of HDAC inhibition in nuclear extracts from HT-29 cells and levels reported in human fecal water and/or fermentations of fecal slurry (Table 2) revealed that some of the compounds tested are relatively potent HDAC inhibitors in a concentration range probably found in the colon. However, none of the compounds tested, except butyrate, is likely to reach levels in the colon which were able to inhibit HDAC in intact HeLa Mad 38 cells to a relevant extent.

4. Discussion

The class of human HDACs comprises 18 known members, which have been divided into three enzyme families based on their phylogenetic homology to yeast enzymes [2,22]. HDACs of class I are mostly found in the nucleus, while HDACs of class II are supposed to shuttle between the nucleus and the cytoplasm [5]. Both classes comprise a highly conserved catalytic domain which is supposed to activate a water molecule via a zinc atom. The latter is coordinated with histidine and aspartate residues and binds the acetate moiety. The activated water attacks the carbonyl function of the acetylated lysine, which then decomposes into free acetate and a free lysine residue [2,3,22]. HDACs of class III are NAD^+-dependent Sir2 deacetylases which are not sensitive to typical inhibitors of class I or class II enzymes [5].

HDACs are not considered to be able to bind directly to histones but need to be recruited by histone-binding proteins such as Sin3 or NuRD and other stabilizing factors [2,5,22].

HDAC inhibitors have shown great potential as anticancer drugs [3]. Mainly inhibitors of class I and II enzymes can act by competitive or noncompetitive mechanisms. Effective inhibitors have been found among the derivatives of hydroxamic acid (including TSA), carboxylic acids, benzamide and cyclic peptides [5,6]. Furthermore, SCFAs such as butyrate, phenylbutyrate or valproic acid [4] are active as HDAC inhibitors. They are likely to act in a noncompetitive manner and usually show a lower potency than TSA [7,22]. The potency of HDAC inhibitors in cancer therapy has been reviewed, e.g., by Kouraklis and Theocharis [23]. So far, promising findings have been obtained with butyrate-releasing pro-drugs and butyrate derivatives rather than with butyrate itself. There have also been a number of reports using HT-29 cells investigating the links between HDAC inhibition, altered gene expression and apoptosis [14,24,25].

The chemopreventive effect of a diet rich in fiber and slowly digestible carbohydrates has been attributed, among a variety of factors, to an enhanced butyrate formation in the colon [5]. Hinnebusch et al. [14] found a relationship between the growth-inhibitory effect of butyrate in colon cancer cell lines and histone hyperacetylation, while other SCFAs such as propionate or valerate were less effective; acetate was inactive. It has to be kept in mind, however, that butyrate and phenylbutyrate, and probably a number of other compounds tested here, can also inhibit phosphorylation and methylation of proteins and DNA methylation, and may thus also influence gene expression via other mechanisms [26].

Marks et al. [5] described phenylacetate and phenylbutyrate as HDAC inhibitors which are of particular interest since these compounds can be formed from polyphenols in fruits and vegetables during the intestinal passage [15].

In our study, we investigated butyrate and a variety of other SCFAs in two different HDAC inhibitor assays, nuclear extracts from HT-29 carcinoma cells incubated with a standard HDAC substrate and a reporter gene assay designed to measure HDAC inhibition in intact HeLa cells. The finding that butyrate was more effective than all other SCFAs tested is in agreement with the data published by Hinnebusch et al. [14] and Kiefer et al. [13]. Interestingly, propionate was about twofold less potent than butyrate as HDAC inhibitor in the nuclear extract but almost 10-fold less potent in the whole-cell assay. These findings suggest that not only the interaction of butyrate with HDACs but also its uptake and intracellular bioavailability/metabolism play an important role for its outstanding inhibitory effects on HDACs. Acetate was ineffective as has been reported by others [13].

Among the polyphenol metabolites tested, only 3-OH-phenylpropionate and 3-(4-OH)-phenylpropionate exhibited a relevant increase in reporter gene activity, at a relatively high concentration range of 10–20 mM, however. In contrast, 3-phenylacetate and 3,4-di-OH-phenylacetate, which were both inactive or suppressed reporter gene activity in the reporter gene assay, were more effective HDAC inhibitors in the nuclear extract from HT-29 cells. The most effective HDAC inhibitors in this assay were *p*-coumaric acid, 3-(4-OH-phenyl)-propionate and caffeic acid. Recently, Lea et al. [19] found an IC_{50} of 0.62 mM for phenylbutyrate as HDAC inhibitor in nuclear extracts from DS19 cells. These results are in clear contrast to the results in the reporter gene assay where *p*-coumaric acid and caffeic acid were inactive. In the case of caffeic acid and 3,4-diOH-phenylpropionic acid, the marked cytotoxicity, obviously related to the catecholic position of the OH groups in the aromatic ring, can easily explain the lack of efficacy in the reporter gene assay. In fact, both compounds decreased reporter gene activity at the respective concentration levels.

For the other compounds, the lack of marked inhibitory activity in the whole-cell reporter gene assay is probably due

to a limited uptake or extensive intracellular metabolism which prevents the compounds from reaching effective target concentrations.

A comparison between IC_{50} values of HDAC inhibition and levels reported in human fecal water and/or fermentations [15] of fecal slurry revealed that none of the compounds tested, except butyrate and possibly propionate, is likely to reach levels in the colon which are able to inhibit HDAC in intact HeLa Mad 38 cells to a relevant extent. Even for compounds such as phenylacetate or *p*-coumaric acid which can be found in fecal water at levels in the range of or exceeding those which inhibit HDAC in nuclear extracts, the efficacy in intact cells remains doubtful. In summary, our data demonstrate that butyrate plays an outstanding role as endogenous HDAC inhibitor in the colon, while other carboxylic acids including SCFAs and polyphenolic metabolites are of minor importance.

Acknowledgments

The authors wish to thank Dr. Thorsten Heinzel (Georg-Speyer-Haus, Institute for Biomedical Research, Frankfurt, Germany) for providing HeLa Mad 38 cells. The expert technical assistance by Wilhelmine Daub and Monika Gross is gratefully acknowledged. This work received financial support from the Federal Ministry for Education and Research, Bonn, Germany (Network 'Role of nutrition in the etiology of intestinal disease and perspectives for their prevention via nutrition'/01EA0501), and from the State of Rhineland-Palatinate (Projekt 'Wissen schafft Zukunft').

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